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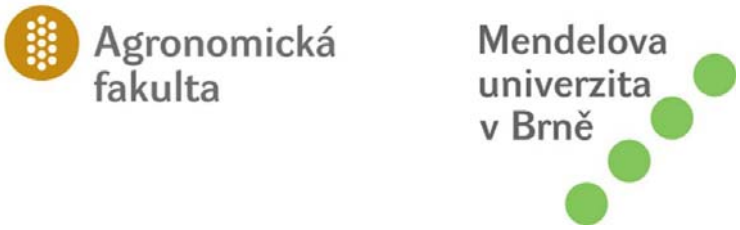

Efekt iontů těžkých kovů na biologické vlastnosti, rezistenci a virulenci

Disertační práce
Studijní obor:4106V017 Zemědělská chemie

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dne …….

podpis…….

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V neposlední řadě bych ráda poděkovala své rodině a přátelům za zázemí, lásku, podporu a nekonečnou energii hnát mě stále kupředu.

Největší dík patří mému příteli za to, že je…

Anotace

Bakteriální infekce s komplikovaným průběhem, a tudíž zdlouhavou, mnohdy i neřešitelnou léčbou způsobenou vznikající rezistencí vůči antibiotickým léčivům, jsou v současnosti stále častějším problémem řady medicínských oborů. Předkládaná práce s názvem „Efekt iontů těžkých kovů na biologické vlastnosti, rezistenci a virulenci“ byla primárně zaměřena na studium vlivu vybraných iontů kovů (Ag^{+}, Cd^{2+}, Cu^{2+}, Pb^{2+} a Zn^{2+}) na biochemické vlastnosti bakteriálních kmenů, zejména na pozorování jejich vlivu na tvorbu volných kyslíkových radikálů, vznik oxidačního stresu a růstové vlastnosti. Průkaz vlivu kovů na biochemické parametry, ale především jejich významný antimikrobiální efekt, nasměroval naše další studie na testování antimikrobiální aktivity kovů (nejčastěji stříbra), popřípadě kovů ve formě nanočástic syntetizovaných do komplexů s polymerními látkami (chitosanem a kyselinou hyaluronovou). Dosažené výsledky jednoznačně prokazovaly formu nanočástic jako nejvhodnější, proto byly následně porovnávány účinky prokazatelně antimikrobiálních nanočástic stříbra s nanočásticemi polokovu selenu, se kterým jsme dosáhli ještě slibnějších výsledků v oblasti antimikrobiální aktivity. V neposlední řadě byl testován vliv dlouhodobého působení iontů kovů na vznikající rezistenci nebo tzv. multirezistenci v případě zkřížené rezistence kovů a antibiotických léčiv.

Klíčová slova: antibiotika, bakterie, kovy a polokovy, nanočástice kovů, oxidační stres, polymerní látky, rezistence

Annotation

Bacterial infections with complicated process and therefore lengthy, often unsolvable treatment due to emerging resistance to antibiotic drugs are currently increasingly frequent problem of many medical fields. The presented thesis entitled "Effect of heavy metal ions on the biological properties, resistance and virulence" was primarily focused on the study of selected metal ions (Ag^{+}, Cd^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+}) on the biochemical properties of bacterial strains, in particular observing their influence on free radical formation, creation of oxidative stress and growth properties. Detection of the influence of metals not only on biochemical parameters, but mainly their significant antimicrobial effect directed our further studies to testing of antimicrobial activity of metals (usually silver) or metal nanoparticles synthesized in complexes with polymer substances (chitosan, hyaluronic acid). The obtained results clearly showed the nanoparticles as the best form, so the effects of proven antimicrobial silver nanoparticles were subsequently compared with metalloid selenium nanoparticles, which even surpassed the previous results of antimicrobial activity. Finally, the effect of long-term action of metal ions on the emerging resistance or so-called multiresistance in the case of cross-resistance of metals and antibiotic drugs was tested.

Key words: antibiotics, bacteria, metals and semimetals, metal nanoparticles, oxidative stress, polymeric substances, resistance

OBSAH

1 ÚVOD

Píše se rok 1676 a Antoni van Leeuwenhoek jako první pozoruje bakteriální kmeny pod mikroskopem, který si sám sestrojil. Od té doby je objevena celá řada dalších bakteriálních kmenů, které svým evolučním vývojem postupně mění své vlastnosti a stále častěji se začínají stávat odolnějšími vůči původně nevyhovujícím, mnohdy likvidačním podmínkám vnějšího prostředí. Mikroorganismy, zejména ty patogenní, se tak stávají závažnou komplikací léčby různých onemocnění.

Rozvoj a následné šíření bakteriálních infekcí je velmi snadné. Bakterie si v těle hostitele najdou vhodné prostředí pro jejich život, zde se usadí a nekontrolovatelně se množí. Problematika bakteriálních infekcí trápí zejména nemocniční zařízení, kde v zemích Evropské unie podle statistik v souvislosti s bakteriálními infekcemi umírá ročně až 37 tisíc lidí. Problematika původců onemocnění disponujících rezistencí vůči konvenční antibiotické léčbě však není zdaleka ojedinělá. Česká republika v rámci Evropy hlásí výskyt některých multirezistentních nemocničních bakterií mnohem častěji než v jiných evropských zemích. Výjimečně, ale přesto, se můžeme setkat i s tzv. panrezistentními bakteriemi, na které nejsou dostupná dokonce vůbec žádná účinná antibiotická léčiva.

O rezistenci se začíná mluvit v době, kdy léčba antibiotiky přestává účinkovat, bakterie nelze likvidovat nebo alespoň inhibovat jejich růst. Jsou známy základní mechanismy jejího vzniku, jako je přenos genů, kódujících rezistenci plasmidy nebo chromozomovými kazetami. Tato problematika je však stále opředena řadou nejasností. Vznik rezistence majoritně závisí na četnosti podávání antibiotik, jestli je pacient opakovaně nebo dlouhodobě hospitalizován s infekcí a musí tedy antibiotickou léčbu neprodleně podstoupit. Dalšími faktory, které výrazně přispívají zvýšenému výskytu rezistence u bakterií je zvyšující se využívání invazivních technik léčby, operování starších a imunokompromitovaných pacientů, jejichž léčba následně pokračuje na jednotkách intenzivní péče. Rezistentní bakteriální kmeny výrazným způsobem zhoršují prognózu léčby pacientů a v řadě případů mohou vést i k nutnosti náročných chirurgických zákroků.

Vůči některým typům antibiotik je vznik rezistence rychlý a snadný, jako je tomu například u rezistence vůči penicilinu u stafylokoků, která vznikla hned po několika letech po zavedení antibiotika do praxe. Naproti tomu účinnost penicilinu na

streptokoky je celosvětově velmi výrazná, rezistence u tohoto mikroorganismu doposud nebyla rozpoznána. Problematika rezistence je obecně největším problémem u širokospektrých antibiotik s větším selekčním potenciálem, tedy schopností více likvidovat citlivé mikroorganismy a opomíjet tak mikroorganismy odolnější.

Mikrobiologie se tak musí snažit s touto hrozbou vyrovnat. Je proto nezbytné využívat nové techniky na poli molekulární biologie nebo proteomiky pro rychlou identifikaci mikroorganismů. Ruku v ruce s identifikací jde pečlivé studium bakteriálních infekcí, jejich vzniku a snaha o nalezení vhodných alternativ antibiotik, které by mohly na dobu dalších několik desítek let výskyt bakteriálních infekcí účinně řešit, nebo ho alespoň výrazně eliminovat.

2 CÍLE PRÁCE

- Sumarizovat informace o bakteriích, zejména *Staphylococcus aureus*, jejich vlastnostech a především rezistenci vůči těžkým kovům
- Porovnat nerezistentní kontrolní kmeny *Staphylococcus aureus* s kmeny *Staphylococcus aureus* s rezistencí indukovanou expozicí těžkým kovům nebo antibiotickým léčivům
- Sledovat změny metabolismu na genové a proteinové úrovni
- Testovat alternativní cesty na bázi nanotechnologií pro léčbu bakteriálních infekcí

3 LITERÁRNÍ PŘEHLED

3.1 Bakteriální kmeny – jejich vlastnosti, výskyt a šíření

3.1.1 Grampozitivní a gramnegativní bakterie z morfologického pohledu

Nejvýznamnějším rozdílem z hlediska morfologické stavby zástupců jednobuněčných prokaryotických organismů, grampozitivních (G^+) a gramnegativních (G^-) bakterií, je bezpochyby buněčná stěna, která je jejich typickou součástí spolu s jadernou oblastí, DNA, plasmidy a prokaryotickým typem ribozomů.

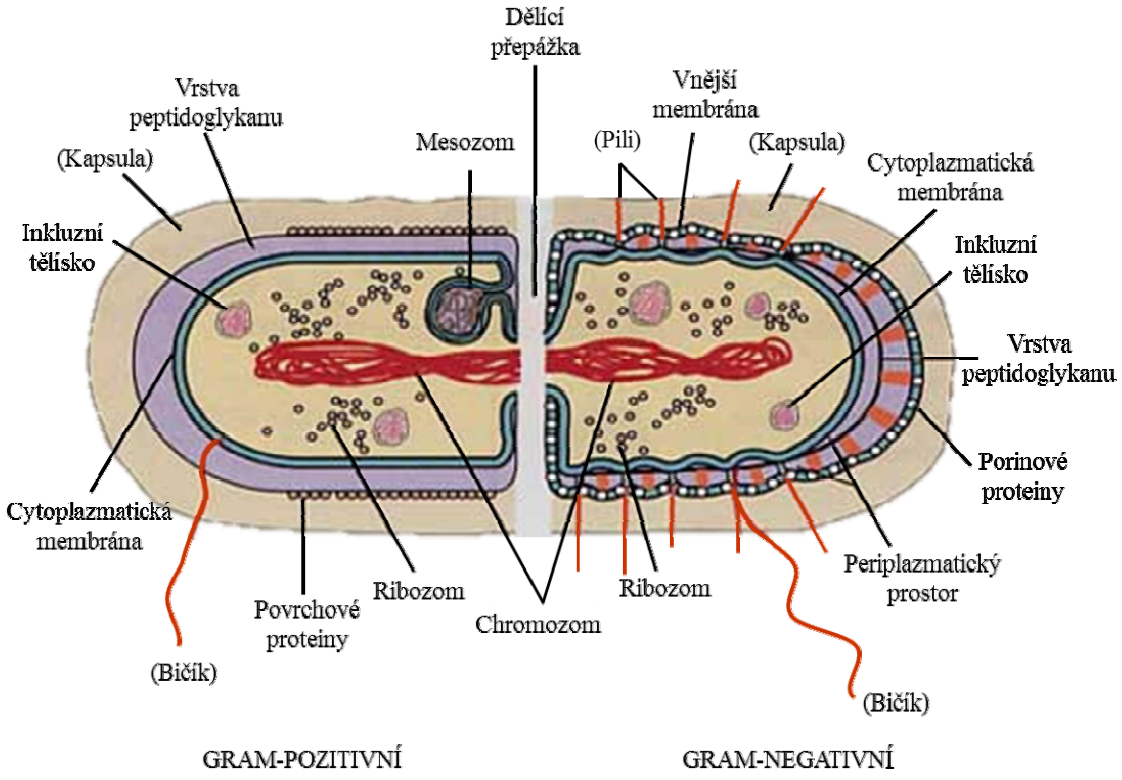
Buněčná stěna G^+ bakterií je složena z vysokého obsahu peptidoglykanu a zároveň postrádá vnější membránu a vrstvu lipopolysacharidů. Tato skutečnost pak ovlivňuje výsledek diagnostického barvení dle Grama, kdy G^+ bakterie pod mikroskopem můžeme pozorovat modrofialové [3-5].

G^- bakterie oproti tomu disponují buněčnou stěnou tvořenou převážně lipopolysacharidy a základní buněčnou membránu překrývá druhá membrána. Struktura buněčné stěny pak po Gramově barvení způsobuje zbarvení růžové [5]. Lipopolysacharidy jsou pravděpodobným důvodem vyšší patogenity kmenů G^- bakterií v porovnání s G^+. Rozdílný je i počet aminokyselin, G^- bakterie obsahují až 17 aminokyselin, a to včetně aromatických, zatímco G^+ pouze 10 či méně [3, 4] (Obr. 1).

Některé G^- bakterie tvoří pouzdra nebo pochvy, G^+ bakterie termorezistentní endospóry. Hlavní funkcí pouzder je zpevňování povrchu bakterií a snazší přichycení k substrátu díky jejich polysacharidové nebo proteinové skladbě. Tato vrstva odděluje buňku od vnějšího prostředí a u různých druhů bakterií se liší právě svým chemickým složením (polysacharidové složení vyskytující se například u streptokoků, polypeptidové u aerobních bakterií) [3, 6].

Endosporami obecně jsou myšleny spóry vznikající uvnitř buněk. Termorezistentní endospóra vznikne v buňce vždy jen jedna. Hlavními znaky endospór je jejich vznik pomocí vegetativního rozmnožování a schopnost přetrvávat nepříznivé podmínky prostředí. Nejprve se buňka zduří, postupem času dojde k jejímu rozpuštění a uvolnění samotné spóry. Sporulace je geneticky řízený proces, kdy exprese genů bývá vyvolána různými faktory prostředí. Vzniklá spóra má stejnou genetickou informaci jako předchozí buňka, její morfologické a fyziologické znaky jsou však zcela odlišné.

Ke vzniku spór dochází nejčastěji na konci exponenciální fáze růstu bakterií a je pro něj nezbytná přítomnost kyslíku a dostatečné množství živin [3, 6].



Obr. 1 Morfologie grampozitivních a gramnegativních bakterií. Popisky uvedené v závorce značí nepřítomnost u některých bakterií. Převzato z http://micro.digitalproteus.com/morphology2.php.

3.1.2 Bakteriální infekce v medicíně

Bakteriální infekce způsobované G^+ a G^- bakteriemi představují jednu z nejzávažnějších komplikací při léčbě celé řady onemocnění, jako jsou onemocnění onkologická nebo onemocnění v oblasti cévní chirurgie [7]. Bakteriální infekce a následné komplikace spojené s cévní chirurgií bývají nejčastěji zapříčiněny zvyšující se frekvencí využívání umělých cévních náhrad [8]. Infekce postihují pacienty nejčastěji v závislosti na operované části těla, věku, typu operace nebo odolnosti organismu vůči infekcím [9-13]. Komplikace vedou k oslabení imunitního systému, nutnosti opětovných chirurgických zákroků a v nejzávažnějších případech k amputacím, septickému šoku nebo dokonce smrti pacienta [14-16].

Některé druhy patogenních bakterií, a to zejména methicilin-rezistentní *Staphylococcus aureus* (MRSA), jsou zodpovědné za více než polovinu všech infekcí v oblasti cévní chirurgie [17, 18] a jsou obecně považovány za vůbec nejčastější příčinu všech infekcí [17-20].

Eliminace výskytu infekčních onemocnění na bázi různých technických a technologických opatření probíhá nejčastěji s využitím řady antibakteriálních komponent netoxických pro lidský organismus. Použití antibiotik v klinické praxi naráží na celou řadu komplikací, mezi které lze zařadit například vznik odolnosti bakterií vůči jejich působení a taktéž jejich možná toxicita [21, 22], pro necílené tkáně a orgány jsou často velmi toxické [23].

3.1.3 *Staphylococcus aureus* jako nejčastější původce komplikací léčby infekcí

Stafylokoky se v současnosti řadí mezi jedny z nejdůležitějších bakteriálních patogenů. V případě nozokomiálních infekcí (infekce vzniklé v souvislosti s pobytem osob ve zdravotnickém zařízení) tvoří spolu s enterokoky etiologické agens (patogeny) více než polovinu případů. Jako nejčastější infekční patogen bývá uváděna G^+ bakterie *Staphylococcus aureus* (*S. aureus*) [19, 20]. Je spojován s infekcemi kůže, měkkých tkání, infekčními ranami a dalšími vážnými následky onemocnění jako jsou sepse, infekce močových cest, osteomyelitida nebo endokarditida [24-27]. Navíc je tento mikroorganismus hrozbou díky své rezistenci [17, 18].

Běžně využívaná methicilinová antibiotika způsobují tvorbu MRSA se zvýšenou schopností adheze. Tento kmen je obecně odolný proti β-laktamovým antibiotikům. Všechny MRSA nesou získaný genetický faktor (*mecA* nebo *mecC*), který kóduje nízkou afinitu penicilin vázajícího proteinu (PBP2A nebo PBP2A´), která může vést ke katalýze transpeptidace peptidoglykanu v přítomnosti vysokých koncentrací β-laktamových antibiotik [28].

Vysokomolekulární penicilin vázající proteiny bakterií katalyzují v oddělených oblastech činnosti transglykosyláz a transpeptidáz nezbytných pro biosyntézu peptidoglykanového polymeru, který obsahuje bakteriální buněčná stěna. *MecA* je přítomen na stafylokokové chromozomální kazetě mec (SCCmec). SCCmec je genomový ostrov, který soustřeďuje geny β-laktamové rezistence vůči antibiotikům a jiné geny rezistence [29].

Výskyt rezistentních kmenů bakterií je výrazným problémem stále více lékařských praxí. Zde se běžně vyskytují CA-MRSA (komunitní methicilin-rezistentní *S. aureus*), které se často objevují mezi skupinami lidí, jako jsou sportovci nebo vojáci a HA-MRSA (nozokomiální (nemocniční) methicilin-rezistentní *S. aureus*), způsobující bakteriální infekce a zvýšenou antibiotickou rezistenci [30].

3.2 Metody identifikace bakteriálních kmenů

Využití moderních technik vede k velmi rychlé identifikaci bakteriálních druhů v neznámém vzorku. Včasná a přesná identifikace bakterií v klinických materiálech je nutnou a nezbytnou fází při léčbě bakteriálních infekcí, navržení vhodných léčebných postupů a eliminaci šíření těchto infekcí.

3.2.1 Selektivní a neselektivní kultivace

K selektivní kultivaci je nejčastěji využíváno čtyř agarových živných půd, které zajišťují specifické podmínky pro růst určitých skupin nebo druhů bakteriálních kmenů.

První selektivní půdou využívanou pro roztěr vzorků je krevní agar s 10% obsahem NaCl. Obsažená sůl slouží k selekci halotolerantních bakterií, mezi které řadíme stafylokoky a cyanobakterie. V klinických vzorcích se cyanobakterie běžně nevyskytují, proto je na krevním agaru s 10% obsahem NaCl nejočekávanější nárůst stafylokoků [31, 32].

Druhou živnou půdou je krevní agar bez selekčních komponent. Tato půda slouží k pomnožení veškerých G^+ i G^- přítomných ve vzorku [33, 34].
Třetí agarovou živnou půdou je Endův agar, který svým složením (pepton, laktóza, fosforečnan draselný a siřičitan sodný) umožňuje růst pouze G^- bakterií. U klinických materiálů se do této skupiny řadí koliformní bakterie (*Escherichia*, *Enterobacter*, *Klebsiella*, *Citrobacter*) a bakterie rodů *Pseudomonas*, *Proteus*, *Legionella* [35].
Poslední, čtvrtou selektivní živnou půdou, je krevní agar obsahující antibiotikum amikacin, sloužící k inhibici růstu G^- bakterií a stafylokoků a tím k selekci a nárůstu streptokoků a enterokoků, disponujících rezistencí vůči amikacinu [36].
Petriho misky s roztěry klinických vzorků obsahujících bakteriální kultury jsou inkubovány za vhodných podmínek. Inkubace krevního agaru s NaCl a Endova agaru probíhá za aerobních podmínek při 37 °C po dobu 48 hodin, pro inkubaci krevního agaru bez selekčních komponent je nutné dodání zvýšené tenze CO_2, pro kultivaci krevního agaru s amikacinem je pak nutné zajištění mikroaerofilního prostředí [37].

Neselektivní kultivace je dosaženo využitím obecných kultivačních médií, jejichž složení je nejčastěji tvořeno obecnými živinami nezbytnými pro růst mikroflóry (monosacharidy, proteiny, živočišné komponenty) [38, 39]. Jejich složení lze dále modifikovat mnoha způsoby, jako je například modifikace aminokyselinami, uhlíkem, grafenem, magnetickými částicemi nebo látkami indikujícími biochemické změny [40-43].

3.2.2 Biochemické a imunologické konfirmační testy

Morfologie bakterií a jejich růst na selekčních kultivačních agarech zdaleka nestačí pro přesnou identifikaci bakteriálních kmenů. Pro bližší taxonomické zařazení bakterií běžně slouží tzv. konfirmační (potvrzující) testy [44, 45] na bázi biochemických a imunologických metod [46].
Nejčastěji používanými konfirmačními technikami jsou biochemické testy. Největší výhodou těchto testů je jejich snadná použitelnost a stálost biochemických vlastností bakterií [47]. Mezi základní sledované biochemické parametry pro přiřazení bakterií k jednotlivým rodům a druhům patří průkaz katalázy, oxidázy, plazmakoagulázy, laktózy, zkvašování cukrů, ureázy, dekarboxylázy a lyzinu, popřípadě tvorba indolu,

plynu, sirovodíku nebo schopnost redukce dusičnanů na dusitany [5, 48]. Pomocí biochemických testů je zjišťována například schopnost fermentace různých cukrů, schopnost využití substrátů jako zdroje uhlíku, hydrolýza substrátů, aktivita enzymů, citlivost ke žluči a deoxycholátu, produkce specifických metabolitů, hemolýza, apod.

Příkladem konfirmačních testů je např. kvasná zkouška u koliformních bakterií (její obdobou je teplotní test u termotolerantních koliformních bakterií). K biochemickým testům se řadí cytochromoxidázový test u koliformních bakterií [49], stanovení aktivity β-D-glukuronidázy, zkvašování cukrů [50], IMViC test (test tzv. pestré řady, kdy se zjišťuje reakce na indol) [51], methylenová červeň Voges-Proskauerova reakce, reakce na citrát (díky výsledkům dochází k identifikaci *Escherichia coli*, *Citrobacter* sp. nebo *Aerobacter aerogenes*).

Kromě běžných fyziologických vlastností, jako je pohyblivost nebo vztah k O_2, jsou biochemické vlastnosti jednotlivých druhů bakterií dále posuzovány na základě skupiny produkovaných enzymů [48]. Tyto enzymy jsou pro bakterie základem ke štěpení živin nebo mohou být využívány k syntéze stavebních součástí bakterií. Aktivita enzymů bývá prokazována interakcí bakteriálních enzymů se stanovovanými reakčními látkami a je vizualizována změnou barvy roztoku obsahujícího látky rozložitelné příslušnými enzymy typickými pro danou bakterii [52]. Jedná se o metody přímého stanovení pomocí fluorogenních substrátů, které jsou založeny na enzymatických reakcích a přítomnosti specifických enzymů. Základem testů je schopnost bakteriálních druhů štěpit specifickým enzymem substrát.

Důkazy imunologickými metodami jsou založeny na identifikaci bakteriálních antigenů pomocí specifických protilátek [53]. Tato stanovení se provádí pomocí metod jako je latexová aglutinace, enzymatická imunoanalýza (ELISA = enzyme linked immunosorbent assay) a přímá či nepřímá imunofluorescence (IFA = immunofluorescence assay) [54]. Přednostmi těchto metod je rychlost, jednoduchost a citlivost.

V době stále častější miniaturizace technik a laboratorních procesů se v oboru mikrobiologie začínají využívat sady tzv. mikrotestů (diagnostických souprav), které usnadňují provedení jednotlivých analýz. Příkladem jsou testy API-systém s několika řadami mikrozkumavek s testy v jedné vylisované destičce [55]; ENTEROTUBE-systém představovaný speciální plastovou trubicí rozdělenou přepážkami, kdy je

středem trubice unášen vzorek pomocí očkovací jehly [56]; mikro-LA-test obsahující diagnostická média umístěná v jamkách mikrotitračních destiček, kterým můžeme identifikovat až 8-12 kmenů (ENTEROtest pro identifikaci střevních bakterií [57], NEFERMtest pro identifikaci nefermentujících tyčinek [58], STAPHYtest pro identifikaci stafylokoků [59] nebo STREPTOtest pro streptokoky [60].

3.2.3 Průkaz přítomnosti specifických genů

Bakterie a jejich genetická informace je odlišná pořadím nukleotidů, které dohromady tvoří DNA. Pořadí nukleotidů zároveň kóduje specifické vlastnosti jednotlivých bakterií. Bakterie mají na rozdíl od eukaryot jen jeden chromozom (jsou haploidní), který je kruhový, množí se pouze asexuálně dělením a bakterie jsou proto náchylné ke genetické nestabilitě (snadno získávají nebo ztrácejí různé vlastnosti, jakoukoli změnu předávají na potomstvo). Amplifikace dílčích genů ve vzorku pomocí specifických primerů a jejich pořadí vede k identifikaci bakteriálních kmenů ve vzorku.

Přítomnost specifických genů bakterií je určována metodou polymerázové řetězové reakce (PCR) [61], kdy dochází k namnožení genů pomocí primerů (oligonukleotidů komplementárních ke stanovovanému genu). Úspěšné provedení PCR reakce a namnožení produktu je zobrazováno pomocí gelové elektroforézy, při které dochází k separaci makromolekul DNA. Principem gelové elektroforézy je pohyb fragmentů makromolekul gelem s různými velikostmi pórů v závislosti na velikosti, uspořádání a náboji fragmentu [62].

3.2.4 Hmotnostní spektrometrie

Vědecký pokrok a snaha o zdokonalování metod pro identifikaci bakterií vedly k objevu účinnějších metod pro tyto účely, mezi které lze zařadit i hmotnostní spektrometrii s matricí asistovanou laserovou desorpcí/ionizací a analyzátorem doby letu (MALDI-TOF MS). MALDI-TOF MS je technika schopná identifikace bakteriálních kmenů již do jedné hodiny od jejich kultivace a mohla by se tedy v budoucnu stát referenční metodou v této oblasti.

Hmotnostní spektrometrie s laserovou desorpcí/ionizací za účasti matrice ve spojení s průletovým hmotnostním analyzátorem je univerzální a rychlou metodou pro analýzu

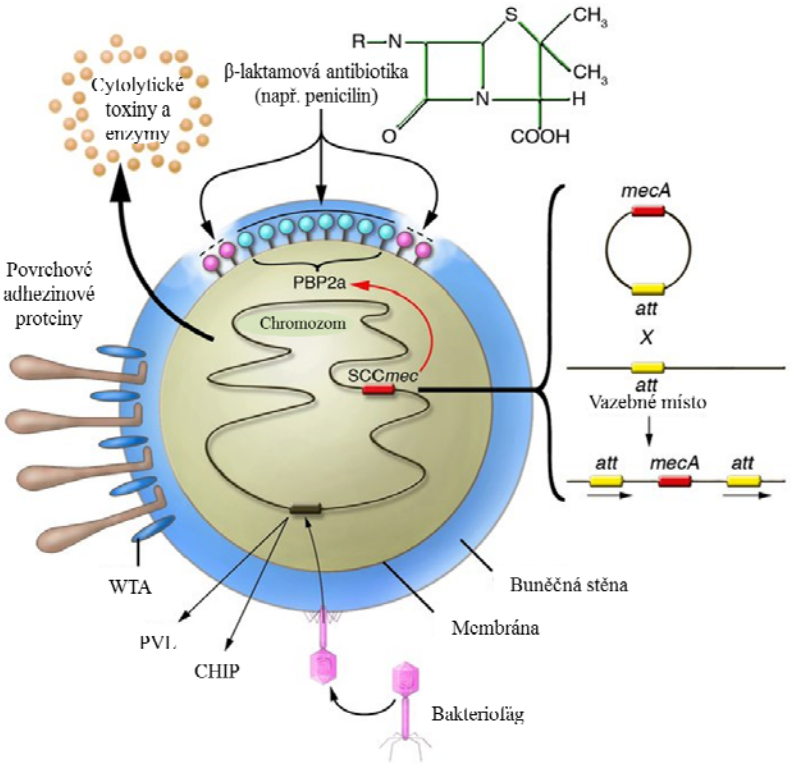
biomolekul i pro identifikaci mikroorganismů [63, 64]. Vlastní identifikace spočívá buď v přímém nanesení bakteriální kolonie na MALDI destičku, nebo je bakteriální kultura extrahována kyselinou mravenčí a acetonitrilem, kdy je následně analyzován 1 μl této suspenze. Vzorky jsou následně na MALDI destičce smíchány s matricí, nejčastěji kyselinou hydroxyskořicovou [65] a jsou změřena hmotnostní spektra. Výsledné záznamy jsou porovnávány s databází hmotnostních spekter známých bakterií. Na základě shodnosti lze s velkou pravděpodobností vzorek identifikovat.

V případě neshody hmotnostního spektra vzorku s databází, je nutné vzorek s bakteriálním kmenem identifikovat klasickými metodami. Po úspěšné základní identifikaci lze naměřená hmotnostní spektra bakterií nahrát do databáze a tím ji aktualizovat pro další a přesnější analýzy.

3.3 Biochemická podstata rezistence bakterií a virulence

3.3.1 Odolnost bakteriálních kmenů vůči vlivům vnějšího prostředí

Rezistence bakteriálních kmenů je závažným celosvětovým problém v mnoha oblastech, zejména v oblasti zdravotnictví a nemocniční péče [66]. Týká se téměř všech hlavních bakteriálních patogenů, které se velmi snadno šíří pomocí kapénkové infekce [67]. V dnešní době se napříč odbornou zdravotnickou veřejností diskutuje o antibiotické rezistenci jako o zásadním problému, který je nutno řešit. Nejprobádanějším bakteriálním kmenem z hlediska rezistence vůči antibiotikům je bezpochyby methicilin-rezistentní *Staphylococcus aureus* a mechanismus vzniku rezistence vůči β-laktamovým antibiotikům (Obr. 2).



Obr. 2 Schéma znázorňující vznik rezistence k methicilinu u bakterie *S. aureus* a jeho schopnost exprimovat různé faktory virulence. Bakterie exprimuje povrchové proteiny adheziny a vylučuje mnoho toxinů a enzymů aktivací chromozomálních genů. Rezistence k methicilinu je získána vložením horizontálně převáděného DNA elementu zvaného SCCmec. Pět různých SCCmec elementů může integrovat ve stejném místě na chromozomu pomocí Campbell mechanismu zahrnujícího místně specifické rekombinace. *MecA* gen kóduje nové penicilin vázající protein citlivý k β-laktamům (PBP2A), který pokračuje v syntéze nové buněčné stěny peptidoglykanu dokonce i tehdy, kdy normální penicilin vázající proteiny jsou inhibovány. Některé faktory virulence, jako Panton-Valentine leukocidin (PVL) a inhibiční bílkoviny chemotaxe (CHIP) jsou kódovány geny umístěnými na lyzogenních bakteriofázích. Převzato z [2].

V případě iontů kovů výzkum vychází ze základních poznatků, kterých již bylo dosaženo, jako princip vstupu kovu do buňky a mechanismus, kterým buňka kov eliminuje [68]. Dalším cenným zdrojem informací jsou poznatky z oboru ekologie, kde bylo vynaloženo velkých prostředků na výzkum bakteriálních kmenů v oblastech znečištěných těžbou kovů apod. Antibiotická i kovová rezistence jsou obě geneticky podmíněné, jejich informace je uložena v některé z buněčných částí. Geny uložené v chromosomech, plasmidech nebo transpozomech kódují rezistenci vůči specifickému kovu [69].

Většina kovů není esenciální a jsou pro bakterie potenciálně toxické. Při vysoké koncentraci jsou i však esenciální kovy pro buňku toxické a mohou narušovat cytoplazmatickou membránu [70].

V životním prostředí jsou vytvářeny stresové podmínky pro bakteriální kmeny přítomností kovů v půdě. Tento proces vede k postupnému vývoji rezistence k téměř všem toxickým kovům [71]. Bakteriální rezistence vůči kovům je heterogenní jak v biochemické, tak genetické podstatě. Od 70. let minulého století bylo identifikováno několik mikroorganismů rezistentních vůči kovům. Tyto výsledky se týkaly především aerobních bakterií *Staphylococcus* sp., *Escherichia coli*, *Pseudomonas aeruginosa* a *Bacillus* sp. [72-76]. Systém kovové rezistence se pravděpodobně vyvinul krátce po vzniku prokaryotického života a v současnosti se nachází téměř u všech bakteriálních druhů [77]. Kovová rezistence je v mnoha případech spojena s rezistencí antibiotickou [72, 78-80]. Tyto dvě rezistence jsou distribuovány mezi jinými populacemi konjugací nebo transdukcí [74, 81].

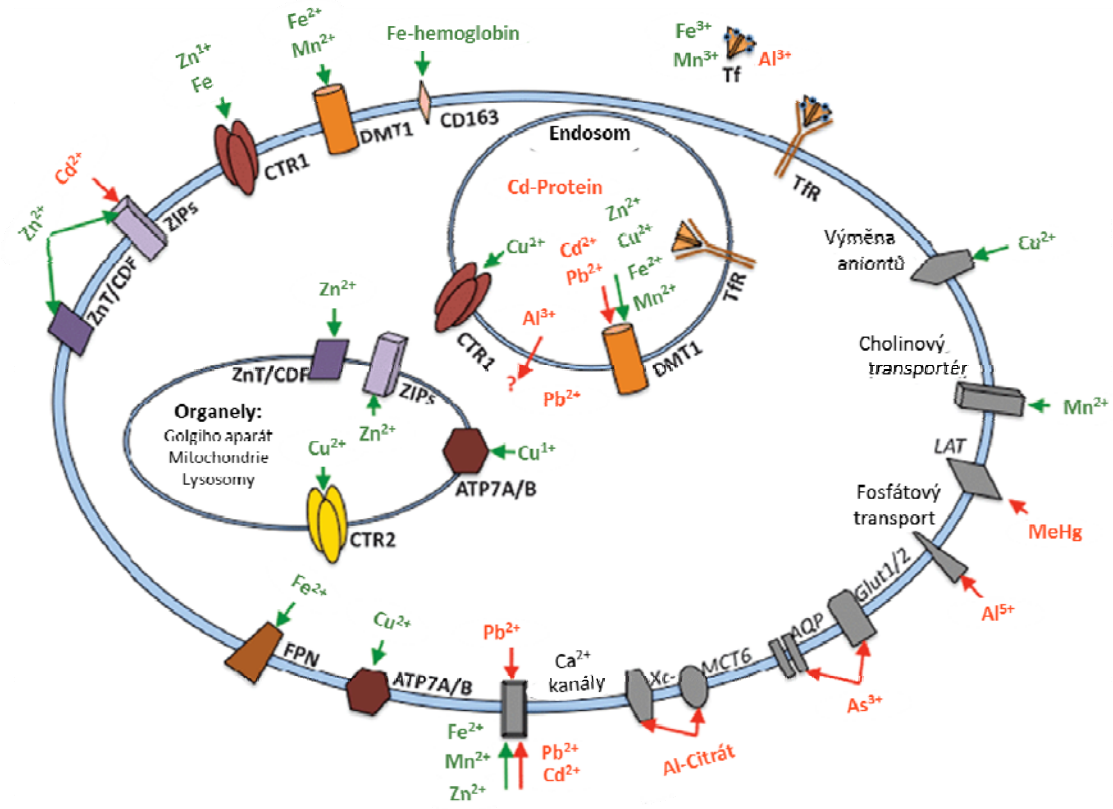
3.3.2 Mechanismy vzniku rezistence

3.3.2.1 Mechanismus toxicity kovu a vstup kovu do buňky

Mechanismus toxicity kovů v buňce je dán interakcí konkrétního kovu se specifickým biologickým druhem [71]. Kovy vstupují do buňky dvěma způsoby [82]. Prvním způsobem, skrze nespecifické transportéry, se do buňky dostávají nejen esenciální, ale i těžké kovy [83]. Tato cesta umožňuje rychlý transport kovů do buňky přes cytoplazmatickou membránu, je řízena chemoosmotickým gradientem buňky [84]. K transportu kovu do buňky však dochází i ve stavu přebytku kovu v buňce. Pro tuto vlastnost se systém nazývá „open gate“ a vysvětluje také příčinu toxicity těžkých kovů.

Druhý způsob, specifický transport iontů, který je oproti prvně jmenovanému pomalejší, spotřebovává energii a probíhá pouze v situaci potřeby, například při nedostatku výživových látek [85]. Hlavním mechanismem toxicity je lipidová peroxidace, která zahrnuje přímou reakci kyslíku a polynenasycenými mastnými kyselinami, částmi lipidové membrány, která vede k tvorbě radikálových meziproduktů. Tyto radikály snadno reagují s částmi buňky a dochází k poškození těchto částí. Železo nebo měď mohou fungovat jako katalyzátory reakcí, při kterých dochází k tvorbě radikálů. Ve výsledku dochází k tvorbě toxických hydroxylových radikálů. Tyto radikály jsou vysoce reaktivní a interagují s jakýmikoliv biologickými molekulami v bezprostředním okolí. Kovové ionty snižují nebo zvyšují enzymatickou aktivitu nebo mění enzymovou specifitu pomocí indukce změn konformace enzymu.

Některé kovy, esenciální, jsou důležité pro výživu buňky [77]. Působení kovu na buňku tedy není pouze negativní, esenciální kovy (např. kobalt, měď a nikl) fungují ve stopovém množství, jako redoxní činitelé k stabilizaci molekul při elektrostatické interakci jako součást enzymů pro regulaci osmotického tlaku.



Obr. 3 Buněčný transport a homeostáza esenciálních a neesenciálních kovů. Na schématickém znázornění transportu kovů do intracelulárního prostoru jsou červeně označeny transportní mechanismy specifické pro neesenciální kovy (Al, As, Hg, Pb, Cd) a zeleně jsou znázorněny možnosti transportu esenciálních kovů (Fe, Mn, Zn, Cu). Převzato z [1].

3.3.2.2 Obecné mechanismy rezistence

Mezi rezistencí zprostředkovanou chromosomy a plasmidy jsou značné rozdíly. Gen rezistence na chromosomu obvykle zodpovídá za regulaci nadbytku esenciálních kovů a mechanismus je složitější než u plasmidu. Gen rezistence obsažený v plasmidu pak odpovídá za ochranu před ionty neesenciálních toxických kovů, jedná se o efluxní mechanismus. Efluxní mechanismus přenášený plasmidem zajišťuje snadnou mobilitu v buňce a přenos mezi jednotlivými jedinci, zároveň pro buňku neznamenají zátěž v podobě dalších genů a jsou použity pouze v případech potřeby [86]. Mechanismy rezistence neseny pomocí DNA plasmidu jsou nejběžnější, velmi specifické a byly nalezeny u téměř všech studovaných skupin eubakterií [87, 88]. Rezistence vůči kovu a zároveň antibiotiku může být zprostředkována jedním plasmidem [72].

Rezistence je zprostředkována jedním nebo více geny kódovanými pomocí chromosomů, plasmidů nebo transposomů. Buňka zajišťuje rezistenci vůči kovům zabráněním nebo omezením přístupu kovu k citlivým částem buňky nebo snížením citlivosti daných částí buňky. Je stanoveno 6 obecných biochemických mechanizmů, které se podílejí na výsledné rezistenci bakterií [71, 86]:

- vyloučení kovu pomocí propustné bariéry,
- vyloučení kovu pomocí aktivního transportu z buňky,
- vnitrobuněčné fyzické odloučení kovu pomocí obalení kovu proteinem, vedoucí k zabránění narušení části buňky, které jsou vůči kovům citlivé,
- mimobuněčná sekvestrace,
- průběh chemické reakce, v níž se toxická aktivita kovu sníží,
- snížení citlivosti cílových orgánů buňky, které jsou náchylné vůči působení kovů.

Chemická reakce vedoucí ke snížení toxicity dané buňky probíhá zvýšením aktivity alternativní metabolické cesty, která zajišťuje relativní rezistenci vůči kovům. Různé mechanismy rezistence mohou působit samostatně nebo mohou participovat na rezistenci vůči různým kovům v jednom druhu [89]. Specifická obrana těchto citlivých buněk může být dosažena čtyřmi způsoby:

- mutací citlivých částí buňky vedoucí k snížení senzitivity bez výrazného ovlivnění jejich běžné funkce,
- navýšením množství struktur náchylných na kov, buňka tak vytvoří prostor, kde toxická aktivita působí, ale nezničí prostor celý,
- opravou buněčných komponent, v praxi možné pouze pro DNA [90],
- upravením metabolické cesty za využití plasmidu obsahujícího genetickou informaci pro rezistenci, tak aby toxická aktivita nepůsobila na chromosomální složku, jež odpovídá za slabost buňky [91]. Tento mechanismus je analogický antibiotické rezistenci proti trimetoprimu [81].

3.3.3 Faktory virulence

Virulence je jednou ze základních vlastností bakterií určujících úroveň patogenity jednotlivých kmenů, tedy schopnosti individuálních kmenů bakterií poškozovat hostitelskou buňku [92, 93]. Faktory virulence bakterií jsou nejen schopné zapříčinit vznik bakteriální infekce, ale zároveň disponují schopností odolávat imunitní odpovědi hostitelských buněk. Virulence však není pro životaschopnost bakteriálních kmenů sama o sobě nutná [93, 94].

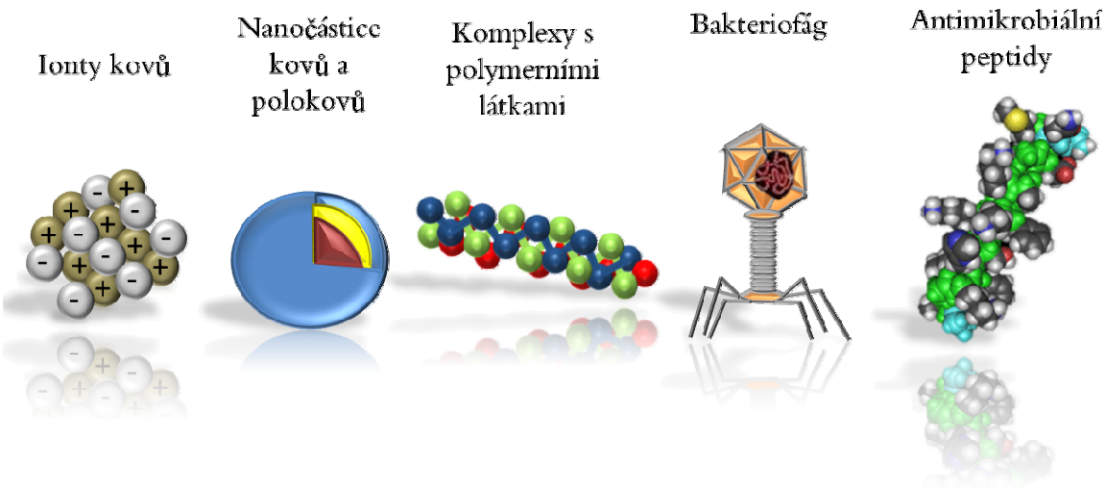
Virulence může být zesilována nebo naopak snižována. K zesílení dochází opakovaným přenosem kmene bakterií na stejném druhu hostitele. Snižování (tzv. atenuace) vede ke tvorbě kmenů se sníženou virulencí, někdy i úplně vymizelou virulencí (tzv. avirulencí), kterých bývá využito při přípravě očkovacích látek [95].

Faktorů virulence existuje hned několik. Základními faktory je množství přítomných adhezinů, invazinů, impedinů, modulinů a agresinů, toxinů, enzymů nebo bičíky a chemotaxe [92].

Základním předpokladem vývoje virulence je její pokles s dobou koexistence bakteriálního kmene a hostitelského organismu. Na základě empirických dat je však tato teorie spíše vyvrácena [96].

3.4 Alternativní cesty léčby bakteriálních infekcí

Alternativy konvenčních antibiotických léčiv se v posledních letech dostaly do popředí zájmu ve vědě a výzkumu [97]. Mezi alternativy využívající antibakteriální efekt k prevenci a eliminaci bakteriálních infekcí můžeme zařadit široké spektrum antibakteriálních činitelů, jako jsou v této oblasti asi nejlépe probádané kovy ve formě iontů nebo nanočástic, ale i jejich kombinace s polymerními látkami, dále antimikrobiální peptidy nebo přirození bakteriální parazité, tzv. bakteriofágové (Obr. 4).



Obr. 4 Možné alternativy antibiotických léčiv při léčbě bakteriálních infekcí. 3D struktura antimikrobiálního peptidu magaininu převzata z http://scienceblogs.com/.

3.4.1 Ionty kovů s prokázanými antibakteriálními účinky

Kovy z pohledu jejich antimikrobiálních vlastností jsou známé již z dávné minulosti [98]. Nejčastěji testovaným kovem je bezpochyby stříbro. Tento kov byl pro své vlastnosti využíván již 4000 let př. n. l. k potlačení bakteriálních infekcí a plísní [99]. Zároveň je však stříbro známé i silnou toxicitou k mikroorganismům [100, 101]. Antibakteriální efekt stříbra je zabezpečen několika mechanismy působení na buňku [102, 103] a je podmíněn jeho biologickou dostupností, ve formě iontů (dusičnan stříbrný, thiosíran stříbrný), nanočástic stříbra nebo stříbra oxidujícího v médiích vytvářejících nanočástice [104-106].

Stříbrné ionty způsobují inhibici buněčného dělení, interagují s nukleovými kyselinami a thiolovými skupinami aminokyselin a proteinů [107]. Z těchto důvodů jsou stříbrné

ionty využívány pro kontrolu bakteriálního růstu v řadě lékařských aplikací i mimo ně [108-111].

Mechanismus působení stříbrných iontů je založen na inaktivaci membránových proteinů, kdy je narušen systém transportu elektronů a na inhibici dýchacích enzymů pro podporu tvorby reaktivních forem kyslíku (ROS = reactive oxygen species). Obecně platí, že zvýšená koncentrace stříbrných iontů vede ke zvýšení oxidačního stresu, který je jedním z ukazatelů, který umožňuje sledování toxických účinků těžkých kovů na mikroorganismy. Tento účinek je založen na vazbě iontů stříbra do bakteriální buněčné stěny a plazmatické membrány, což vede k inhibici dýchacího procesu bakterií [110, 112, 113].

Bakterie mohou obrannými mechanismy účinně eliminovat vytvořené ROS, a tím i eliminovat toxické účinky iontů stříbra [114, 115]. Účinek ROS umožňuje sledování oxidačního stresu na základě stanovení antioxidační kapacity s využitím spektrofotometrických metod, jako je ABTS (2,2´-azino-bis(3-ethylbenzotiazolin-6-sulfonová) kyselina), DPPH (2,2-difenyl-1-pikrylhydrazyl), FRAP (antioxidační potenciál redukce železa) [116, 117] nebo elektrochemických metod za použití cyklické voltametrie [118-121].

3.4.2 Kovy a polokovy ve formě nanočástic

Nanotechnologie jsou jedním z nejpokrokovějších vědních oborů současnosti. Cílem je tvorba nanočástic, které budou v souladu s požadavky na velikost, tvar, strukturu a distribuci v organismu. Kovové nanočástice o velikosti nejčastěji v řádech 1-200 nm mohou být modifikovány za použití vrstev různých sloučenin, jako jsou biopolymery [122]. Nanočástice připravené tímto způsobem ukazují modifikované fyzikální a chemické vlastnosti ve srovnání s nanočásticemi pouze na bázi kovů samotných [123, 124].

Díky specifickým vlastnostem se nanočástice používají jako katalyzátory a důležité součásti senzorů na bázi optických nebo elektrochemických metod. Našly také široké využití v oblasti biomedicínských aplikací, kde je vyžadována bakteriální sterilita [125]. Vysoká katalytická schopnost díky velkému povrchu nanočástic a schopnosti vytvářet reaktivní formy kyslíku [126] způsobuje vysokou reaktivitu nanočástic, které se tak stávají více toxické pro bakterie [127].

Využitím moderních postupů lze nanočástice kovů funkcionalizovat, a tím zvyšovat jejich antimikrobiální aktivitu [98]. Díky svým vlastnostem vykazují nanočástice v organismu vyšší retenci, čímž lze snáze docílit požadovaného distribučního účinku [128].

Zejména při transplantacích dochází ke zvýšenému riziku vzniku bakteriálních infekcí. Jedním z potenciálních řešení, díky kterému lze snížit počet infekcí při operacích, se stala možnost aplikace stříbrných nanočástic [129, 130]. Nanočástice stříbra jsou běžně používány v textilním průmyslu a lékařství [131]. Až do nedávné doby byly nanočástice stříbra běžně používány v implantovaných materiálech, které snižovaly výskyt pooperačních komplikací, nicméně postupem času byly tyto aplikace často spojovány s potenciálním rizikem vzhledem k jejich toxicitě [132].

Ionty stříbra ovlivňují proces proliferace buněk a stříbrné ionty a nanočástice tak našly mimo jiné uplatnění v dermatologii pro usnadnění hojení ran a v širokém spektru terapeutického využití [133].

Mechanismus účinku stříbrných nanočástic zůstává stále nejasný. Nicméně jsou navrženy možnosti účinku stříbrných nanočástic založené na inhibici enzymů, změně integrity membrány, průniku do bakteriální cytoplazmy a akumulaci v periplazmatickém prostoru nebo na tvorbě reaktivních forem kyslíku. Bylo potvrzeno,

že složení buněčné stěny a plazmatické membrány hraje významnou roli v prostupu nanočástic do buňky. U G^+ bakterií je prostup výrazně pomalejší než je tomu u bakterií G^- [134].

Další, novější možností z pohledu nanotechnologií a bakteriálních infekcí jsou nanočástice selenu. Selenové nanočástice byly zkoumány pro různé medicinské aplikace a jako potenciální materiál pro ortopedické implantáty [135]. Nyní jsou k dispozici také studie, které poukazují právě na schopnost sloučenin selenu inhibovat růst bakterií a tvořit bakteriální biofilmy [135]. U řady sloučenin selenu (jako je například 2,4,6-tri-para-methoxyfenylselenopyrylium chlorid, 9-para-chlorofenyloktahydroselenoxanthen nebo perhydroselenoxanthen) byla prokázána antibakteriální aktivita *in vitro*, zejména proti *S. aureus*. Nicméně účinky elementárních nanočástic selenu zůstávají doposud z velké části neznámé [136, 137].

3.4.3 Tvorba komplexů s polymerními látkami

Aplikace iontů kovů nebo jejich nanočástic v kombinaci s dalšími substancemi s antimikrobiálními vlastnostmi je další cestou, jak zvýšit antimikrobiální aktivitu [138, 139]. Při minimalizaci nebezpečí vzniku bakteriálních infekcí byla potvrzena jako velmi efektivní právě kombinace nanočástic s určitými polymerními látkami [140, 141]. Komplexy polymerních materiálů s nanočásticemi stříbra jsou široce využívány jako inhibitory růstu G^+ a G^- bakterií [142] a jako látky využívané pro aplikaci při regeneraci poúrazových a pooperačních tkání [143, 144].

První z nejčastěji využívaných polymerních látek je chitosan, disponující řadou užitečných vlastností [141]. Chitosan je lineární polysacharid, složený z 2-amino-2-deoxy-D-glukopyranózových a 2-acetamido-2-deoxy-D-glukopyranózových jednotek [145]. Syntetizuje se buď v těle živočichů nebo průmyslově částečnou deacetylací chitinu, což je hlavní složka schránek mušlí a korýšů [146]. Jedná se o nejrozšířenější biopolymerní látku vůbec [147]. Díky netoxickým vlastnostem, biokompatibilitě a biodegradabilitě je využíván v oblasti cílené aplikace léčiv v lidském těle [148]. Chitosan zároveň vyniká svými antimikrobiálními vlastnostmi. Z tohoto důvodu je běžně využíván jako látka pro zajištění sterility umělých implantátů [149, 150]. Polymerní strukturou chitosanu je dosaženo snadné tvorby komplexů s kovy [150-152].

Druhou, neméně významnou polymerní látkou využívanou při tvorbě komplexů s kovy je kyselina hyaluronová. Kyselina hyaluronová (HA), nebo její sůl hyaluronan, je

lineárním polysacharidem z disacharidových jednotek kyseliny D-glukuronové a N-acetylglukosaminu [153]. Je přirozeně přítomna u savců, v extracelulárním matrixu, svalových a nervových tkáních, očním sklivci a kůži [154]. Je kompatibilní s lidským organismem, biodegradabilní a podílí se na tvorbě biopolymerů [155-158]. K její syntéze dochází v plazmatické membráně fibroblastů [159]. Kyselina hyaluronová v intracelulárním matrixu váže vodu na proteiny, podílí se na výstavbě extracelulární matrice, a také hraje důležitou roli při organizaci komplexů proteoglykanů v měkkých pojivových tkáních [160, 161]. Největší výskyt tohoto polymeru můžeme pozorovat v lidském organismu v oblastech s proliferujícími buňkami. Vzhledem ke svému uspořádání je kyselina hyaluronová využívána v oční chirurgii, tkáňovém inženýrství a kosmetice, kde slouží k urychlení regenerace tkání [162-166].

Interakce biopolymerních látek s kovy jsou založeny na vazbě kovových iontů s aminoskupinami chitosanu pomocí komplexotvorných mechanismů [167]. Podobné mechanismy byly popsány i v souvislosti s vazbou iontů kovů nebo nanočástic s kyselinou hyaluronovou [168].

3.4.4 Antimikrobiální peptidy

Antimikrobiální peptidy (AMP) se nacházejí v široké škále organismů, jako jsou rostliny, hmyz a obratlovci, a poskytují obranný mechanismus proti napadajícím mikrobiálním druhům [169, 170]. Tyto peptidy obvykle vykazují selektivitu vůči prokaryotickým patogenním buňkám přes hostitelské buňky [171, 172]. Některé vykazují selektivitu pro houby, rakovinné buňky a parazity [173, 174]. Pravděpodobnost vzniku rezistence na AMP je mnohem nižší než pravděpodobnost tvorby rezistence u tradičních antibiotických léčiv, což jim předurčuje vysoký potenciál pro klinické využití [175].

V současné době existuje již mnoho známých antimikrobiálních peptidů s prokázaným terapeutickým potenciálem. Jejich přesný mechanismus účinku však stále zůstává kontroverzní záležitostí [176].

AMP interagují s cytoplazmatickou membránou, při čemž hraje významnou roli jejich amfipatická struktura. Jednoduché modely penetrace membrány zahrnují tvorbu póru a rozpouštění membrány v detergentu [177, 178]. Tyto procesy vedou ke zhroucení transmembránového potenciálu způsobujícího vylití buněčného obsahu a následně buněčnou smrt. Mechanismus antibakteriálního účinku pravděpodobně zahrnuje

rozpoznání a specifickou interakci s bakteriální buněčnou membránou vyvolávající shlukování lipidů nebo separaci lipidové fáze [179, 180].

Experimenty s několika antimikrobiálními peptidy prokázaly jejich schopnost translokace přes buněčnou stěnu a liposomovou membránu, což je vlastnost, kterou sdílejí spolu s peptidy pronikajícím do buňky (tzv. cell penetrating peptides) [181]. Pochopení procesu translokace a tvorba pórů by mohly mít dalekosáhlé důsledky. Dosavadní výsledky však velký průlom nepřinesly, zejména z vysoké výrobní ceny peptidů a jejich občasné necílové toxicity.

3.4.5 Bakteriofágové a nanotechnologie

Bakteriofág je virus, který je schopen infikovat bakterie. Svými rozměry je výrazně menší než bakterie, které ničí – obvykle mezi 20 a 200 nm. Genetický materiál může tvořit jak DNA, tak RNA, a to jednovláknová i dvouvláknová, kružnicová i lineární, dlouhá 5000 až 500 000 nukleotidů [182].

Bakteriofágové patří mezi nejrozšířenější organismy planety, které můžeme najít na všech místech obývaných jejich hostiteli [183]. Jednotlivé druhy mají své označení (číselné nebo písmenné), např. M13, P70, phi29 aj. Každý druh bakteriofága má širší nebo užší rozmezí druhů hostitelů, množí se a infikuje pouze buňky určitých bakterií. Například bakteriofág phi29 infikuje bakterii *Bacillus subtilis* [184], UZ1 infikuje *Enterobacter aerogenes* [185].

Po řadu let byly užívány jako alternativa k antibiotikům. Dnes je dle [186] vidíme jako možnou léčbu onemocnění polyrezistentními bakteriálními kmeny. Nejčastěji se používají v molekulární biologii a v genetickém inženýrství pro přenos genů [186].

Biologické systémy obecně obsahují vysoce uspořádané makromolekulární struktury [187] s různými funkcemi, které inspirují k využití v nanotechnologiích [188]. V budoucnu by se tak i bakteriofágové mohli uplatnit zejména v těchto oblastech. Výzkumů a studií zabývajících se využitím bakteriofágů v oblasti nanotechnologií je velké množství [187, 189, 190].

Základním předpokladem pro využití bakteriofágů v jakékoliv nanotechnologické aplikaci je jednak studium pochopení mechanismu virové infekce, tedy napadení bakterie bakteriofágem [184], stejně tak pokroky v oblasti strukturálních a funkčních studií zvyšují chápání molekulárního základu biologického pohybu [191].

Vláknití bakteriofágové, dlouhá a tenká vlákna, která jsou vylučována z hostitelských buněk, aniž by je zabíjela, jsou protikladem ke standardnímu pohledu na bakteriofágy jako vraždící stroje [189]. V případě vláknitých fágů mohou hloubkové studie genetiky a struktury a vývoj zobrazovacích technik dopomoci k pokroku ve vývoji nových nanomateriálů a následného využití bakteriofágů ve zdravotnictví [192]. Sloučení zobrazovacích technologií s nanotechnologiemi se pak ukazuje jako slibné pro využití v oblastech medicíny a techniky, jako je lékařská diagnostika a monitorování, molekulární zobrazování, vývoj vakcín a tkáňových náhrad [192]. Viry jsou stále více využívány ve vědě, inženýrství a nanotechnologiích jako nástroje a stavební bloky pro elektroniku, chemii a biomedicínské vědy [193].

Struktura a stavba bakteriofágů z hlediska přenosu DNA do hostitelské bakterie hraje významnou roli. Schránka bakteriofágů chrání během přenosu virovou DNA [194], vláknité fágy obsahují ve vnějším plášti tisíce kopií bílkovin využitelné pro výběr fágových sond specifických pro zkušební antigeny [195]. Peptidy jsou mnohem menší molekuly, proto mohou být lépe využitelné k léčbě rakovinových buněk [196]. Použití vybraných peptidů jako terapeutického činidla by nejen usnadnilo přenos konkrétního genu, ale zároveň by umožnilo dlouhodobé podávání léčiv, zabránilo by se tedy tvorbě protilátek z opakované léčby chronických onemocnění [197].

Při studiu bakteriofágů se významně uplatňují různé typy mikroskopie a spektroskopie. Při zkoumání povrchové struktury a procesu tvorby filmu z nanokrystalů sulfidu zinečnatého modifikovaného bakteriofágem M13 byla využita mikroskopie atomárních sil (AFM = atomic force microscopy) a skenovací (rastrovací) elektronová mikroskopie (SEM = scanning electron microscopy) [198]. Při širším testování Fd bakteriofága (vláknitého viru) se uplatnila tzv. spektroskopie nukleární magnetické rezonance (NMR = nuclear magnetic resonance) [199], přičemž tento bakteriofág je dle [199] virem široce používaným v bio- a nanotechnologiích. Využití biologických objektů (např. virů) jako nanošablon pro výrobu nanostrukturovaných prvků popisuje ve své studii Tsen a kol. [200], kdy například M13 fág a virus tabákové mozaiky se úspěšně využívá jako biologická šablona pro syntézu polovodičů a kovových vláken. M13 fág zde byl studován pomocí Ramanovy spektroskopie [200]. Ramanovy spektroskopie pak opět využil Tsen a kol. [201] pro studium akustické vibrace na bakteriofágovi M13.

Nově vznikající oblastí výzkumu se v posledních letech stává využití RNA inženýrství pro nanotechnologické a lékařské aplikace [202], kdy roste zájem o RNA

nanotechnologie především pro možnost využití RNA nanočástic jako terapeutik [203]. Ve studii [202] popsány různé přístupy k diagnostice, prevenci a léčbě lidských onemocnění se zaměřením na již zmíněné RNA inženýrství. RNA nanotechnologie mohou také pomoci řízenému shlukování nanočástic pro jejich specifický přenos z terapeutických molekul do nádorové buňky [204]. Například DNA bakteriofága phi29 obsahuje šest kopií molekul pRNA, jejichž struktura je flexibilní. A právě RNA monomery, dimery a trimery s proměnnou délkou jsou potenciálními díly pro vznik nanomateriálů [205], proto studie bakteriofága phi29 může sloužit jako stavební kámen pro výzkum a využití nanomateriálů v praxi [203].

Bakteriofágové mohou být velice dobrým prostředkem pro transport léčiv. Bakteriofág potažený polymerem je schopen v sobě zapouzdřit protinádorové léky (např. doxorubicin) a přenášet tak tato léčiva [206]. Studie Suthiwangcharoena a kol. [206] nabízí nový způsob, jak vyrobit transportéry léků.

4 MATERIÁL A METODIKA

4.1 Chemikálie

Všechny chemikálie (v ACS čistotě) byly pořízeny od firmy Sigma-Aldrich (USA), pokud není uvedeno jinak.

4.2 Metody

4.2.1 Kultivace *Staphylococcus aureus*

S. aureus (NCTC 8511) byl získán z České sbírky mikroorganismů, Přírodovědecké fakulty Masarykovy univerzity, Brno, Česká republika. Kmeny byly uchovávány jako suspenze spór ve 20% (*v/v*) glycerolu při teplotě -20 °C. Před jejich použitím byly kmeny rozmrazeny a glycerol byl odstraněn promytím destilovanou vodou. Složení Luria Bertani média pro kultivaci bakteriální kultury bylo následující: trypton 10 g/l, kvasničný extrakt 5 g/l, NaCl 5 g/l, sterilní MiliQ voda. pH kultivačního média bylo před sterilizací upraveno na 7.4. Sterilizace média byla prováděna při teplotě 121 °C po dobu 30 minut ve sterilizátoru (Tuttnauer 2450EL, Izrael). Připravená kultivační média byla naočkována bakteriální kulturou v Erlenmeyerově baňce do celkového objemu 25 ml. Po inokulaci byly bakteriální kultury kultivovány po dobu 24 hodin na třepačce (Biosan OS-10, Litva) při 600 rpm a teplotě 37 °C. Bakteriální kultura kultivovaná za těchto podmínek byla následně ředěna kultivačním médiem na OD600 = 0.1 pro použití v dalších experimentech.

4.2.2 Příprava rezistentních kmenů *Staphylococcus aureus*

Rezistentní kmeny *S. aureus* byly vytvořeny v naší laboratoři. 2mM zásobní roztoky iontů těžkých kovů (Ag^{+}, Cd^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}) byly přidány k nerezistentní bakteriální kultuře *S. aureus* kultivované v Luria Bertani médiu. Nejnižší aplikovaná koncentrace kovových iontů v médiu s bakteriální kulturou byla 50 μM, poté se koncentrace iontů těžkých kovů zvyšovala vždy o 50 μM do maximální možné dávky pro následnou regeneraci *S. aureus*.

4.2.3 Stanovení antibakteriálních vlastností

Za účelem stanovení antimikrobiálního účinku látek (kovů, nanočástic kovů, polokovů) bylo využito měření inhibičních zón na Petriho miskách. Povrch agaru v Petriho misce (stejné složení a příprava jako v případě kultivačního média, pouze s přídavkem 15 g/l bakteriologického agaru) byl pokryt směsí 100 µl bakteriální kultury *S. aureus* rostoucí po dobu 24 hodin se 3 ml Luria Bertani média. Přebytečný objem z Petriho misek byl odsát. Z textilie pro tvorbu umělých cévních náhrad vyrobených Výzkumným ústavem pletařským v Brně byly vystřiženy čtverce o velikosti 1 cm^2. V mikrozkumavkách byla textilie smísena s testovanými komponenty a vždy po dvou čtvercích křížovým způsobem položena na Petriho misku s bakteriální kulturou. Petriho misky byly zaizolovány proti možné vnější kontaminaci a vysychání vzorku a inkubovány v termostatu při 37 °C po dobu 24 hodin. Po inkubaci byly změřeny vzniklé inhibiční zóny a byla provedena fotodokumentace.

4.2.4 Stanovení růstových křivek

K vyhodnocení antimikrobiálního účinku testovaných sloučenin a jejich kombinací bylo využito měření absorbance pomocí přístroje Multiskan EX (Thermo Fisher Scientific, Německo) a následné analýzy ve formě růstových křivek. Totožná kultura jako v předchozím postupu byla naředěna Luria Bertani médiem na spektrofotometru SPECORD 210 (Analytik Jena, Německo) při vlnové délce 600 nm na absorbanci 0,1 AU. V mikrotitrační destičce byla bakteriální kultura smísena s různými koncentracemi testovaných komponent (nejčastěji 0, 10, 25, 50, 125, 250 a 300 µM). Celkový objem mikrotitrační destičky byl vždy 300 µl. Měření bylo prováděno vždy v čase 0, poté v půlhodinových intervalech po dobu 24 hodin při teplotě 37 °C a vlnové délce 600 nm. Dosažené hodnoty byly zobrazeny do grafické podoby růstových křivek pro každou koncentraci testované látky zvlášť.

4.2.5 MALDI-TOF hmotnostní spektrometrie

500 µl vzorku kultury *S. aureus* (OD600 = 0.1) kultivované přes noc bylo centrifugováno při 14000 × g po dobu 2 minut. Následně byl supernatant odstraněn a pelet resuspendován v 300 µl deionizované vody. Poté bylo ke vzorku přidáno 900 µl etanolu. Po centrifugaci při 14000 × g po dobu 2 minut byl opět supernatant odstraněn a

pelet byl sušen na vzduchu. Vysušený pelet byl rozpuštěn v 70% kyselině mravenčí (*v/v*) a 25 µl acetonitrilu a promísen. Vzorky byly centrifugovány opět při 14000 × g po dobu 2 minut a 1 µl čirého supernatantu byl nanesen na MALDI destičku (MTP 384 destička z leštěné oceli) a vysušen na vzduchu při laboratorní teplotě. Každý spot byl následně překryt 1 µl matrice α-kyano-4-hydroxyskořicové kyseliny (HCCA) nasycené organickým rozpouštědlem (50% acetonitril a 2,5% kyselina trifluoroctová, obě *v/v*) a zcela vysušen na vzduchu k provedení laserové desorpce/ionizace laserem za účasti matrice v kombinaci s detektorem doby letu (MALDI-TOF MS, Bruker Daltonics, Brémy, Německo). Spektra byla měřena v rozsahu m/z 2000 Da až 20000 Da a každé spektrum byl výsledek akumulace 1000 laserových pulsů získaných z deseti různých oblastí téhož vzorku. Spektra byla analyzována pomocí softwaru pro analýzu Flex (verze 3.4). Spektra byla vložena do MALDI BioTyper^{TM} 3.1 (Bruker Daltonik GmbH, Brémy, Německo), kde byla následně porovnána s existující databází.

4.2.6 Izolace DNA, polymerázová řetězová reakce fragmentu genu 16S rRNA a polymorfismus délky restrikčních fragmentů

2 ml bakteriální kultury *S. aureus* byly centrifugovány při 5000 × g při 20 °C po dobu 10 minut. K peletům bylo přidáno 400 µl lyzačního roztoku (6 M guanidin hydrochlorid a 0,1 M octan sodný) a byla provedena lýza buněk při 20 °C, 600 rpm po dobu 10 minut. Izolace genomové DNA byla provedena pomocí MagNA Pure Compact (Roche, Německo), Nucleic Acid Isolation Kit I dle protokolu pro DNA bakterie v souladu s pokyny výrobce (Roche, Německo).

Gen 16S rRNA byl amplifikován pomocí PCR. Sekvence primerů byly 5'-GAGTTTGATCCTGGCTCAG-3' a 5'-GGTTACCTTGTTACGACTT-3'. PCR reakční směs obsahovala Taq reakční pufr (New England Biolabs, Ipswich, Velká Británie), dNTP, reverse a forward primery (Sigma Aldrich, St. Louis, MO, USA), Taq DNA polymerázu. Podmínky pro PCR byly počáteční denaturace při 94 ° C po dobu 4 minut, 30 cyklů 94 ° C po dobu 30 s, 52 ° C po dobu 30 s a 72 ° C po dobu 1 minuty a konečné prodloužení po dobu 7 minut. Reakcí vznikl fragment o velikosti 1500 párů bazí.

Analýza délky restrikčních fragmentů (RFLP) byla provedena za použití izolované DNA, HaeIII enzymu, HaeIII pufru a sterilní vody při teplotě 37 °C po dobu 2 hodin s následnou inaktivací enzymu při 80 °C po dobu 20 minut.

Výsledky byly zobrazeny metodou gelové elektroforézy na 2 % agarózovém gelu (TAE pufr a ethidium bromid). Elektroforéza (Bio-Rad, Hecules, Kalifornie, USA) probíhala při 60 V a 6 °C po dobu 160 minut. Výsledné bandy byly vizualizovány pomocí UV transluminátoru při 312 nm (Viber-Lourmant, Marne-la-Vallée Cedex 1, Francie).

5 VÝSLEDKY A DISKUSE

Výsledková část předkládané disertační práce je přiložena ve formě publikací v odborných časopisech a dále doplněna o komentáře autorky. U každé práce je vyznačen i podíl autorky na vytvoření publikace, se kterým souhlasí všichni uvedení spoluautoři.

5.1 Sledování biochemických a molekulárně biologických vlastností bakterií jako odpovědi na působení iontů kovů

5.1.1 Vědecký článek I

CHUDOBOVA, D.; DOSTALOVA, S.; RUTTKAY-NEDECKY B.; GURAN, R.; MERLOS RODRIGO, M. A.; TMEJOVA, K.; KRIZKOVA, S.; ZITKA, O.; ADAM, V.; KIZEK, R. The effect of metal ions on *Staphylococcus aureus* revealed by biochemical and mass spectrometric analyses. *Microbiological Research*, in press.
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Problematika vznikající rezistence je stále častějším tématem výzkumných skupin a jejich studií po celém světě. Tato problematika je velmi dobře prozkoumána na úrovni rezistence vůči antibiotickým léčivům [207-212]. V souvislosti s účinkem iontů kovů a vznikající rezistencí bakteriálních kmenů k iontům kovů nejsou mechanismy účinku v buňce v důsledku použití těžkých kovů stále dostatečně a jednoznačně pochopené a popsané.

Cílem práce bylo studium G^+ bakterií *Staphylococcus aureus* rezistentních vůči dlouhodobému působení těžkých kovů na několika úrovních. Tato otázka byla zkoumána zejména prostřednictvím změn vybraných vlastností bakteriálních kmenů, které byly vystaveny působení iontů těžkých kovů (Ag^+, Cd^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}). Vlastnosti byly pozorovány na buněčné a molekulární úrovni. MALDI-TOF hmotnostní spektrometrie byla použita pro kvalitativní analýzu vzorků k identifikaci nerezistentních a rezistentních kmenů *S. aureus*, které byly vystaveny působení již zmíněných pěti těžkých kovů ve formě iontů. Mimo jiné byly provedeny biochemické testy zahrnující aktivitu alaninaminotransferázy, aspartátaminotransferázy, alkalické fosfatázy, γ-glutamyltransferázy. Pro potvrzení pozorovaných jevů bylo použito stanovení

antioxidační aktivity a stanovení hladiny metalothioneinu úzce spojené s oxidačním stresem [213-215].

V této studii byly prokázány modifikace, v souvislosti s biochemickými vlastnostmi a proteinovou skladbou, které byly pozorovány u kmenů rezistentních vůči působení iontů těžkých kovů ve srovnání s kmenem kontrolním, citlivým. Naše výsledky popisují možné cesty analýzy rezistentních kmenů *S. aureus* a mohou tak sloužit jako podklad pro sledování změn v genetické informaci bakterií v důsledku vytváření rezistence vůči těžkým kovům.

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The effect of metal ions on *Staphylococcus aureus* revealed by biochemical and mass spectrometric analyses

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ABSTRACT

In this study, we focused on the effect of heavy metal ions in resistant strains of gram-positive bacteria *Staphylococcus aureus* using biochemical methods and mass spectrometry. Five nitrate solutions of heavy metals (Ag^{+}, Cu^{2+}, Cd^{2+}, Zn^{2+} and Pb^{2+}) were used to create *S. aureus* resistant strains. Biochemical changes of resistant strains in comparison with the non-resistant control strain of *S. aureus* were observed by microbiological (measuring - growth curves and inhibition zones) and spectrophotometric methods (antioxidant activity and alaninaminotransferase, aspartateaminotransferase, alkaline phosphatase, γ-glutamyltransferase activities). Mass spectrometry was employed for the qualitative analysis of the samples (changes in *S. aureus* protein composition) and for the identification of the strains database MALDI Biotyper was employed. Alterations, in terms of biochemical properties and protein composition, were observed in resistant strains compared to non-resistant control strain. Our results describe the possible option for the analysis of *S. aureus* resistant strains and may thus serve as a support for monitoring of changes in genetic information caused by the forming of resistance to heavy metals.

1. Introduction

The issue of emerging resistance is more frequent topic of research groups and their studies around the world. This topic is very well explored at the level of antibiotics resistance (Alm et al., 2014; Machado and Bordalo, 2014; Nedbalcova et al., 2014; Nguyen et al., 2014; Pesavento et al., 2014; Rodriguez et al., 2014). However, in the case of metal resistance there is not a sufficient amount of data for unambiguous understanding of cellular mechanisms due to the application of heavy metals. More interesting is also a problem of cross-resistance (Kaur et al., 2014; Kumari et al., 2014), a combination of resistance to antibiotics and heavy metals (Chudobova et al., 2014). To understand cross-resistance it is necessary to go from the basics and understand the sub-section of metal resistance.

Staphylococcus aureus is a gram-positive commensal bacterium causing nosocomial infections (Baker et al., 2011), and one of the main pathogens associated with skin infections, soft tissue, wound infections and more serious sequelae such as septicaemia, urinary tract infections, osteomyelitis and endocarditis (Duffy et al., 2013; Leucker et al., 2013; Taylor, 2013). Much of the dissimilarity between pathogenic *S. aureus* strains is dependent on the presence of virulence factors encoded mainly by mobile genetic elements, especially heavy metal resistance genes play an important role in virulence (Kahankova et al., 2010).

The mechanism of microorganism inhibition involves the entry of heavy metal ions (Zn^{2+}, Cu^{2+}, Cd^{2+}, Ag^{+}, etc.) to the metabolic system of an organism with consequent formation of secondary metabolites, which are toxic to the organism due to the presence of heavy metals (Lim et al., 2013). It has been shown that the heavy metal stress significantly contributes to the inhibition of bacterial growth (Seniya et al., 2012). However, most bacterial strains are able to create the resistance against the effect of heavy metal ions. Biological resistance is gained by the organisms against adverse effects of internal and external environment, such as the long-term effects of heavy metals from soil and water or widespread use of antibiotics (Ohlsen et al., 2003). In the response to exposition to toxic metals, metal resistance comes mostly in plasmid-encoded bacteria. Resistance genes encode genetic information of microorganisms that is changed by external or internal conditions. There

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are many mechanisms of resistance, as in the multiple-metal-resistant bacterium *S. aureus*, where Cd^{2+} (and probably Zn^{2+}) efflux is catalysed by the membrane-bound CadA protein and P-type ATPase. CadC protein is required for full resistance and CadR protein is hypothesized for regulation of the resistance determinant (Nies, 1992). The non-toxic metals are an important cofactor for many enzymes; however, they can show toxic effects at their high concentrations too. Therefore, bacteria must strictly control the intake of the metals into the cell for use as a cofactor and, more importantly, must limit free intracellular levels to prevent toxicity (Rouch et al., 1995).

Effects of heavy metals on bacterial cell are observed by changes in enzymes' activities, significant growth inhibition, inhibition of replication etc., whereas most of these mechanisms lead to cell lysis (Fig. 1A) (Silver and Ji, 1994). Baker *et al.* suggested that *S. aureus* has one main mechanism for adapting to high levels of environmental copper *via* increased oxidative stress resistance (Baker et al., 2010). Some microorganisms are able to resist the effects of heavy metals by formation of the antioxidant enzyme superoxide dismutase or by reduction of metal ions (Singh et al., 2013; Wiesemann et al., 2013). One of the most important target molecules for intracellular interaction with metals is cysteine-rich protein metallothionein. Its primary function is to detoxify the heavy metals in living organisms, which was the subject of many previous studies (Templeton and Cherian, 1991; Klaassen et al., 2009). Regulation of expression is probably caused by metal binding to the transcription factor MTF-1, although the information about expression is currently still insufficient (Babula et al., 2012).

Furthermore, some changes in genetic information can occur due to the heavy metal influence, like in the gene 16S rRNA. 16S ribosomal RNA, conferred by 16S rDNA, is one of the components of small subunit of prokaryotic ribosomes. This gene is about 1500 bp in length in *S. aureus* and it is often used in phylogenetic studies due to its hypervariable regions useful for identification of bacteria (species or genera). These variable regions result in numerous differences in endonuclease restriction site, which can be studied via restriction fragment length polymorphism (RFLP) and subsequently analysed by gel electrophoresis (Stomeo et al., 2013).

Based on the above mentioned facts, this work is focused on the studying of gram-positive bacteria *S. aureus* resistance to heavy metals at several levels. This issue was studied particularly through the changes of selected properties of bacterial strains, which were exposed to heavy metal ions. These properties were observed on the cellular and molecular levels. MALDI-TOF mass spectrometry was employed to identify non-resistant and resistant strains of *S. aureus* treated with silver, copper, cadmium, zinc and lead ions**.** Moreover, some biochemical assays including activities of alaninaminotransferase, aspartateaminotransferase, alkaline phosphatase, γ-glutamyltransferase, antioxidant activity and content of metallothionein closely connected to oxidative stress (Eckschlager et al., 2009; Babula et al., 2012; Krizkova et al., 2012) were used to confirm the observed phenomena.

2. Material and methods

2.1. Chemicals

Chemicals used in this study (Tryptone, Yeast Extract, NaCl, $AgNO_3$, $CuN_2O_6.3H_2O$, $Pb(NO_3)_2$, $Cd(NO_3)_2.4H_2O$, $Zn(NO_3)_2.6H_2O$) were purchased from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity unless noted otherwise. Heavy metals used for the preparation of *S. aureus* resistant strains were in the form of nitrates ($AgNO_3$, $CuN_2O_6{\cdot}3H_2O$, $Pb(NO_3)_2$, $Cd(NO_3)_2{\cdot}4H_2O$, $Zn(NO_3)_2{\cdot}6H_2O$) and dissolved in 100 ml MilliQ water in 2 mM concentration. Deionized water was prepared using reverse osmosis equipment Aqual 25 (Brno, Czech Republic). Deionized water was further purified using a MilliQ Direct QUV apparatus equipped with UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

2.2. Cultivation of S. aureus

S. aureus (NCTC 8511) was obtained from the Czech Collection of Microorganisms (Faculty of Science, Masaryk University, Brno, Czech Republic). Strains were stored in the form of a spore suspension in 20% (*v*/*v*) glycerol at -20 °C. Prior to use, the strains were thawed and the glycerol was removed by washing with distilled water. The composition of cultivation medium (LB medium) was prepared according to protocol from Sigma Aldrich. Composition was as follows: Tryptone 10 g.l^{-1}, NaCl 5 g.l^{-1}, Yeast Extract 5 g.l^{-1} and sterilized MilliQ water with 18 MΩ. pH of the cultivation medium was adjusted to 7.4 before sterilization. Sterilization of media was carried out at 121 °C for 30 min in sterilizer (Tuttnauer 2450EL, Israel). The prepared cultivation media were inoculated with bacterial culture into 25 ml Erlenmeyer flasks. After the inoculation, bacterial cultures were cultivated for 24 h on a shaker at 600 rpm and 37 °C. Bacterial culture cultivated under these conditions was diluted by cultivation medium on Specord spectrophotometer 210 (Analytic, Jena, Germany) to OD_{600} = 0.1 and used in the following experiments.

2.3. Preparation of Resistant Strains of S. aureus

For this work, we developed a method for creating of resistant strains of *S. aureus* (NCTC 8511). To this bacterial culture 2 mM solutions of heavy metals (Ag, Cu, Cd, Zn and Pb) were added. Lowest resulting concentration of the metal in a medium inoculated with bacterial culture was found to be 50 μM, and then the metal concentration was gradually increased by 50 μM up to the maximum possible dose, in which *S. aureus* was still able to regenerate. It was always possible to revitalize resistant strains using pure medium without addition of metal.

2.4. Determination of Growth Curves

The procedure for the evaluation of an antimicrobial effect of tested heavy metals was based on the *S. aureus* bacterial culture. An apparatus Multiskan EX (Thermo Fisher Scientific, Germany) *via* Ascent Software for Multiskan was used with subsequent analysis in the form of growth curves. The bacterial culture growing overnight was diluted with LB medium to absorbance of 0.1 measured using a Specord spectrophotometer 210 (Analytik, Jena, Germany) at a wavelength of 600 nm. The diluted culture was pipetted into a microplate (total volume of 300 μl) alone as a control variant, or with various concentrations of tested heavy metals. The concentrations of these metals in the well were 0, 10, 25, 50, 75, 150, 225, and 300 μM. Measurements were carried out at time 0, then each half-hour for 24 h at 37 °C, at a wavelength of 600 nm. The measured absorbances were analysed in a graphic form as growth curves for each experimental group individually (Chudobova et al., 2013).

2.5. MALDI-TOF MS Identification of Resistant Strains of S. aureus

The following extraction protocol and sample preparation was based on MALDI Biotyper 3.0 User Manual Revision 2, similar extraction method was used also in (Sauer et al., 2008). A sample of 500 μl *S. aureus* (0.1 OD) culture, cultivated overnight, was centrifuged at 14.000 × g for 2 min. The supernatant was discarded and the pellet was resuspended in 300 μl of deionized water and

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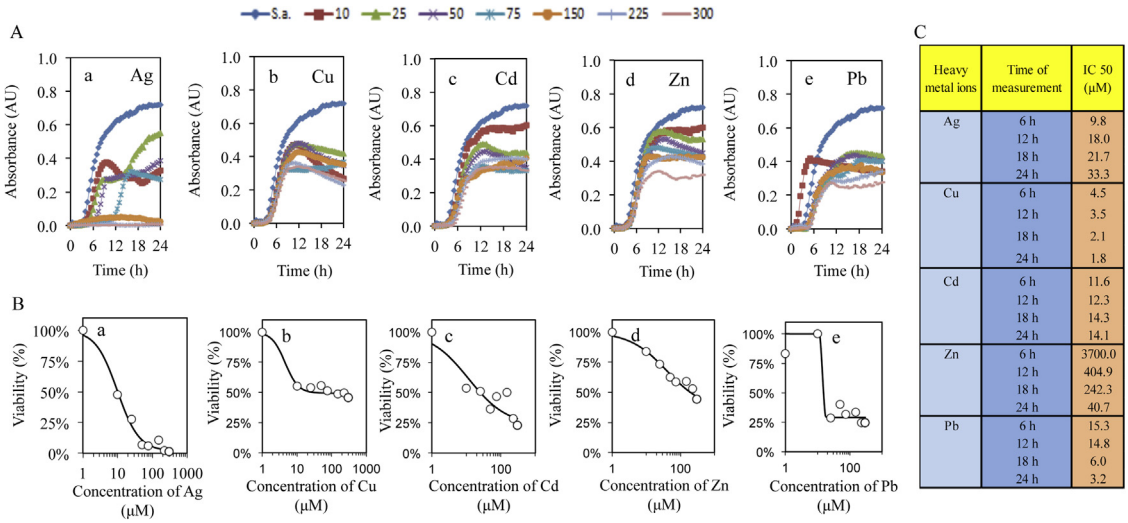

Heavy metal ions	Time of measurement	IC 50 (μM)
Ag	6 h	9.8
	12 h	18.0
	18 h	21.7
	24 h	33.3
Cu	6 h	4.5
	12 h	3.5
	18 h	2.1
	24 h	1.8
Cd	6 h	11.6
	12 h	12.3
	18 h	14.3
	24 h	14.1
Zn	6 h	3700.0
	12 h	404.9
	18 h	242.3
	24 h	40.7
Pb	6 h	15.3
	12 h	14.8
	18 h	6.0
	24 h	3.2

Figure 1. (**A**) Growth curves of *S.aureus* strains resistant to heavy metals (a) RAg, (b) RCu, (c) RCd, (d) RZn, (e) RPb. (**B**) Statistical evaluation of viability after the application of heavy metal ions (a) Ag^{+}, (b) Cu^{2+}, (c) Cd^{2+}, (d) Zn^{2+}, (e) Pb^{2+}. (**C**) Table of inhibition concentrations (IC_{50}) for each of different resistant strains at 6, 12, 18 and 24 hours of measurement.

900 μl of ethanol was added. After centrifugation at 14.000 × g for 2 min, the supernatant was discarded and the obtained pellet was air-dried. The pellet was then dissolved in 25 μl of 70% formic acid (*v*/*v*) and 25 μl of acetonitrile and mixed. The samples were centrifuged at 14.000 × g for 2 min and 1 μl of the clear supernatant was spotted in duplicate onto the MALDI target (MTP 384 target polished steel plate; Bruker Daltonik GmbH, Bremen, Germany) and air-dried at room temperature. Then, each spot was overlaid with 1 μl of saturated α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution in organic solvent (50% acetonitrile and 2.5% trifluoroacetic acid, both *v*/*v*) and air-dried completely prior to MALDI-TOF MS measurement on UltrafleXtreme MS (Bruker Daltonik GmbH, Bremen, Germany). Spectral data were taken in the *m*/*z* range of 2.000 Da to 20.000 Da, and each was a result of the accumulation of at least 1000 laser shots obtained from ten different regions of the same sample spot. These data were analysed with the Flex Analysis software (Version 3.4). Prior to analysis, the mass spectrometer was externally calibrated with a peptide mix of bombesin, angiotensin I, glu-fibrinopeptide B, adrenocorticotropic hormone (ACTH) (18-39), ubiquitin, and cytochrome c. Spectra with peaks outside the allowed average were not considered. Modified spectra were loaded into the MALDI BioTyper™ 3.1 Version (Bruker Daltonik GmbH, Bremen, Germany). Pictures of sample-matrix crystals were taken by built-in camera.

2.6. Spectrophotometric Measurements

Spectrophotometric measurements were carried out using an automated chemical analyzer BS 400 (Mindray, Shenzhen, China) under conditions described in previous publication (Gumulec et al., 2013). The transfer of samples and reagents into cuvettes was performed by a robotic arm equipped with a dosing needle. The cuvette contents were mixed by an automatic mixer. Determination of alaninaminotranspherase (ALT), aspartateaminotranspherase (AST) and alkaline phosphatase (ALP) activity was carried out according to the kit manufacturer's instruction (Greiner, Frickenhausen, Germany) as it is specified in previous publication (Sochor et al., 2012).

2.7. Determination of γ-glutamyltransferase (GMT) activity

150 μl of solution R1 (150 mM Glycylglycine) was pipetted into a plastic cuvette with subsequent addition of 7.5 μl of a sample. This solution was incubated for 270 seconds. Subsequently, 30 μl of solution R2 (6 mM L-Gamma-glutamyl-3-carboxy-4-nitroanilide) was added and the solution was incubated for 90 seconds. The absorbance measurement lasted for 180 seconds. The mean increase of absorbance per minute was calculated.

2.8. Determination of antioxidant activity by the FRAP method

The FRAP method (Ferric Reducing Antioxidant Power) is based on the reduction of complexes of 2,4,6-tripyridyl-*s*-triazine (TPTZ) with ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), which are almost colourless, and eventually slightly brownish. This chemical forms blue ferrous complexes after its reduction. Reagent preparation and analysis conducted by (Sochor et al., 2010). A 150 μl of reagent was pipetted into a plastic cuvette with subsequent addition of 30 μl of a sample. Absorbance was measured at 605 nm for 12 minutes. For the calculation of antioxidant activity, the values before decrease of absorbance (2^{nd} minute of measurement–A_2) and the last one (12^{th} minute of measurement–A_{12}) were used. Resulting value was calculated in accordance with following formula: Differential absorbance $A = A_{12} - A_2$. The calibration curve using gallic acid was prepared as described in previous paper (Sochor et al., 2010). Antioxidant activity was expressed as gallic acid equivalents (GAE).

2.9. Determination of Metallothionein Amount

The pellet of 1 ml of *S. aureus* incubated for 24 hours at 37 °C in LB medium was resuspended in 1 ml of phosphate buffer (pH 7), after that the sample was denaturated in thermoblock at 99 °C for 20 minutes and centrifuged for 20 minutes at 25 000 rpm. Supernatant from the sample was used for electrochemical detection.

Electrochemical detection was carried out using the method of differential pulse voltammetry (Raspor et al., 2001; Sevcikova et al., 2013) (three electrodes consisted of working mercury drop electrode (HMDE), reference silver-chloride electrode Ag/AgCl/3 M KCl and auxiliary carbon electrode). The analysed samples were deoxidized by argon for 120 seconds. As a supporting electrolyte, Brdicka solution, which contained 1 mM $[Co(NH_3)_6]Cl_3$ and 1 M ammonium buffer ($NH_3(aq) + NH_4Cl$, pH 9.6), was used as a supporting electrolyte. This electrolyte was changed after each analysis of sample. The parameters for measurements were following: initial potential -0.7 V, final potential -1.75 V, the time interval 0.2 s, step potential 2 mV, amplitude -250 mV. For the calibration curve of

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metallothionein was used Metallothionein 95%, cat.no. MT_95_P_1 mg (IZKUS PROTEOMICS) in the concentration range 0.156 - 50 μM.

2.10. Isolation of DNA, Polymerase Chain Reaction and Restriction Fragment Length Polymorphism

2 ml of *S. aureus* culture was centrifuged at 5000 x g and 20 °C for 10 minutes. 400 μl of lysis solution (6 M guanidine hydrochloride + 0.1 M sodium acetate) was added to the pellet and the lysis was carried out at 20 °C and 600 rpm for 1 hour. Isolation of genomic DNA was performed using MagNA Pure Compact (Roche, Germany), Nucleic Acid Isolation Kit I, and protocol DNA Bacteria according to the manufacturer's instructions (Roche, Germany).

The *16S* gene was amplified using polymerase chain reaction (PCR) according to (Stomeo et al., 2013). The sequences of forward and reverse primers were 5′-GAGTTTGATCCTGGCTCAG-3′ and 5′-GGTTACCTTGTTACGACTT-3′ respectively. The volume of PCR reaction mixture was 100 μl containing 1× *Taq* reaction buffer (New England Biolabs, Ipswich, Great Britain), 0.2 mM dNTP, 0.5 mM each primer from Sigma-Aldrich (St. Louis, MO, USA) and 1.6 units of *Taq* DNA polymerase (New England Biolabs, Ipswich, Great Britain). The reaction profile was as follows: initial denaturation at 94 °C for 4 min, 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min and a final extension for 7 min. The amplification generated a 1500 bp fragment. Restriction fragment length polymorphism (RFLP) was conducted according to (Stomeo et al., 2013) using 1.6 μg of isolated DNA, 10 units of HaeIII enzyme, 1× HaeIII buffer (New England Biolabs, Ipswich, Great Britain) and sterile water to the final volume of 50 μl, digested at 37 °C for 2 hours, with following enzyme inactivation at 80 °C for 20 min.

Agarose gel (2% *v*/*v*, high melt, Mercury, San Diego, CA, USA) was prepared with 1× TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM ethylenediaminetetraacetic acid) and ethidium bromide (5 μl per 100 ml of the gel) according to (Smerkova et al., 2013). 100 bp DNA ladder (New England BioLabs, Ipswich, MA, USA) within the size range from 100 to 1517 bp was used to monitor the size of the analysed fragment. The electrophoresis (Bio-Rad, Hercules, CA, USA) was running at 60 V and 6 °C for 160 minutes. The bands were visualized by UV transilluminator at 312 nm (Vilber-Lourmant, Marne-la-Vallée Cedex 1, France).

GenomeLab DTCS Quick Start kit (Beckman Coulter, USA) with 16 ng of amplified DNA was used for the sequencing reaction according to (Smerkova et al., 2013). The cycling conditions were as follows: 30 cycles of denaturation at 96 °C for 20 s; annealing at 50 °C for 20 s and 60 °C for 4 min. DNA fragments from this reaction were purified using magnetic particles CleanSEQ (Beckman Coulter, USA). DNA sequencing was performed on Genetic Analysis System CEQ 8000 (Beckman Coulter, USA). After denaturation at 90 °C for 2 min, the fluorescence-marked DNA fragments were separated in 33 cm capillary with 75 μm i.d. (Beckman Coulter, USA), which was filled with a linear polyacrylamide denaturing gel (Beckman Coulter, USA). The separation was performed at capillary temperature of 50 °C and voltage of 4.2 kV for 85 min.

2.11. Statistical Analyses

Mathematical analysis of the data and their graphical interpretation were realized by Microsoft Excel®, Microsoft Word® and Microsoft PowerPoint®. Software STATISTICA (data analysis software system) version 10.0 (Tulsa, Oklahoma, USA) was used for data processing. Half-maximal concentrations (IC_{50}) were calculated from logarithmic regression of sigmoidal dose-response curve.

3. Results

3.1. Growth characteristics

Heavy metal-resistant strains of *S. aureus* were prepared by prolonged cultivation in the increasing concentration of heavy metals (for further details see Experimental Procedures - Preparation of Resistant Strains of *S. aureus*). To characterize the resistant strains, the determination of minimal inhibition concentrations was performed by method of growth curves (Fig. 1A) (Bajpai et al., 2013). Data obtained from this method are transferred to the line graph and interleaved with the growth curve of *S. aureus* without any additives, for more illustrative evaluation of antimicrobial activity (Muller et al., 2013).

Results of microbiological determination showed differences in individual resistant strains after addition of the metal with the control strain of *S. aureus* (blue curve). The most resistant and therefore the least reacting to the addition of metal were the cadmium (RCd) and zinc (RZn) resistant strain. MIC (minimum inhibitory concentration) was determined in all resistant strains after the addition of 10 μM concentration of metal ions, but with different intensity. In the silver (RAg) and lead (RPb) resistant strains, the decrease (43%) of growth (absorbance values) was observed after the application of 10 μM metal ions after 24 hours of measurement (Figs. 1Aa-e, respectively). In copper resistant strain (RCu), the absorbance was decreased to 29% (Fig. 1Ab) and in RCd and RZn only by 14% (Figs. 1Ac and 1Ad, respectively). The relative standard deviation of growth curves determination was found to vary from 5.5 to 8.1% (calculated from three independent measurements). The total inhibition concentration after the addition of silver ions in concentrations of 150, 225 and 300 μM was achieved only in RAg strain (Fig. 1Aa).

These results were also supported by statistical evaluation, called viability of cells (Figs. 1Ba (silver), 1Bb (copper), 1Bc (cadmium), 1Bd (zinc) and 1Be (lead)), its values can be statistically calculated to the IC_{50} (the half maximal inhibitory concentration) value (Fig. 1 C). The IC_{50} showed the highest concentrations for inhibition of the growth of bacterial culture in the RZn strain. IC_{50} values were determined at 6, 12, 18 and 24 hours of measurement (Fig. 1C).

3.2. Spectrometric assays

In our study, ferric reducing ability power (FRAP) assay was used for the assessment of oxidative stress in non-resistant and resistant *S. aureus* (Fig. 2). Besides the oxidative stress, activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GMT) and alkaline phosphatase (ALP) were determined too (Fig. 2). The obtained results were subtracted from those measured in the case of control strain and were divided according to applied metal ion as silver (Fig. 2A), copper (Fig. 2B), lead (Fig. 2C), zinc (Fig. 2D) and cadmium (Fig. 2E).

3.3. Metabolic changes revealed by MALDI-TOF MS

The MALDI-TOF mass spectra of heavy metal resistant *S. aureus* strains are shown in Fig. 3. Moreover; there are shown the pictures corresponding to sample-matrix crystals to illustrate that crystals were formed randomly in relatively homogenous layer. The obtained spectra were analysed in Biotyper™ 3.1 Version software, which identified the samples within the genus *Staphylococcus*, but in different species compared to the control (without addition of metal). The control strain *S. aureus* was identified as a *S. aureus* ssp. *aureus* DSM 4910 (Fig. 3A), *S. aureus* with addition of 950 μM of Cu^{2+} as a *Staphylococcus saprophyticus* ssp. *saprophyticus* CCM 2682 (Fig. 3B), *S. aureus* with addition of 950 μM of Zn^{2+} as a *Staphylococcus felis* DSM 7377 T (Fig. 3C), *S. aureus* with addition of

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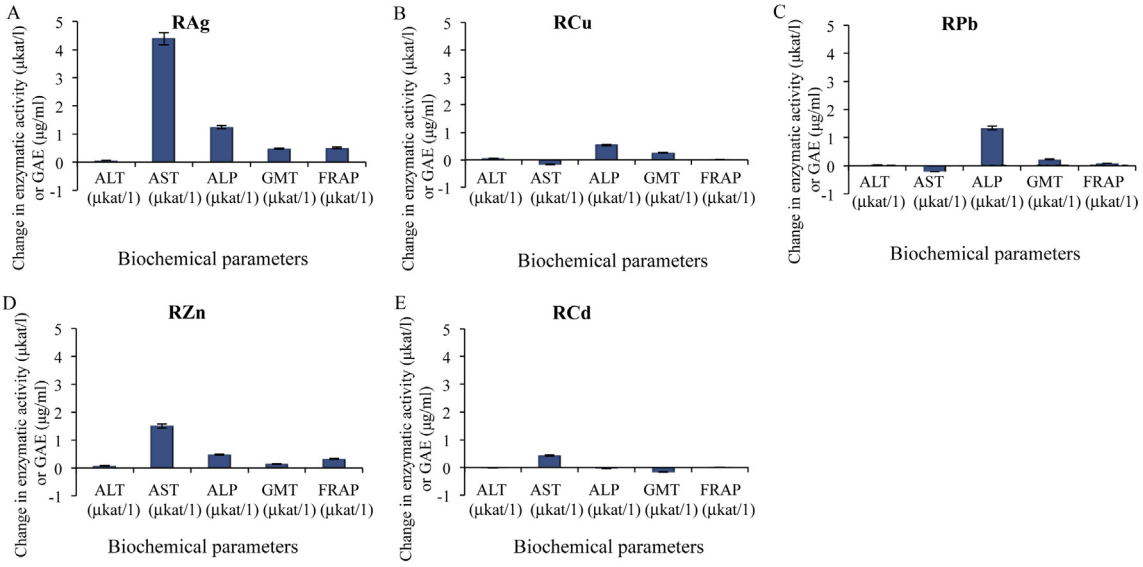

Figure 2. Spectrophotometric analysis of the non-resistant *S. aureus* or *S. aureus* resistant strains to different heavy metals. Determination of enzymatic activity by the ALT, AST, ALP and GMT and antioxidant activity by the FRAP. Results from each analysis were obtained three times and always subtracted from the value of the control measurements non-resistant *S. aureus* (A) *Staphylococcus aureus.* (B) RAg. (C) Resistant strain of *S. aureus* to copper ions. (D) Resistant strain of *S. aureus* to lead ions. (E) Resistant strain of *S. aureus* to zinc ions. (F) Resistant strain of *S. aureus* to Cd.

950 μM of Pb^{2+} as a *Staphylococcus capitis* ssp. *capitis* DSM 20326T DSM (Fig. 3D), *S. aureus* with addition of 950 μM of Cd^{2+} as a *S. aureus* ssp. *aureus* DSM 20491 (Fig. 3E) and *S. aureus* with addition of 350 μM of Ag^{+} a *Staphylococcus condimenti* DSM 11674T DSM (Fig. 3F). It is obvious that the resistant *S. aureus* strains exhibit distinct changes in protein profiles, which can be monitored by MALDI-TOF.

3.4. Metallothionein

Concentrations of thermostable protein metallothionein were higher in heavy metal resistant strains (mainly RCu and RPb) when compared with the control strain (Fig. 4A), whereas voltammograms obtained by the differential pulse voltammetry are shown in Fig. 4B. The high narrow peak (called RS_2Co) detected at potential of -1.25 ± 0.05 V is the specific response of the supporting electrolyte and serves as a control of the proper conditions for catalytic reaction. For evaluation of metallothionein content we determined a height of Cat2 peak (-1.55 ± 0.05 V) as we previously described in our study (Adam et al., 2008).

3.5. Ribosomal RNA

16S ribosomal RNA, conferred by 16S rDNA, is one of the components of small subunit of prokaryotic ribosomes. This sequence is considered as conservative and is used for molecular taxonomy. A significant change in the sequence shows the high level of mutations induced by heavy metals despite increased levels of antioxidants and heavy metals chelators, as it is shown by FRAP and MT analysis. It is interesting that in all cases this is the same mutation as shown by sequence and RFLP. At the same time, after prolonged exposure to heavy metals there were changes in the

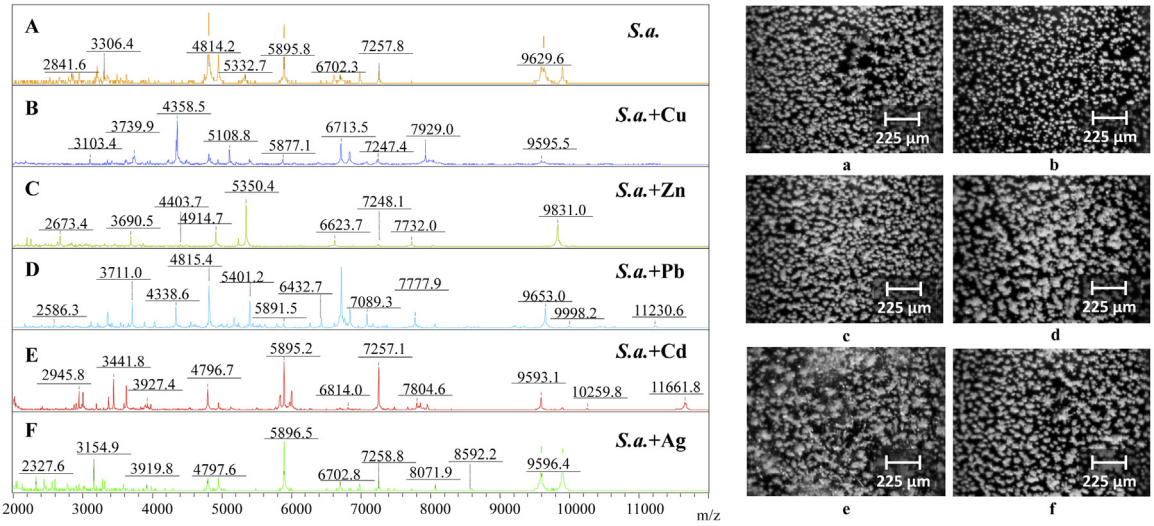

Figure 3. MALDI-TOF mass spectra of protein fingerprints for the identification of non-resistant strains of *S. aureus* and resistant strains of *S. aureus*. Data were collected in the m/z 2000-20000 range after processing 1 ml of *S. aureus*. (**A**) Control strain of *S. aureus* identified. *S. aureus* with addition of (**B**) 950 μM Cu^{2+}. (**C**) 950 μM of Zn^{2+}. (**D**) 950 μM Pb^{2+}. (**E**) 950 μM Cd^{2+}. (**F**) 350 μM Ag^{+}. The results were compared with the library software MALDI BioTyper™ 3.1 Version and were completed with photos of crystals.

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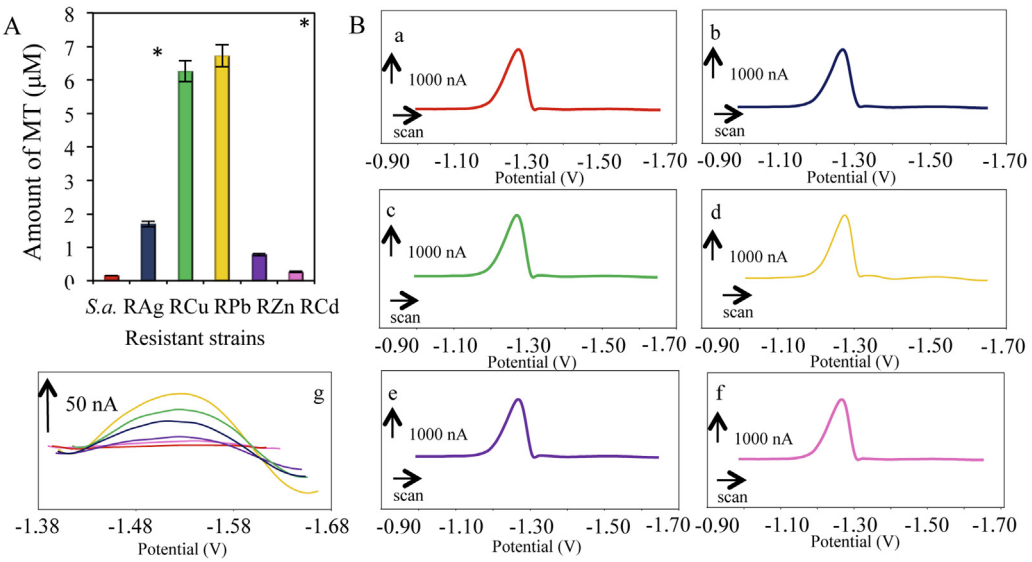

Figure 4. Electrochemical analysis of the non-resistant *S. aureus* or *S. aureus* resistant strains to different heavy metals. (A) Determination of amount of metallothionein (MT). (B) Comparison of DP voltammograms of extracts obtained from various resistant strains and non-resistant one: a) non-resistant strain of *S. aureus*, b) RAg, c) RCu, d) RPb, e) RZn, f) RCd, g) cut out of the range in potentials from -1.38 to -1.68 V, where the significant changes in the peak height were found.

biochemical profile of bacteria and changes were evident even by MALDI-TOF. This shows that the biochemical profile was not changed due to the inhibition or activation of the enzyme by heavy metals, but there was also a significant change in protein expression. For the digestion of this gene, endonuclease HaeIII, originally isolated from *Haemophilus aegypticus*, was used. The restriction site for this endonuclease is 5′-GG'CC-3′ (manufacturer's manual).

Fig. 5A showed the presence of 1500 bp PCR product of 16S rRNA gene amplification both in control and resistant strains. Results of RFLP analysis (Fig. 5B) showed two restriction fragments in control strain of *S. aureus* and additional fragments in all resistant strains compared to control sample. This is a result of the single base substitution in 16S rRNA gene sequence, probably caused by developed resistance. Sequencing data analysed by software BLAST show one restriction site at 326 bp in control strain and the exact lengths of restriction fragments are 326 and 1243 bp. Three transitions occurred in resistant strains at 349-bp the transition from thymine to cytosine, at 992-bp the transition from adenine to guanine and at 1461-bp the transition from thymine to cytosine. These transitions result in forming three additional restriction sites and the lengths of restriction fragments are 326, 22, 644, 469 and 108 bp (Fig. 5C). The 22-bp fragment is not visible on agarose gel due to its small size. These results support the theory that there are changes in genetic information of *S. aureus* resistant to metals.

4. Discussion

4.1. Metal detoxification mechanisms

Different types of organisms are able to intake contaminants limiting their adverse effects due to various types of detoxification mechanisms. Into these types we can also include bacterial strains. Various microorganisms have successfully adapted to the presence of several metals by use of chromosome, plasmid, or transposon-encoded, metal-resistance mechanisms. Most of these resistance mechanisms are plasmid-mediated and are highly specific to a particular cation or anion (Bruins et al., 2000). Inside the cell, metals may have various effects, according to their concentration and chemical properties (Bruins et al., 2000). When concentration levels are too elevated bacteria usually react by the expression of specific metal resistance systems such as P-type ATPases, metallothioneins, RND efflux pumps and/or CDF transporters (Nies, 2003). The resistance genes are located on chromosomes, plasmids or transposons and can therefore be transferred very efficiently to other community members (Bruins et al., 2000; Sorensen et al., 2005). An observation of an increased level of a specific metal resistance system in bacterial cell is good for monitoring of bioavailability of heavy metals from environment (Roosa et al., 2014).

From these facts, it is clear that the resistance can not only formed but also transferred inside the bacterial community. The emergence of resistance based on changes in morphology and biochemical properties can be observed using a number of basic microbiological and spectrophotometric methods. Spectrophotometry-based monitoring of the bacterial growth is suitable for studying of bacterial cultures influenced by various agents (Turner et al., 2012). Therefore, determination of growth dependences ("growth curves") (Rufian-Henares and Morales, 2008; Borneman et al., 2009; Fernandez-Saiz et al., 2010), where the 50% inhibitory concentration induced inhibition of growth - IC_{50} (Wong and Lee, 2014), was used. Our results show the highest resistance (Fig. 1A) and viability (Fig. 1B) was found at *S. aureus* strains treated with Cd^{2+} and Zn^{2+}. Resistance to Cd^{2+} is probably caused by the efflux pumps, the most prominent metal system (Bruins et al., 2000). *S. aureus* carried out cadmium resistance through efflux mechanism consisted from the P-type ATPase transport system (Nies and Silver, 1995). $Zn^{2+}/Cd^{2+}/Pb^{2+}$-translocating ATPase in *S. aureus (cadA)* is determined with location on plasmid, pI258 (Nies and Silver, 1995; Naik et al., 2013). The mechanism for zinc resistance utilizes on the one hand the same CadA system as for Cd^{2+} efflux and on the other hand zinc resistance is based on numerous other processes. One of them is the ability of *S. aureus* to build an effective shield on the bacteria surface thereby to damage antibacterial action of ZnO (Ann et al., 2014). Next is Zn^{2+} transportation by CorA system, which was found in many bacteria and archaea. Another potential chemiosmotically driven mechanism is by the MgtE proteins (Smith et al., 1995), that also probably transports Zn^{2+}. Next transport mechanism is MgtA, a P-type ATPase (Townsend et al., 1995). MgtA is regulated by magnesium starvation (Tao et al., 1995) and zinc may interfere with this process. However, this ATPase is not high-specific for zinc. In *Streptococcus pneumonia* (Dintilhac et al., 1997) and *gordonii* (Kolenbrander et al., 1998) ABC transporter was found to take place in metal resistance.

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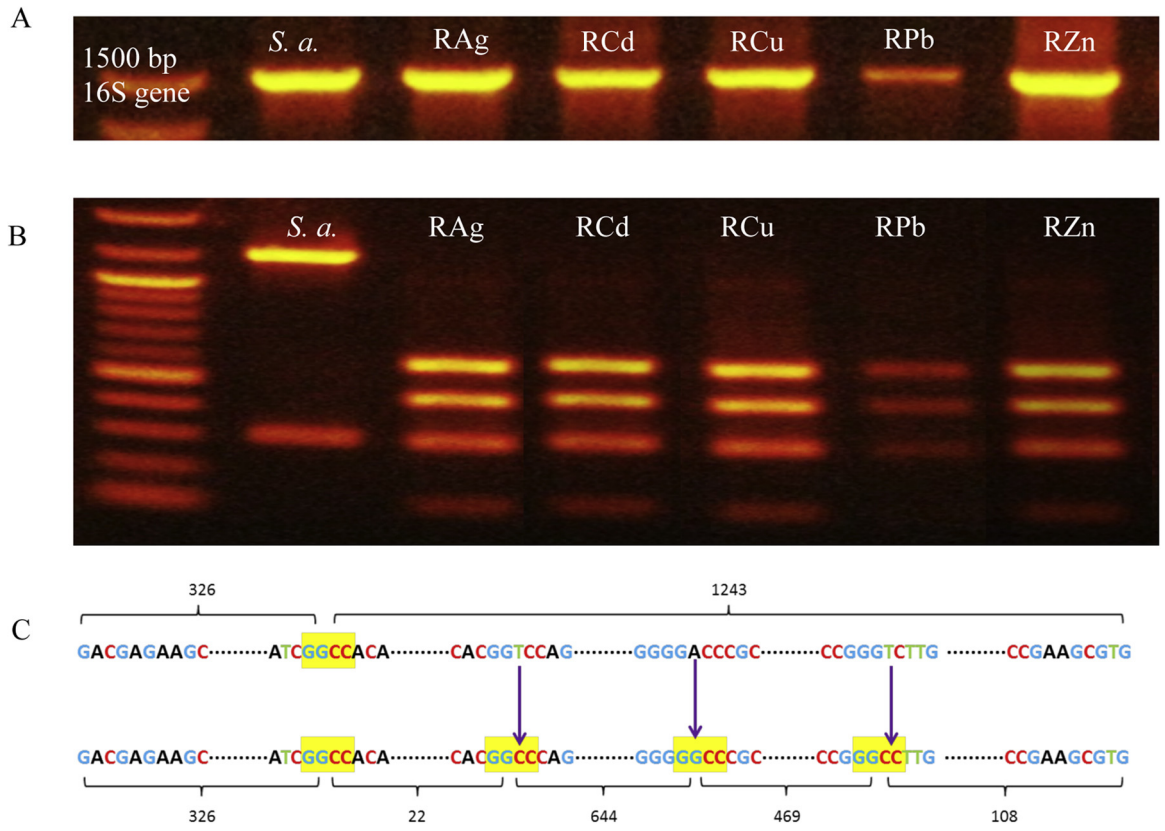

Figure 5. (A) Amplification of 16S rRNA gene in *S. aureus* resistant to 950 μM of different metals. Samples were as follows: control strain of *S. aureus*, RAg, RCd, RCu, RPb and RZn. (B) Amplification of 16S rRNA gene in *S. aureus* resistant to 950 μM of different metals using RFLP *via* HaeIII. Samples were as follows: control strain of *S. aureus*, RAg, RCd, RCu, RPb and RZn. (C) Genetic changes demonstrated by RFLP *via* HaeIII in control strain of *S. aureus* and generally in resistant strains.

These synergic facts, including fact that zinc has great biological importance and is present in all organisms, could explain high viability and resistance (Fig. 1Bd, 1Ad) of bacterial strain treated with Zn^{2+}.

Moreover, *S. aureus* shows lower tolerance to lead (Fig. 1Ae, 1Be) presumably caused by the same gene *cadA* like the resistance to cadmium. This gene encodes multipurpose P-type ATPase (Bruins et al., 2000; Blaszak and Bienkowska, 2009). In addition to membrane transport pumps intra- and extracellular binding of lead ions evade toxicity of free lead ions by precipitating lead as a phosphate salt (Levinson et al., 1996). The lowest resistance and viability was detected for *S. aureus* treated with Ag^{+} and Cu^{2+} (Fig. 1Aa, 1Ba, 1Ab, 1Bb). Copper is probably very toxic because of easy interaction with radicals. Its radical character makes copper very toxic (Nies, 1999). The explanation of toxicity mechanism could be based on the experiment on gram-positive bacterium strain *Enterococcus hirae* (Odermatt et al., 1993; Odermatt et al., 1994). *E. hirae* contains a *cop* operon with two structural genes that encode P-type ATPase. It is assumed that CopA is responsible for copper uptake and copper nutrition, CopB is responsible for copper efflux and detoxification. Both operons can probably transport copper as well as silver. *Gupta et al.* demonstrated that resistance of silver is encoded and is determined by plasmid pMG101 (Gupta et al., 1999). Silver also can be removed from bacteria through metabolic efflux (Schreurs and Rosenberg, 1982) and its toxic effect is caused by interfering with DNA. All results show that studied metals could be divided to four groups with different resistant mechanism, (i) zinc like biogenic element receiving help of blocking membranes, (ii) cadmium, lead and copper are mainly detoxified by ATPases that are unspecific and bind metals according to affinity of individual structures, (iii) copper has Cad and Cop transporters (this fact is probable reason of different viability and resistance as a comparison with cadmium and lead), (iv) silver, which is probably mainly influenced by the efflux mechanism.

4.2. Oxidative stress

Spectrophotometric methods like FRAP, determination of antioxidant enzymes like ALT, AST, ALP or GMT are the most common methods for determination of the oxidative stress in cells and whole tissues extracts (He et al., 2013; Kaiser et al., 2013). Spectrophotometric methods are necessary for determination of antioxidant capacity and oxidative stress in bacterial lysates (Barriere et al., 2001; Bir et al., 2012).

The most synthesized enzymes are AST, ALP and GMT (Figs. 2A - 2E). Only low change in enzymatic activity was observed in the case of ALT. The highest enzymatic activity was found in RAg. The high enzymatic activity was observed also in RZn, RPb and RCu strain. The lowest enzymatic activity was found in RCd strain. On the other hand regarding to antioxidant activity measured by FRAP method RAg and RZn only had the increased antioxidant activity, which was not observed in the case of RPb, RCu and RCd. This phenomenon could be connected with low resistance to Ag^{+} or large amount of Zn^{2+} necessary to form resistance of *S. aureus* strain. Regarding to the antioxidant activity of RCd measured by FRAP, showing a higher antioxidant activity, because level of all measured stressed enzyme is the lowest. Strains RAg, RPb and RZn showed the highest increases in enzymatic or antioxidant activity. An increase in enzymatic or antioxidant activity likely helps to cope better with oxidative stress, which is caused by the presence of heavy metals. Generally it could be suggested a theory that ROS generation by metals is responsible for the strong antibacterial activity. This was observed on *S. aureus* exposed to Ag nanoparticles (Kim et al., 2007).

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4.3. Protein profile

Modifications at the protein level, thus continuing changes in metabolism in response to the effect of heavy metal ions onto a cell, can be monitored using MALDI-TOF MS. The changes in protein profiles were investigated using mass spectrometry method for its ability to achieve high sensitivity, fast analysis and precise results (Elased et al., 2005; Elased et al., 2006; Ahsan et al., 2009; Song et al., 2013). MALDI-TOF MS-based identification was shown to be a fast and accurate technology in the identification of a variety of *S. aureus* strains (Szabados et al., 2010; van Veen et al., 2010; Bohme et al., 2012; Charyulu et al., 2012). MALDI spectra of *S. aureus* and metal resistance strains are shown in Fig. 3A. *S. aureus* has four typical peaks (4814, 5896, 6702 and 9630), which are also presented in spectra of resistance strains. Other peaks in spectra are probably connected with the changes in protein morphology due to the metal influence. During bacteria identification control sample of *S. aureus* had the value of log(score) higher than 2 only. This value determines high conformity with the reference spectrum in the database. Other samples (metal resistant *S. aureus*) reached log(score) lower than 2 and probably conformity was defined only in the bacteria genus, however we were not able to identify the specific specie. Gopal *et al.* observed changes in MALDI spectra after the exposure to Pt nanoparticles (PtNPs) in gram-negative *Pseudomonas aeruginosa* (Gopal et al., 2013). They studied the bacterial toxicity of different sizes of PtNPs and the results show that PtNPs 1 - 3 nm sized have bacterio-toxic properties while PtNPs higher (4–21 nm) show bacterio-compatible properties. Pictures in the Fig. 3a–f are finger prints of each *S. aureus* strain evaporated on the target for MALDI analysis.

4.4. Metallothionein

Several genetic mechanisms are known in bacteria maintaining intracellular homeostasis of essential metals and regulating resistance against toxic metals. Metal efflux, intracellular sequestration by metal binding proteins, extracellular sequestration by exopolysaccharides, cell surface biosorption by negative groups, bioprecipitation and redox reactions are the resistance mechanisms, which are present in microorganisms to counteract heavy metal stress (Naik et al., 2012). Intracellular sequestration is mostly based on the presence of metallothionein. Metallothionein is an intracellular low molecular mass protein containing the amino acids rich in sulphur (Adam et al., 2005; Vasak, 2005; Krizkova et al., 2007), which is synthesized in the response to the presence of metal ions. Occurrence of metallothionein was observed in bacteria (Coyle et al., 2002; Henkel and Krebs, 2004; Vasak, 2005), plants (Coyle et al., 2002; Henkel and Krebs, 2004; Vasak, 2005), invertebrates (Ivankovic et al., 2005; Erk et al., 2008) and also in vertebrates (Coyle et al., 2002; Henkel and Krebs, 2004; Vasak, 2005; Dragun et al., 2013).

Metallothionein is involved in many cellular functions, particularly in the transport, storage and detoxification of metals, metabolism of essential metals and uptake of the free radicals (Babula et al., 2010; Krizkova et al., 2012; Ryvolova et al., 2012; Pekarik et al., 2013). Formation of metallothionein-metal complexes protects the organism mainly against acute toxic effects of metals (Waalkes et al., 1984). Therefore, an increased concentration of metallothionein can be important for homeostasis and detoxification of metals to acquire resistance to heavy metal in *S. aureus* strains. Therefore, the ability of *S. aureus* express metallothionein (MT) due to the expose of heavy metals compared to control *S. aureus* was tested (Fig. 4A). Figs. 4Ba-g show voltammograms of resistant strains measured in Brdicka electrolyte. The highest level of MT was detected in RCu > RPb » RAg > RZn > RCd. The explanation is connected with mechanism of metal ability to enter to bacteria (Lebrun et al., 1994) and with different MT affinity to metals (Toriumi et al., 2005). Lebrun *et al.* studied *Listeria monocytogenes*, which is similar to *S. aureus*. *L. monocytogenes* has cadmium resistance gene (*pLm74*) and this gene is like plasmid pI258 in *S. aureus* and prevents accumulation of Cd^{2+} in the bacteria by an ATPase efflux mechanism. Results showed cadmium-inducible synthesis of *L. monocytogenes cadAC* RNA (Lebrun et al., 1994). Moreover, the relationship between metallothionein-3 and metals was observed on the organism *E. coli* and it was indicated as follows Cu > Cd > Zn, same as metallothionein-1 and 2. The affinity of metallothionein-3 to Cu was much higher compared with other isoforms (Toriumi et al., 2005). According to Silver *et al.* the MT synthesis is regulated by gene *smtB* in gram-positive bacteria *Enterococcus hirae* during metal exposure. In addition to rapid response to toxic metals by growth depression, two long-term mechanisms of increasing *smtA* MT gene expression was found. After exposure by metal ions three levels of gene regulation can occur (a) by depression of the SmtB repressor, (b) by amplification of the gene, and (c) by deletion of the repressor gene between fixed chromosomal points (Silver and Phung, 1996).

4.5. Gene expression

The 70S ribosome consists of a small 30S and a larger 50S subunit. 16S ribosomal RNA and 21 proteins form the 30S subunit, while 23S RNA and 34 proteins form the 50S subunit. In order to create the 70S bacterial ribosome both subunits must be present and both are essential for translation, which uses specific activity centres in each subunit (Beach and Champney, 2014). There is a lot of information about ribosomal RNA in relation to antibiotics. Antibiotics targeting the 30S and 50S subunits inhibit both bacterial protein synthesis and subunit assembly, indicating that inhibition of ribosomal subunit assembly may be a synergistic process with translational inhibition (Poehlsgaard and Douthwaite, 2005). Antibiotics as well as heavy metals have a bacteriostatic activity and have had success in treating staphylococcal infections, however, resistance formation has continued to plague advances made in the field (Beach and Champney, 2014).

16S rRNA gene sequence is used for bacterial identification and molecular taxonomy. This sequence is highly conservative within single species and the level of similarity of 16S rRNA gene sequence is used as indicator of the phylogenetic distance within the species (Fig. 5A). The RFLP profile (Fig. 5B) shows formation of three new restriction sites for HaeIII at all resistants. Fig. 5C shows the sequences of *S. aureus* and heavy metal resistant strains with observed mutations. The point mutations formed (Fig. 5C, marked by arrows) are likely to be created by thymine tautomerization as a consequence of direct interaction with heavy metals or adenine oxidative deamination. In our work we show that heavy metal-resistant strains of *S. aureus* differ not only in metabolic and protein profiles, but also in influence of genetic information. Three new restriction sites for HaeIII, minimally three point mutations in 1500 bp long PCR product, indicate a high level of mutations induced by heavy metals despite the increased level of antioxidants and heavy metals chelators, as shown by FRAP, glutathione and MT analysis. Changes both in biochemical and protein profiles of heavy metal-resistant *S. aureus* indicate that the observed changes in biochemical profiles are caused mainly by change in protein expression and minor by heavy metals-caused enzymes inhibition or activation. The obtained data manifest the speed of change of genetic information and therefore protein and metabolic profiles of *S. aureus* even after cultivation in pure culture without the possibility of horizontal gene transfer, which is given by

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both high mutagenity of heavy metals and their lethality and high selection pressure.

5. Conclusions

Resistance of bacterial strains to various types of environmental factors, mainly their formation and consequences, are still under investigations of numerous scientists worldwide. Therefore, some new concepts and methodologies are still looked for. In this study, we did an overview of the effects of some metal ions on *S. aureus* with the emphasis on the occurrence of the resistance to the presence of these ions. Our results describe the possible option for the analysis of *S. aureus* resistant strains and may thus serve as a support for monitoring of changes in genetic information caused by the forming of resistance to heavy metals. The analysis was done by resistance curve and viability, enzyme activity, metallothionein (as metal binding protein), protein profile and detection of mutants by RFLP. Resistance and viability results show that studied metals could be divided to three groups with different resistant mechanism, (i) zinc, (ii) cadmium, lead and copper and (iii) silver. Changes both in biochemical and protein profiles of heavy metal-resistant *S. aureus* indicate that the observed changes in biochemical profiles are caused mainly by modification in protein expression and minor by heavy metals-caused enzymes inhibition or activation. Changes in protein expression are caused by the great presence of point mutations by heavy metal-resistant *S. aureus*.

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References

Adam V, Baloun J, Fabrik I, Trnkova L, Kizek R. An electrochemical detection of metallothioneins at the zeptomole level in nanolitre volumes. Sensors 2008;8:2293–305.
Adam V, Petrlova J, Potesil D, Zehnalek J, Sures B, Trnkova L, et al. Study of metallothionein modified electrode surface behaviour in the presence of heavy metal ions-biosensor. Electroanalysis 2005;17:1649–57.
Ahsan N, Renaut J, Komatsu S. Recent developments in the application of proteomics to the analysis of plant responses to heavy metals. Proteomics 2009;9:2602–21.
Alm EW, Zimbler D, Callahan E, Plomaritis E. Patterns and persistence of antibiotic resistance in faecal indicator bacteria from freshwater recreational beaches. J Appl Microbiol 2014;117:273–85.
Ann LC, Mahmud S, Bakhori SKM, Sirelkhatim A, Mohamad D, Hasan H, et al. Antibacterial responses of zinc oxide structures against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes*. Ceram Int 2014;40:2993–3001.
Babula P, Kohoutkova V, Opatrilova R, Dankova I, Masarik M, Kizek R. Pharmaceutical importance of zinc and metallothionein in cell signalling. Chim Oggi-Chem Today 2010;28:18–21.
Babula P, Masarik M, Adam V, Eckschlager T, Stiborova M, Trnkova L, et al. Mammalians' metallothioneins and their properties and functions. Metallomics 2012;4:739–50.
Bajpai VK, Sharma A, Baek KH. Antibacterial mode of action of *Cudrania tricuspidata* fruit essential oil, affecting membrane permeability and surface characteristics of food-borne pathogens. Food Control 2013;32:582–90.
Baker J, Sengupta M, Jayaswal RK, Morrissey JA. The *Staphylococcus aureus* CsoR regulates both chromosomal and plasmid-encoded copper resistance mechanisms. Environ Microbiol 2011;13:2495–507.
Baker J, Sitthisak S, Sengupta M, Johnson M, Jayaswal RK, Morrissey JA. Copper Stress Induces a Global Stress Response in Staphylococcus aureus and Represses sae and agr Expression and Biofilm Formation. Appl Environ Microbiol 2010;76:150–60.
Barriere C, Leroy-Setrin S, Talon R. Characterization of catalase and superoxide dismutase in *Staphylococcus carnosus* 833 strain. J Appl Microbiol 2001;91:514–9.
Beach JM, Champney WS. An examination of the inhibitory effects of three antibiotics in combination on ribosome biosynthesis in *Staphylococcus aureus*. Arch Microbiol 2014;196:249–60.
Bir F, Khireddine H, Touati A, Sidane D, Yala S, Oudadesse H. Electrochemical depositions of fluorohydroxyapatite doped by Cu2+, Zn2+, Ag+ on stainless steel substrates. Appl Surf Sci 2012;258:7021–30.
Blaszak M, Bienkowska D. Effect of soil pollution on bacterial resistance to lead ions: an experimental approach. Pol J Ecol 2009;57:555–60.
Bohme K, Morandi S, Cremonesi P, No ICF, Barros-Velazquez J, Castiglioni B, et al. Characterization of *Staphylococcus aureus* strains isolated from Italian dairy products by MALDI-TOF mass fingerprinting. Electrophoresis 2012;33:2355–64.
Borneman DL, Ingham SC, Ane C. Mathematical Approaches To Estimating Lag-Phase Duration and Growth Rate for Predicting Growth of *Salmonella* Serovars, *Escherichia coli* O157:H7, and *Staphylococcus aureus* in Raw Beef, Bratwurst, and Poultry. J Food Prot 2009;72:1190–200.
Bruins MR, Kapil S, Oehme FW. Microbial resistance to metals in the environment. Ecotox Environ Safe 2000;45:198–207.
Charyulu EM, Gnanamani A, Mandal AB. Identification and Discrimination of Methicillin Resistant *Staphylococcus aureus* Strains Isolated from Burn Wound Sites Using PCR and Authentication with MALDI-TOF-MS. Indian J Microbiol 2012;52:337–45.
Chudobova D, Nejdl L, Gumulec J, Krystofova O, Rodrigo MAM, Kynicky J, et al. Complexes of Silver(I) Ions and Silver Phosphate Nanoparticles with Hyaluronic Acid and/or Chitosan as Promising Antimicrobial Agents for Vascular Grafts. Int J Mol Sci 2013;14:13592–614.
Chudobova D, Dostalova S, Blazkova I, Michalek P, Ruttkay-Nedecky B, Sklenar M, et al. Effect of Ampicillin, Streptomycin, Penicillin and Tetracycline on Metal Resistant and Non-Resistant *Staphylococcus aureus*. Int J Environ Res Public Health 2014;11:3233–55.
Coyle P, Philcox JC, Carey LC, Rofe AM. Metallothionein: The multipurpose protein. Cell Mol Life Sci 2002;59:627–47.
Dintilhac A, Alloing G, Granadel C, Claverys JP. Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. Mol Microbiol 1997;25:727–39.
Dragun Z, Marijic VF, Kapetanovic D, Valic D, Smrzlic IV, Krasnici N, et al. Assessment of general condition of fish inhabiting a moderately contaminated aquatic environment. Environ Sci Pollut Res 2013;20:4954–68.
Duffy J, Dumyati G, Bulens S, Namburi S, Gellert A, Fridkin SK, Lessa FC. Community-onset invasive methicillin-resistant *Staphylococcus aureus* infections following hospital discharge. Am J Infect Control 2013;41:782–6.
Eckschlager T, Adam V, Hrabeta J, Figova K, Kizek R. Metallothioneins and cancer. Curr Protein Pept Sci 2009;10:360–75.
Elased KM, Cool DR, Morris M. Novel mass spectrometric methods for evaluation of plasma angiotensin converting enzyme 1 and renin activity. Hypertension 2005;46:953–9.
Elased KM, Cunha TS, Gurley SB, Coffman TM, Morris M. New mass spectrometric assay for angiotensin-converting enzyme 2 activity. Hypertension 2006;47:1010–7.
Erk M, Muyssen BTA, Ghekiere A, Janssen CR. Metallothioneins and cytosolic metals in Neomysis integer exposed to cadmium at different salinities. Mar Environ Res 2008;65:437–44.
Fernandez-Saiz P, Soler C, Lagaron JM, Ocio MJ. Effects of chitosan films on the growth of *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella* spp. in laboratory media and in fish soup. Int J Food Microbiol 2010;137:287–94.
Gopal J, Hasan N, Manikandan M, Wu HF. Bacterial toxicity/compatibility of platinum nanospheres, nanocuboids and nanoflowers. Sci Rep 2013;3:1–8.
Gumulec J, Raudenska M, Hlavna M, Stracina T, Sztalmachova M, Tanhauserova V, et al. Determination of oxidative stress and activities of antioxidant enzymes in guinea pigs treated with haloperidol. Exp Ther Med 2013;5:479–84.
Gupta A, Matsui K, Lo JF, Silver S. Molecular basis for resistance to silver cations in Salmonella. Nat Med 1999;5:183–8.
He RJ, Zou BQ, Fang H, Zhang Y, Wu YR. Free Radical Scavenging Activities of beta-Sitosterol Extracted from *Abies yuanbaoshanensis*. Asian J Chem 2013;25:3507–8.
Henkel G, Krebs B. Metallothioneins: Zinc, cadmium, mercury, and copper thiolates and selenolates mimicking protein active site features - Structural aspects and biological implications. Chem Rev 2004;104:801–24.
Ivankovic D, Pavicic J, Erk M, Filipovic-Marijic V, Raspor B. Evaluation of the *Mytilus galloprovincialis* Lam. digestive gland metallothionein as a biomarker in a long-term field study: Seasonal and spatial variability. Mar Pollut Bull 2005;50:1303–13.
Kahankova J, Pantucek R, Goerke C, Ruzickova V, Holochova P, Doskar J. Multilocus PCR typing strategy for differentiation of *Staphylococcus aureus* siphoviruses reflecting their modular genome structure. Environ Microbiol 2010;12:2527–38.
Kaiser A, Kammerer DR, Carle R. Impact of blanching on polyphenol stability and antioxidant capacity of innovative coriander (*Coriandrum sativum* L.) pastes. Food Chem 2013;140:332–9.
Kaur G, Singh TP, Malik RK, Bhardwaj A, De S. Antibacterial efficacy of nisin, pediocin 34 and enterocin FH99 against *L. monocytogenes*, *E. faecium* and *E. faecalis* and bacteriocin cross resistance and antibiotic susceptibility of their bacteriocin resistant variants. J Food Sci Technol-Mysore 2014;51:233–44.
Kim JS, Kuk E, Yu KN, Kim JH, Park SJ, Lee HJ, et al. Antimicrobial effects of silver nanoparticles. Nanomed-Nanotechnol Biol Med 2007;3:95–101.
Klaassen CD, Liu J, Diwan BA. Metallothionein protection of cadmium toxicity. Toxicol Appl Pharmacol 2009;238:215–20.
Kolenbrander PE, Andersen RN, Baker RA, Jenkinson HF. The adhesion-associated sca operon in *Streptococcus gordonii* encodes an inducible high-affinity ABC transporter for Mn2+ uptake. J Bacteriol 1998;180:290–5.
Krizkova S, Ryvolova M, Hrabeta J, Adam V, Stiborova M, Eckschlager T, Kizek R. Metallothioneins and zinc in cancer diagnosis and therapy. Drug Metab Rev 2012;44:287–301.

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Krizkova S, Adam V, Petrlova J, Zitka O, Stejskal K, Zehnalek J, et al. A suggestion of electrochemical biosensor for study of platinum(II)-DNA interactions. Electroanalysis 2007;19:331–8.
Kumari H, Balasubramanian D, Zincke D, Mathee K. Role of Pseudomonas aeruginosa AmpR on beta-lactam and non-beta-lactam transient cross-resistance upon pre-exposure to subinhibitory concentrations of antibiotics. J Med Microbiol 2014;63:544–55.
Lebrun M, Audurier A, Cossart P. Plasmid-borne cadmium resitance genes in *Listeria-monocytogenes* are similar to Cada and Cadc of *Staphylococcus aureus* and are induced by cadmium. J Bacteriol 1994;176:3040–8.
Leucker TM, Reddy SV, Moffett BK. Methicillin-resistant *Staphylococcus aureus* Induced Reactive Arthritis Treated With Systemic Corticosteroid. Am J Med Sci 2013;346:172–3.
Levinson HS, Mahler I, Blackwelder P, Hood T. Lead resistance and sensitivity in *Staphylococcus aureus*. FEMS Microbiol Lett 1996;145:421–5.
Lim CK, Hassan KA, Penesyan A, Loper JE, Paulsen IT. The effect of zinc limitation on the transcriptome of *Pseudomonas protegens* Pf-5. Environ Microbiol 2013;15:702–15.
Machado A, Bordalo AA. Prevalence of antibiotic resistance in bacteria isolated from drinking well water available in Guinea-Bissau (West Africa). Ecotox Environ Safe 2014;106:188–94.
Muller P, Alber DG, Turnbull L, Schlothauer RC, Carter DA, Whitchurch CB, Harry EJ. Synergism between Medihoney and Rifampicin against Methicillin-Resistant *Staphylococcus aureus* (MRSA). Plos One 2013;8:1–9.
Naik MM, Shamim K, Dubey SK. Biological characterization of lead-resistant bacteria to explore role of bacterial metallothionein in lead resistance. Curr Sci 2012;103:426–9.
Naik MM, Dubey SK, Khanolkar D, D'Costa B. P-type ATPase and MdrL efflux pump-mediated lead and multi-drug resistance in estuarine bacterial isolates. Curr Sci 2013;105:1366–72.
Nedbalcova K, Nechvatalova K, Pokludova L, Bures J, Kucerova Z, Koutecka L, Hera A. Resistance to selected beta-lactam antibiotics. Vet Microbiol 2014;171:328–36.
Nguyen HNK, Van TTH, Nguyen HT, Smooker PM, Shimeta J, Coloe PJ. Molecular characterization of antibiotic resistance in *Pseudomonas* and *Aeromonas* isolates from catfish of the Mekong Delta, Vietnam. Vet Microbiol 2014;171:397–405.
Nies DH. Resistance to cadmium, cobalt, zinc, and nickel in microbes. Plasmid 1992;27:17–28.
Nies DH. Microbial heavy-metal resistance. Appl Microbiol Biotechnol 1999;51:730–50.
Nies DH. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev 2003;27:313–39.
Nies DH, Silver S. Ion efflux systems involved in bacteria metal resistances. J Indust Microbiol 1995;14:186–99.
Odermatt A, Krapf R, Solioz M. Induction of putative copper ATPases, CopA and CopB, of *Enterococcus-hirae* by Ag+ and Cu2+, and Ag+ extrusion by CopB. Biochem Biophys Res Commun 1994;202:44–8.
Odermatt A, Suter H, Krapf R, Solioz M. Primary structure of 2-P-type ATPases involved in copper homeostasis in *Enterococcus-hirae*. J Biol Chem 1993;268:12775–9.
Ohlsen K, Ternes T, Werner G, Wallner U, Loffler D, Ziebuhr W, et al. Impact of antibiotics on conjugational resistance gene transfer in *Staphylococcus aureus* in sewage. Environ Microbiol 2003;5:711–6.
Pekarik V, Gumulec J, Masarik M, Kizek R, Adam V. Prostate cancer, miRNAs, metallothioneins and resistance to cytostatic drugs. Curr Med Chem 2013;20:534–44.
Pesavento G, Calonico C, Ducci B, Magnanini A, Lo Nostro A. Prevalence and antibiotic resistance of *Enterococcus* spp. isolated from retail cheese, ready-to-eat salads, ham, and raw meat. Food Microbiol 2014;41:1–7.
Poehlsgaard J, Douthwaite S. The bacterial ribosome as a target for antibiotics. Nat Rev Microbiol 2005;3:870–81.
Raspor B, Paic M, Erk M. Analysis of metallothioneins by the modified Brdicka procedure. Talanta 2001;55:109–15.
Rodriguez C, Taminiau B, Avesani V, Van Broeck J, Delmee M, Daube G. Multilocus sequence typing analysis and antibiotic resistance of *Clostridium difficile* strains isolated from retail meat and humans inBelgium. Food Microbiol 2014;42:166–71.
Roosa S, Wattiez R, Prygiel E, Lesven L, Billon G, Gillan DC. Bacterial metal resistance genes and metal bioavailability in contaminated sediments. Environ Pollut 2014;189:143–51.
Rouch DA, Lee BTO, Morby AP. Undestanding cellular-responses to toxic agents - a model for mechanism - choice in bacteria metal resistance. J Indust Microbiol 1995;14:132–41.
Rufian-Henares JA, Morales FJ. Microtiter plate-based assay for screening antimicrobial activity of melanoidins against *E-coli* and *S-aureus*. Food Chem 2008;111:1069–74.
Ryvolova M, Adam V, Kizek R. Analysis of metallothionein by capillary electrophoresis. J Chromatogr A 2012;1226:31–42.
Sauer S, Freiwald A, Maier T, Kube M, Reinhardt R, Kostrzewa M, Geider K. Classification and Identification of Bacteria by Mass Spectrometry and Computational Analysis. Plos One 2008;3:1–10.
Seniya C, Verma SK, Trivedia SS, Verma R, Vijayarti HS, Vyas S. Metal Stress and Antibiotic Susceptibility Profile of Some Bacterial and Fungal Strains. J Pure Appl Microbiol 2012;6:1727–34.
Sevcikova M, Modra H, Kruzikova K, Zitka O, Hynek D, Adam V, et al. Effect of Metals on Metallothionein Content in Fish from Skalka and Zelivka Reservoirs. Int J Electrochem Sci 2013;8:1650–63.
Schreurs WJA, Rosenberg H. Effect of silver ions on transport and retention of phosphate by *Escherichia coli*. J Bacteriol 1982;152:7–13.
Silver S, Ji GG. Newer system for bacterial resistances to toxic heavy-metals. Environ Health Perspect 1994;102:107–13.
Silver S, Phung LT. Bacterial heavy metal resistance: New surprises. Annu Rev Microbiol 1996;50:753–89.
Singh P, Raghukumar C, Parvatkar RR, Mascarenhas-Pereira MBL. Heavy metal tolerance in the psychrotolerant *Cryptococcus* sp isolated from deep-sea sediments of the Central Indian Basin. Yeast 2013;30:93–101.
Smerkova K, Dostalova S, Skutkova H, Ryvolova M, Adam V, Provaznik I, Kizek R. Isolation of Xis Gen Fragment of lambda Phage from Agarose Gel Using Magnetic Particles for Subsequent Enzymatic DNA Sequencing. Chromatographia 2013;76:329–34.
Smith RL, Thompson LJ, Maguire ME. Cloning and characterization of MgTe, a putative new class of Mg^{2+} transporter from *Bacillus-firmus* OF4. J Bacteriol 1995;177:1233–8.
Sochor J, Pohanka M, Ruttkay-Nedecky B, Zitka O, Hynek D, Mares P, et al. Effect of selenium in organic and inorganic form on liver, kidney, brain and muscle of Wistar rats. Cent Eur J Chem 2012;10:1442–51.
Sochor J, Ryvolova M, Krystofova O, Salas P, Hubalek J, Adam V, et al. Fully Automated Spectrometric Protocols for Determination of Antioxidant Activity: Advantages and Disadvantages. Molecules 2010;15:8618–40.
Song YF, Cui J, Zhang HX, Wang GP, Zhao FJ, Shen ZG. Proteomic analysis of copper stress responses in the roots of two rice (*Oryza sativa* L.) varieties differing in Cu tolerance. Plant Soil 2013;366:647–58.
Sorensen SJ, Bailey M, Hansen LH, Kroer N, Wuertz S. Studying plasmid horizontal transfer in situ: A critical review. Nat Rev Microbiol 2005;3:700–10.
Stomeo F, Valverde A, Pointing SB, McKay CP, Warren-Rhodes KA, Tuffin MI, et al. Hypolithic and soil microbial community assembly along an aridity gradient in the Namib Desert. Extremophiles 2013;17:329–37.
Szabados F, Woloszyn J, Richter C, Kaase M, Gatermann S. Identification of molecularly defined *Staphylococcus aureus* strains using matrix-assisted laser desorption/ionization time of flight mass spectrometry and the Biotyper 2.0 database. J Med Microbiol 2010;59:787–90.
Tao T, Snavely MD, Farr SG, Maguire ME. Magnesium transport in *Salmonella-typhimurium* - MgtA encodes a P-type ATPase and is regulated by Mg2+ in a manner similar to that of MgtB P-type ATPase. J Bacteriol 1995;177:2654–62.
Taylor AR. Methicillin-Resistant *Staphylococcus Aureus* Infections. Primary Care 2013;40:637–54.
Templeton DM, Cherian MG. Toxicological significance of metallothionein. Method Enzymol 1991;205:11–24.
Toriumi S, Saito T, Hosokawa T, Takahashi Y, Numata T, Kurasaki M. Metal binding ability of metallothionein-3 expressed in Escherichia coli. Basic Clin Pharmacol Toxicol 2005;96:295–301.
Townsend DE, Esenwine AJ, George J, Bross D, Maguire ME, Smith RL. Cloning of the MgtE Mg^{2+} transporter from *Providencia-stuartii* and the distribution of MgtE in gram-negative and gram-positive bacteria. J Bacteriol 1995;177:5350–4.
Turner PE, Draghi JA, Wilpiszeski R. High-throughput analysis of growth differences among phage strains. J Microbiol Methods 2012;88:117–21.
van Veen SQ, Claas ECJ, Kuijper EJ. High-Throughput Identification of Bacteria and Yeast by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry in Conventional Medical Microbiology Laboratories. J Clin Microbiol 2010;48:900–7.
Vasak M. Advances in metallothionein structure and functions. J Trace Elem Med Biol 2005;19:13–7.
Waalkes MP, Harvey MJ, Klaassen CD. Relative in vitro affinity of hepatic metallothionein for metals. Toxicol Lett 1984;20:33–9.
Wiesemann N, Mohr J, Grosse C, Herzberg M, Hause G, Reith F, Nies DH. Influence of Copper Resistance Determinants on Gold Transformation by *Cupriavidus metallidurans* Strain CH34. J Bacteriol 2013;195:2298–308.
Wong SG, Lee MY. Comparison of the IC50-shift and % inhibition assay to evaluate time-dependent inhibition. Drug Metab Rev 2014;45:115.

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5.2 Studium oxidačního stresu působením iontů stříbra u bakteriálních kultur s využitím biochemických markerů

5.2.1 Vědecký článek II

CHUDOBOVA, D.; DOBES, J; NEJDL, L.; MASKOVA, D.; MERLOS RODRIGO, M. A.; RUTTKAY-NEDECKY, B.; KRYSTOFOVA, O.; KYNICKY, J.; KONECNA, M.; POHANKA, M.; HUBALEK, J.; ZEHNALEK, J.; KLEJDUS, B.; KIZEK, R.; ADAM, V. Oxidative Stress in *Staphylococcus aureus* Treated with Silver (I) Ions Revealed by Spectrometric and Voltametric Assays. *International Journal of Electrochemical Sciences*, 2013, roč. 8. č. 4, s. 4422-4440. ISSN 1452-3981.

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Oxidační stres je jedním z ukazatelů, který umožňuje monitoring toxických účinků těžkých kovů obecně, zejména však stříbrných iontů, na mikroorganismy [114, 216]. Tento toxický účinek je založen na vazbě stříbrných iontů k bakteriální buněčné stěně a membránám, což vede k inhibici dýchacího procesu [110, 113, 217]. Díky schopnosti stříbrných iontů vyvolat nadměrnou produkci volných kyslíkových radikálů (ROS), které ovlivňují téměř všechny biomolekuly, jsou zároveň schopny vyvolat metabolickou toxicitu. Organismy mají ochranné mechanismy, kterými mohou volné radikály účinně odstraňovat a tím eliminovat jejich toxické účinky [114, 115]. Na druhé straně, tyto ochranné mechanismy mají omezené možnosti založené na proteosyntetických a obecně biosyntetických schopnostech. Kromě toho, nadbytek ROS umožňuje sledování oxidačního stresu na základě stanovení antioxidační kapacity [218, 219].

Práce byla zaměřena na studium oxidačního stresu u bakteriální kultury *Staphylococcus aureus* po aplikaci iontů stříbra. Byl sledován vliv sedmi koncentrací $AgNO_3$ (10, 25, 50, 75, 150, 225 a 300 μM). Bylo provedeno testování antibakteriálního účinku stříbrných iontů na růst bakteriální kultury *S. aureus* pomocí mikrobiologických metod – měření inhibičních zón a růstových křivek. Dále byla pozorována antioxidační aktivita bakteriálních lyzátů *S. aureus* samotného a inkubovaného v přítomnosti různých koncentrací $AgNO_3$ za podmínek zvýšeného oxidačního stresu způsobeného přidáním peroxidu vodíku (4, 5, 6, 7, 8, 9 a 10 mM). Oxidační stres u bakteriálních lyzátů byl studován spektrofotometricky za použití dvou různých metod stanovení antioxidační aktivity ABTS (2.2´-azino-bis(3-ethylbenzotiazolin-6-sulfonová kyselina) a DPPH

(2.2´difenyl-1-pikrylhydrazyl). Oxidační stres byl taktéž studován metodami elektrochemickými pomocí cyklické voltametrie.

Byl prokázán zvýšený oxidační stres jasně patrný se zvyšující se koncentrací iontů stříbra a peroxidu vodíku. Výsledky objasňují roli stříbrných iontů při vzniku oxidačního stresu bakterií a ukazují na možnost použití uvedených analytických metod v mikrobiologii ke stanovení těchto parametrů.

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Article

Oxidative Stress in *Staphylococcus aureus* Treated with Silver(I) Ions Revealed by Spectrometric and Voltammetric Assays

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We were focused on the studying oxidative stress in bacterial culture of *Staphylococcus aureus* induced by silver ions. The effect of the seven concentrations of $AgNO_3$ (10, 25, 50, 75, 150, 225 and 300 μM) in bacterial culture was studied. Further, antioxidant activity of bacterial lysates of *Staphylococcus aureus* alone and incubated with different concentrations of $AgNO_3$ under conditions of increased oxidative stress caused by the addition of hydrogen peroxide (4, 5, 6, 7, 8, 9 and 10 mM) was observed. Oxidative stress in bacterial lysates was studied spectrophotometrically using two different methods for the determination of antioxidant activity ABTS (2.2´-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) and DPPH (2.2-diphenyl-1-picrylhydrazyl). Oxidative stress was also studied electrochemically using cyclic voltammetry. Increasing oxidative stress was clearly noticeable with increasing concentrations of silver ions and hydrogen peroxide. Our results indicate the possibility of the application of above-mentioned analytical techniques in microbiology for determination of oxidative stress in bacterial cultures.

Keywords: *Staphylococcus aureus*; Silver; Oxidative Stress; Hydrogen Peroxide, Electrochemical Analysis; Screen Printed Electrode; Differential Pulse Voltammetry; Cyclic Voltammetry

1. INTRODUCTION

The bacterial growth is greatly inhibited due to silver(I) ions [1,2]. Silver(I) ions are stored in vacuoles and cell walls as granules inhibiting cell division, interacting with nucleic acids [3] and thiol

groups in enzymes and proteins essentials for the vital cell functions [4]. For these reason, the silver(I) ions are used to control bacterial growth in medical and non-medical applications [1,5-7]. The mechanism of action of silver(I) ions are inactivation of membrane proteins, interference with electron transport system and inhibition of respiratory enzymes to promote the generation of reactive oxygen species, especially superoxide-radical, and consequent bactericidal activity [2,8]. The silver nanoparticles have been also applied as antimicrobial agents. These particles have a low toxicity to human cells, and a far lesser probability to cause microorganism resistance than antibiotics [9]. Silver nanoparticles reduce the expression of some enzymes and inhibiting respiratory chain. When silver nanoparticles enter into bacteria cells, they condense DNA to prevent DNA from replicating and cells from reproducing [6]. Under aerobic conditions silver(I) ions exhibit higher bactericidal activity against *Staphylococcus aureus*, a model strains for gram positive bacteria.

Oxidative stress is one of the markers, which enables monitoring of toxic effects of generally heavy metals including silver(I) ions on microorganisms [2,10]. This toxic effect is based on the binding of silver(I) ions to the bacterial cell wall and membranes, which leads to inhibition of the respiratory process [6,11,12]. Due to ability of silver(I) ions to induce excessive production of ROS that affect almost all biomolecules, they are also able to cause metabolic toxicity. Organisms have the protective mechanisms that can effectively eliminate formed free radicals and thus eliminate their toxic effects [2,13]. On the other hand, these protective mechanisms have limited capabilities based on the proteosynthetic and generally biosynthetic abilities. Moreover, excess of ROS enables the monitoring of oxidative stress based on determination of antioxidant capacity [14,15].

Numerous methods for determination of antioxidant activity have been developed in the field of chemical analysis and biological evaluation of antioxidant characteristics [16-29]. Their diversity is given by the fact that low molecular weight antioxidants may act by different mechanisms, most often by the direct reaction with free radicals (quenching, trapping). A more precise definition of the chemical mechanism of their effect is often issue. Therefore, the procedures evaluating the antioxidant activity are based on chemically different principles [27,29,30].

Spectrophotometric methods are the most used methods in the determination of antioxidant activity/capacity. We studied oxidative stress in *S. aureus* extracts influenced by silver ions and hydrogen peroxide. In our study for the measurement of oxidative stress we used two different methods– ABTS (2.2´-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) and DPPH (2.2-diphenyl-1-picrylhydrazyl). By these methods we determined the amount of radicals in *S. aureus* lysates. The results of spectrophotometric studies were completed with electrochemical analysis. Oxidative stress in *S.aureus* lysates was also studied by cyclic voltammetry using printed electrode and flow cell.

2. EXPERIMENTAL PART

2.1 Cultivation of Staphylococcus aureus

Staphylococcus aureus (NCTC 8511) was obtained from the Czech Collection of Microorganisms, Faculty of Science, Masaryk University, Brno, Czech Republic. Strains were stored as a spore suspension in 20% (*v/v*) glycerol at -20°C. Prior to use in this study, the strains were thawed

and the glycerol was removed by washing with distilled water. The composition of cultivation medium was as follows: meat peptone 5 g/l, NaCl 5 g/l, bovine extract 1.5 g/l, yeast extract 1.5 g/l (HIMEDIA, Mumbai, India), sterilized MilliQ water with 18 MΩ. pH of the cultivation medium was adjusted at 7.4 before sterilization. Sterilization of media was carried out at 121 °C for 30 min. in sterilizer (Tuttnauer 2450EL, Israel). The prepared cultivation media were inoculated with bacterial culture into 25 ml Erlenmeyer flasks. After inoculation, bacterial cultures were cultivated for 24 hours on a shaker at 600 rpm and 37 °C. Bacterial culture cultivated under these conditions was diluted by cultivation medium to OD_{600} = 0.1 and used in the following experiments.

2.2 Chemicals, preparation of deionised water and pH measurement

Chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, USA) in ACS purity unless noted otherwise. The deionised water was prepared using reverse osmosis equipment Aqual 25 (Czech Republic). The deionised water was further purified by using apparatus MiliQ Direct QUV equipped with the UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

2.3 Determination of protein mass spectra for identification of bacteria Staphylococcus aureus

500 µl *S. aureus* culture, grown overnight, was centrifuged at 14,000 × g for 2 min., the supernatant was discarded and the pellet was suspended in 300 µl of de-ionized water; then, 900 µl of ethanol was added. After centrifugation at 14,000 × g for 2 min, the supernatant was discarded and the pellet was air-dried. The pellet was then dissolved in 25 µl of 70 % formic acid and 25 µl of acetonitrile and mixed. The samples were centrifuged at 14,000 × g for 2 min and 1 µl of the clear supernatant was spotted in duplicate onto the MALDI target (MTP 384 target polished steel plate; Bruker Daltonics, Bremen, Germany) and air-dried at room temperature. Then, each spot was overlaid with 1 µl of α-cyano-4-hydroxy cinnamic acid (HCCA) matrix solution saturated with organic solvent (50 % acetonitrile and 2.5 % trifluoroacetic acid, both *v*/*v*) and air-dried completely prior to matrix-assisted laser desorption-ionization time-of-flight mass spectrometric (MALDI-TOF MS) measurement. The spectra were taken in the m/z range of 2,000 Da to 20,000 Da, and each was the result of the accumulation of at least 1000 laser shots obtained from ten different regions of the same sample spot. Spectra were analysed with the Flex Analysis software (Version 3.4). Prior to analysis, the mass spectrometer was externally calibrated with a peptide mix of bombesin, angiotensin I, glu-fibrinopeptide B, adrenocorticotropic hormone (ACTH) (18-39), ubiquitine, and cytochrome c. Spectra with peaks outside the allowed average were not considered. Modified spectra were loaded into the MALDI BioTyper™ 3.1 Version (Bruker Daltonik GmbH, Bremen, Germany). Software settings for MSP creation were set to: maximal mass error of each single spectrum: 2,000; desired mass error for the MSP: 250. Reference spectra were created automatically by the software and all created spectra were added to the main spectra library as unassigned MSPs. Spectra loaded into MALDI BioTyper™ 3.1 Version were measured at the default settings. Unknown spectra were compared with the created reference library by using a score value, the common decadal logarithm for matching results. Results

were analysed following the score value system according to Bruker Daltonik GmbH (Bremen, Germany). Values from 3.00 to 2.30 indicate reliable species identification.

2.4 Spectrophotometric measurements of antimicrobial activity of silver ions

2.4.1 Zones of inhibition

To determine the antimicrobial effect of silver(I) ions (0, 10, 25, 50, 75, 150, 225 and 300 μM), the measurement of the inhibition zones on Petri dishes was done. Agar surface in Petri dish was covered with a mixture of 100 ml of 24 hour grown culture of *S. aureus* and 3 ml of LB medium (Luria Bertani medium). Excess volume of the mixture of the Petri dishes was aspirated. From the fabrics, which were made by Výzkumný ústav pletařský in Brno, were cut out squares in size 1x1 cm. In Eppendorf tubes these were mixed with different concentration of silver(I) ions. Soaked squares were then laid crosswise on a Petri dish, two squares per dish. Petri dishes were insulated against possible external contamination and placed in a thermostat preset at 37 °C for 24 hours. After 24 hour incubation, for each dish the zones of inhibition were measured and photographed.

2.4.2 Growth curves

A second procedure for the evaluation of the antimicrobial effect of tested compounds and their combinations was measuring the absorbance using the apparatus Multiskan EX (Thermo Fisher Scientific, Germany) and subsequent analysis in the form of growth curves. The same culture as in the previous procedure was diluted with LB medium using Specord spectrophotometer at 210 (Analytik Jena, Germany) at a wavelength of 600 nm to absorbance 0.1. In the microplate *S. aureus* culture was mixed with various concentrations of silver(I) ions, hydrogen peroxide or combination of these substances or *S. aureus* alone as a control for measurements. The concentrations of silver(I) ions were 0, 10, 25, 50, 75, 150, 225 and 300 μM. The concentrations of hydrogen peroxide were 0, 4, 5, 6, 7, 8, 9 and 10 mM. Total volume in the microplate wells was always 300 μl. Measurements were carried out at time 0, then each half-hourly for 24 hours at 37 °C and a wavelength of 620 nm. The values achieved were analysed in graphic form as growth curves for each variant individually.

2.5 Preparation of S.aureus lysates for spectrophotometric and electrochemical measurements

After inoculation, bacterial cultures were cultivated on a shaker for 24 hours at 600 rpm and 37 °C. Then bacterial cultures were mixed with silver(I) ions to reach final concentrations (0, 10, 25, 50, 75, 150, 225 and 300 μM) and incubated for further 3 hours at 37 °C. After incubation with silver(I) ions samples were collected by centrifugation (5000 rpm, 15 min.). Bacterial cells were three times washed with phosphate buffer (pH = 7). Finally, cells were resuspended in phosphate buffer (pH = 7, volume 1.5 ml) and sonicated using the ultrasound needle (Hielscher, Germany) for 2 minutes. Homogenates were vortexed (5 min, BioSan, Riga, Latvia) and centrifuged (1600 rpm, 30 min.). Obtained supernatants of bacterial lysates were used for the spectrophotometric and electrochemical determination of oxidative stress. For experiments with hydrogen peroxide (final concentrations: 0, 4,

5, 6, 7, 8, 9 and 10 mM) and hydrogen peroxide in combination with silver(I) ions, hydrogen peroxide was added 15 minutes prior to measurement.

2.6 Spectrophotometric measurements of oxidative stress

Spectrophotometric measurements of total protein content, oxidative stress (ABTS, DPPH) were carried using an automated chemical analyser BS-400 (Mindray, Shenzhen, China). Reagents and samples were placed on cooled sample holder (4 ± 1 °C) and automatically pipetted directly into plastic cuvettes. Incubation proceeded at 37.0 ± 0.1 °C. Mixture was consequently stirred. The washing steps of pipetting needle with distilled water (18 mΩ) were done in the midst of the pipetting. For detection itself, the following range of wave lengths can be used - 340, 380, 412, 450, 505, 546, 570, 605, 660, 700, 740 and 800 nm. The instrument was operated using the BS-400 software (Mindray).

2.6.1 Determination of total proteins by the pyrogallol red

The pyrogallol red protein assay (Skalab) is based upon formation of a blue protein-dye complex in the presence of molybdate under acidic conditions (pH=2.5). A 150 µl volume of reagent mixture (1:1) R1+R2 (50 mM succinic acid, 3.47 mM sodium benzoate, 0.06 mM sodium molybdate, 1.05 mM sodium oxalate and 0.07 mM pyrogallol red) is pipetted into a plastic cuvette with subsequent addition of 8 µl of sample. Absorbance is measured at $\lambda = 605$ nm after 10 minutes of incubation. Resulting value is calculated from the absorbance value of the pure reagent mixture and from the absorbance value after 10 minutes of incubation with the sample.

2.6.2 Determination of oxidative stress using the DPPH method

The DPPH test is based on the ability of the stable 2,2-diphenyl-1-picrylhydrazyl free radical to react with hydrogen donors. The $DPPH^{\bullet}$ radical displays an intense UV-VIS absorption spectrum. In this test, a solution of radical is decolourized after reduction with antioxidant (AH) or a radical ($R^{\bullet}$) in accordance with the following scheme: $DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet}$, $DPPH^{\bullet} + R^{\bullet} \rightarrow DPPH\text{-}R$ [31]. 150 µl of R1 reagent (0.095 mM 2,2-diphenyl-1-picrylhydrazyl - DPPH) was pipetted into plastic cuvette. Subsequently, volume of 15 µl of sample was added. This method is based on the ability of stable free radical of 2,2-diphenyl-1-picrylhydrazyle to react with donors of hydrogen. DPPH has strong absorption in UV-VIS spectrum, absorbance was measured for 12 min at $\lambda = 505$ nm. To assess the production of free radicals absorbance difference of the reagent without sample and reagent with sample after ten-minute incubation was taken. Then, the absorbance difference was recalculated per gram of proteins determined in the sample. After subtraction of the blank value the biggest absorbance difference was taken as 100% of amount of free radicals. The higher was the amount of free radicals, the higher was oxidative stress.

2.6.3 Determination of oxidative stress using the ABTS method

The ABTS radical method is one of the most used assays for the determination of the concentration of free radicals. It is based on the neutralization of a radical-cation arising from the one-

electron oxidation of the synthetic chromophore 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS): ABTS – e- ABTS+. This reaction is monitored spectrophotometrically by the change of the absorption value. Procedure for the determination was adopted from publication of Sochor et al. [32]. 150 µl of R1 reagent (7 mM ABTS (2,2-azinobis(3-ethylbenzothiazolin-6-sulphonic acid and 4.95 mM potassium peroxydisulphate) was pipetted into plastic cuvette. Subsequently, volume of 3 µl of sample was added. Absorbance was measured for 12 min at λ = 660 nm. To assess the production of free radicals absorbance difference of the reagent without sample and reagent with sample after ten-minute incubation was taken. Then, the absorbance difference was recalculated per gram of proteins determined in the sample. After subtraction of the blank value the biggest absorbance difference was taken as 100% of amount of free radicals. The higher was the amount of free radicals, the higher was oxidative stress.

2.7 Electrochemical determination of silver(I) ions using sensor array

Differential pulse voltammetric (DPV) measurements were performed using PalmSens (PalmSens, The Netherlands) potentiostat connected with sensor array (BUT, Czech Republic) through control box (BUT, Czech Republic). For smoothing and baseline correction the PalmSens software supplied by PalmSens was employed. As the supporting electrolyte acetate buffer (0.2 M CH_3COOH; 0.2 M CH_3COONa) was used. Applied volume of sample which was pipetted on one sensor array position was 50 µl. DPV conditions was as follows: start potential -0.2 V, end potential 0.5 V, amplitude 0.05 V, time of accumulation 60 s.

2.8 Cyclic voltammetry

Printed electrodes (three-electrode system) and the flow cell were used in this study. The sample was injected (20 µl) into the flow cell. Change of oxidative signal was recorded with potentiostat PGSTAT 101 (Methrohm, Switzerland). The experimental conditions were as it follows: scan rate 50 mV/s; potential step 0.005 V; start potential 0 V; upper vertex potential 1 V, lover vertex potential -1 V and stop potential 0 V. Screen-printed sensor was fabricated using Aurel C880 semiautomatic screen-printer (Aurel Automation, Italy) and fired using fast BTU furnace fire for thick-film processing (BTU, USA). The conductive layer was fabricated from AgPdPt based paste (ESL 9562-G). The protective layer was fabricated from a dielectric paste (ESL 4917). AE was fabricated from Pt based paste (ESL 5545). All cermet pastes were obtained from ESL ElectroScience Europe, UK and fired at 850 °C according to the recommended values in products datasheets. WE was screen-printed using special carbon paste electrodes for electrochemical sensors of (DuPont BQ221) from DuPont Company (DuPont, USA) and cured at 130 °C for 10 minutes according to datasheet. RE was screen-printed using special polymer Ag/AgCl paste (DuPont 5874, Ag: AgCl ratio 65:35) and dried at 120 ° C for 5 minutes [33-35].

3. RESULTS AND DISCUSSION

3.1 Mass spectrometric characterization of the used bacterial strain

The aim of our study was to investigate oxidative stress in *S. aureus* culture influenced by silver(I) ions. To obtain reliable results, we verified each step. We verified the purity of *S. aureus* culture, how much of the added silver was bound to *S. aureus* and both minimal and total silver inhibitory concentration. First, we verified whether we have a pure culture of *S. aureus* without admixture of other microorganisms using MALDI-TOF technique. MALDI-TOF is a technique that combines a soft, matrix-assisted, ionization process and a TOF analyser to separate the generated ions [36,37]. In MALDI-TOF mass spectra, the mixture of a biological sample with an energy-absorbing matrix allows the genesis of mostly intact-single-charged biomolecules. This tool is routinely used to identify bacterial species in clinical samples [25,38,39] and has been extensively used in biology to search biomarkers and to monitor protein post-translational modifications [40]. The invention of MALDI-TOF mass spectra enormously contributed to the understanding of protein chemistry and cell biology [41]. MALDI-TOF mass spectra applied for the classification of *S. aureus* strains. All isolated strains were clearly identified as *S. aureus* by their spectral fingerprints [42-44].

MALDI-TOF MS-based identification has been shown to be a fast and accurate technology in the identification of a variety of *S. aureus* [42,44-46]. The obtained mass spectrum is shown in Fig. 1A. A correct species diagnosis was calculated in *S. aureus* strains with a mean score of > 2.30 according to a score cut-off value of 2.0 into the MALDI BioType 3.1 (Fig. 1B). The minimum score was 1.958 and the maximum score was 2.322 (Fig. 1C). Therefore, we can ensure that our study was conducted with pure cultures of *S. aureus*, and eliminated any possibility of contamination.

3.2 Electrochemical determination of silver(I) ions

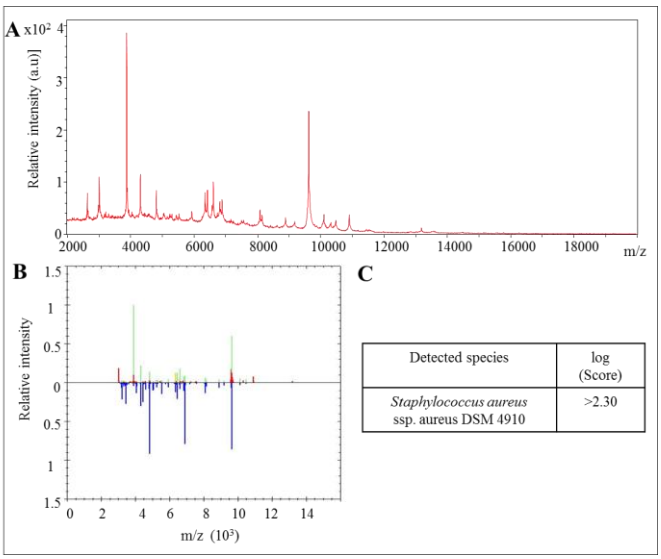

Figure 1. (**A**) MALDI/TOF mass spectra protein fingerprints for the identification of *Staphylococus aureus*. Data were collected in the m/z 2000–20000 range after processing 1 ml of *Staphylococus aureus.* (**B**) Comparison of spectra between biological samples and MSP library by MALDI Biotyper3 and (**C**) species identification by MALDI Biotyper3 using HCCA as a matrix.

To verify how much of the applied silver was bound to *S.aureus* and how much of the silver remained in the medium an electrochemical method was used. For electrochemical determination of heavy metal ions differential pulse voltammetry (DPV) is typically used [47] and can be coupled to carbon paste electrodes [48,49]. To be able to determine the concentration of a predetermined substance, it is firstly necessary to measure and construct the calibration curve, which always precedes own measurements of samples [50,51]. The calibration curve is shown in Fig. 2A with typical DP voltammogram in inset. We used this method for quantification of silver(I) ions and found that the largest quantity of applied metal was bound into bacteria *S.aureus*. Metals in the medium were measured in higher concentrations than free metals (Fig. 2B).

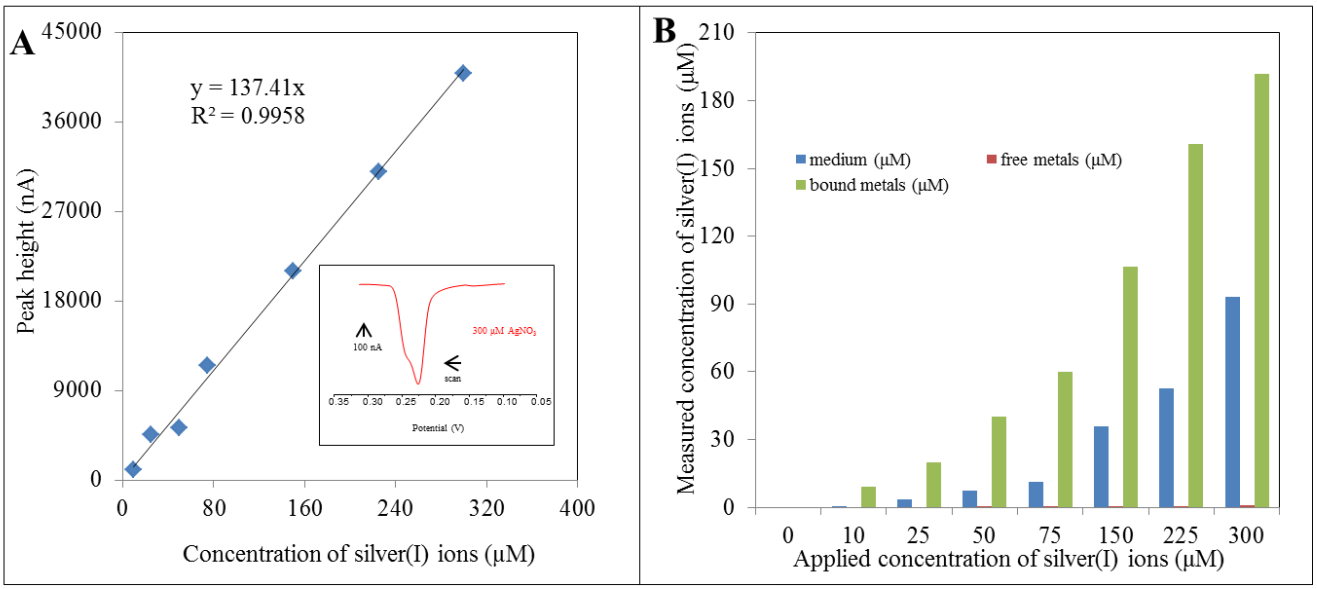

Figure 2. (**A**) Electrochemical analysis of *Staphylococus aureus*. Calibration curve of silver(I) ions concentrations (0, 10, 25, 50, 75, 150, 225 and 300 µM); in inset: DP voltammogram of 300 µM silver(I) ions. DPV parameters were as follows: start potential -0.2 V, end potential 0.5 V, amplitude 0.05 V, time of accumulation 60 s. (**B**) Electrochemical analysis of *Staphylococus aureus* and silver ions in medium, free metals and bound metals. Comparison of added amount of silver(I) ions concentrations (0, 10, 25, 50, 75, 150, 225 and 300 µM) and measured quantity.

3.3 Inhibition of bacterial growth revealed by growth curves and antioxidant activity determination

It is generally known that silver(I) ions inhibit the growth of microorganisms, thus, these ions are suitable substance for incorporation into a variety of materials where there is requirement for antimicrobial activity [52,53]. One of the ways to determine the antibacterial effect of silver ions on bacteria is the growth curves method [54-56]. Using the growth curves method for *S. aureus*, we determined the minimal inhibition concentration (MIC) [57] and total inhibition concentration (TIC) [58]. Measurements were made both in seven added concentrations of silver(I) ions (concentration of $AgNO_3$ were 0, 10, 25, 50, 75, 150, 225 and 300 µM). Minimal inhibition concentration (MIC) of *S. aureus* and $AgNO_3$ resulted from concentration of 10 µM (Fig. 3A), total inhibition concentration was

then achieved with silver(I) ions at concentration of 150 µM. Increasing inhibition of *S. aureus* growth which was caused by the addition of silver(I) ions was also confirmed by the dependency graph of the growth rate of bacteria on the applied concentration of $AgNO_3$ (Fig. 3B). In experimental groups of *S. aureus* with the addition of various concentrations of silver(I) ions, growth curve regression equations were determined from the initial six hours. From these equations, the bar chart was compiled for each variant, which tells us the growth speed of *S. aureus* in the first six hours after the addition of $AgNO_3$ concentrations (Fig. 3B).

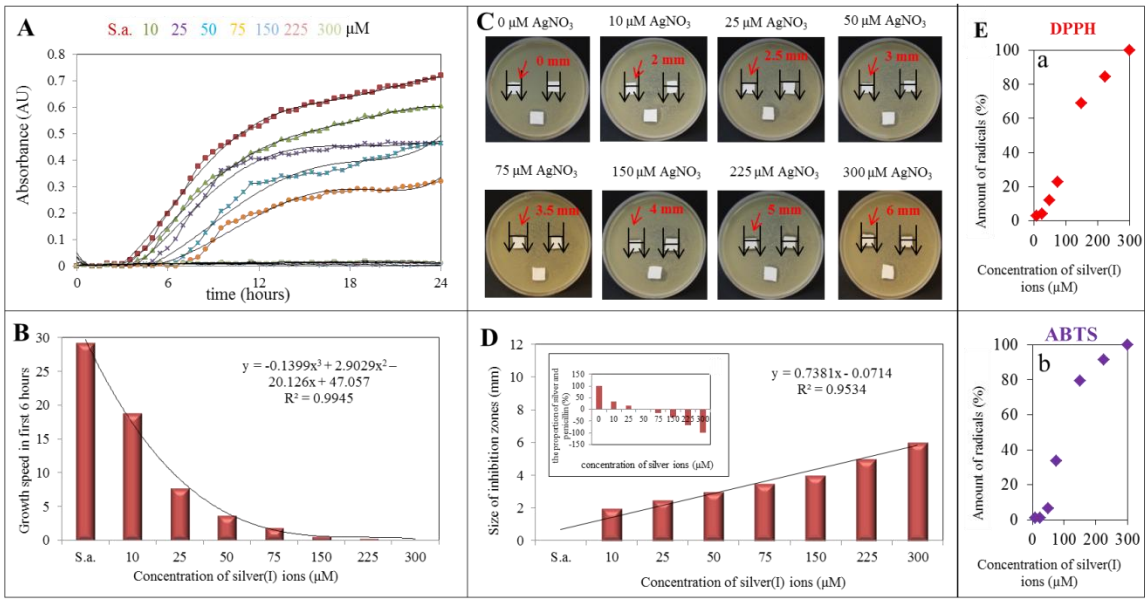

Figure 3. (**A**) Spectrophotometric analysis of the *S. aureus* growth with applied silver(I) ions concentrations 0, 10, 25, 50, 75, 150, 225 and 300 µM in 24 hours. (**B**) Spectrophotometric analysis of the *S. aureus* growth speed with applied silver(I) ions concentrations 0, 10, 25, 50, 75, 150, 225 and 300 µM in 6 hours. (**C**) Zones of inhibition of the *Staphylococus aureus* with applied silver(I) ions concentrations 0, 10, 25, 50, 75, 150, 225 and 300 µM. (**D**) The dependence of the size of the inhibition zones on the applied concentration of silver(I) ions (0, 10, 25, 50, 75, 150, 225 and 300 µM); in inset: The percentage of silver and penicillin. Comparison of the size of the zones of inhibition caused by treatment with various concentrations of silver(I) ions and a constant concentration of penicillin (300 µM). (**E**) Spectrophotometric analysis of the *S. aureus* oxidative stress using analytic methods (a – DPPH, b – ABTS). Dependence of amount of radicals (%) on applied silver(I) ions concentrations (0, 10, 25, 50, 75, 150, 225 and 300 µM).

The antimicrobial properties of silver and other heavy metals on microorganisms (*Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, etc.) have been tested by many studies [59]. To compare the results of antimicrobial properties of silver(I) ions on the bacterium *S. aureus*, obtained by growth curves method, a method of determining the inhibition zones on Petri dishes was used [60-62]. With this method, after 24 hours of cultivation in thermostat at 37 °C, zones of inhibition with increasing intensity in response to increased concentrations of added silver(I) ions were determined (Fig. 3C). Size of the inhibition zones thus ranged from 2 mm to 6 mm, with the widest inhibition zone of a combination of *S. aureus* with 300 µM $AgNO_3$. From the measured size of the

inhibition zones the dependence of the applied concentration of silver(I) ions on the size of these zones was compiled. With upward trend of this graph, we therefore confirmed that the size of the inhibition zones increased with increasing concentration of $AgNO_3$ (Fig. 3D). Moreover, it is shown in inset in Fig. 3D a comparison of the size of the inhibition zones after exposure to different concentrations of silver(I) ions and a constant concentration of penicillin. By comparing the inhibition zones of different concentrations of silver(I) ions with the inhibition zones of control antibiotics penicillin (applied in constant concentration 300 μM) we concluded that the same size of inhibition zones of silver(I) ions has been already achieved at concentration of 50 μM, thus they showed greater antibacterial effects than antibiotics.

Microorganisms, such as *Staphylococcus* species are used as starter cultures in fermented meat products, reduce the level of volatile substances produced by oxidation of lipids. For these, and other micro-organisms is necessary to determine their antioxidant capacity using spectrophotometric methods [50,63]. In this study, we focused on determination of oxidative stress in bacterial lysates caused by silver(I) ions and hydrogen peroxide. Effect of silver(I) ions on oxidative stress in bacteria *S.aureus* is shown in Fig. 3Ea and Eb. Oxidative stress in *S. aureus* lysates prepared after incubation with different concentrations of silver(I) ions was examined by two different methods for the determination of antioxidant activity DPPH and ABTS. The amount of radicals ($DPPH^{\bullet}$ or $ABTS^{+\bullet}$) and their loss after the addition of *S. aureus* lysates was observed spectrophotometrically (Fig. 3Ea and Eb, respectively). Oxidative stress was expressed by the percentual amount of free radicals. The results of measurement of oxidative stress obtained by both methods are similar, i.e. with the increasing silver concentration oxidative stress in *S. aureus* lysates was also increased. The lowest oxidative stress was observed at lower concentrations of added silver (10-50 μM). The largest increase in oxidative stress was observed at silver concentration ranging from 75 to 225 μM, when there was a substantial growth inhibition of *S. aureus*. The highest oxidative stress reached *S. aureus* lysates at silver concentration of 300 μM.

3.4 Hydrogen peroxide and oxidative stress

Next, effect of hydrogen peroxide on oxidative stress in *S. aureus* lysates was investigated using spectrophotometric and electrochemical methods (Fig. 4). Seven different concentrations of hydrogen peroxide (4, 5, 6, 7, 8, 9, 10 mM) was added to the bacterial culture of *S. aureus*. Using the growth curves method it was found that hydrogen peroxide caused *S. aureus* growth inhibition like silver(I) ions, but at much higher concentrations. Minimal inhibition concentration of *S. aureus* and H_2O_2 resulted from concentration of 4 mM, total inhibition concentration was then achieved with hydrogen peroxide at concentration of 10 mM (Fig. 4A). Similarly to the previous experiment with silver(I) ions only, the dependence of bacterial growth speed in the first six hours of measurement was compiled (Fig. 4B). Oxidative stress in *S. aureus* lysates with addition of different concentrations of hydrogen peroxide was again examined by two different methods for the determination of antioxidant activity DPPH and ABTS (Figs. 4C and D). The results of measurement of oxidative stress obtained by both methods are similar. With increasing hydrogen peroxide concentration oxidative stress in *S. aureus* lysates was increased as well, which was also confirmed electrochemically (Fig. 5A) by

electrochemical index determined according to the previously published papers [64-66]. Voltammograms obtained by analysis of each experimental group are shown in Figs. 5B, C, D, E, F, G, H and I.

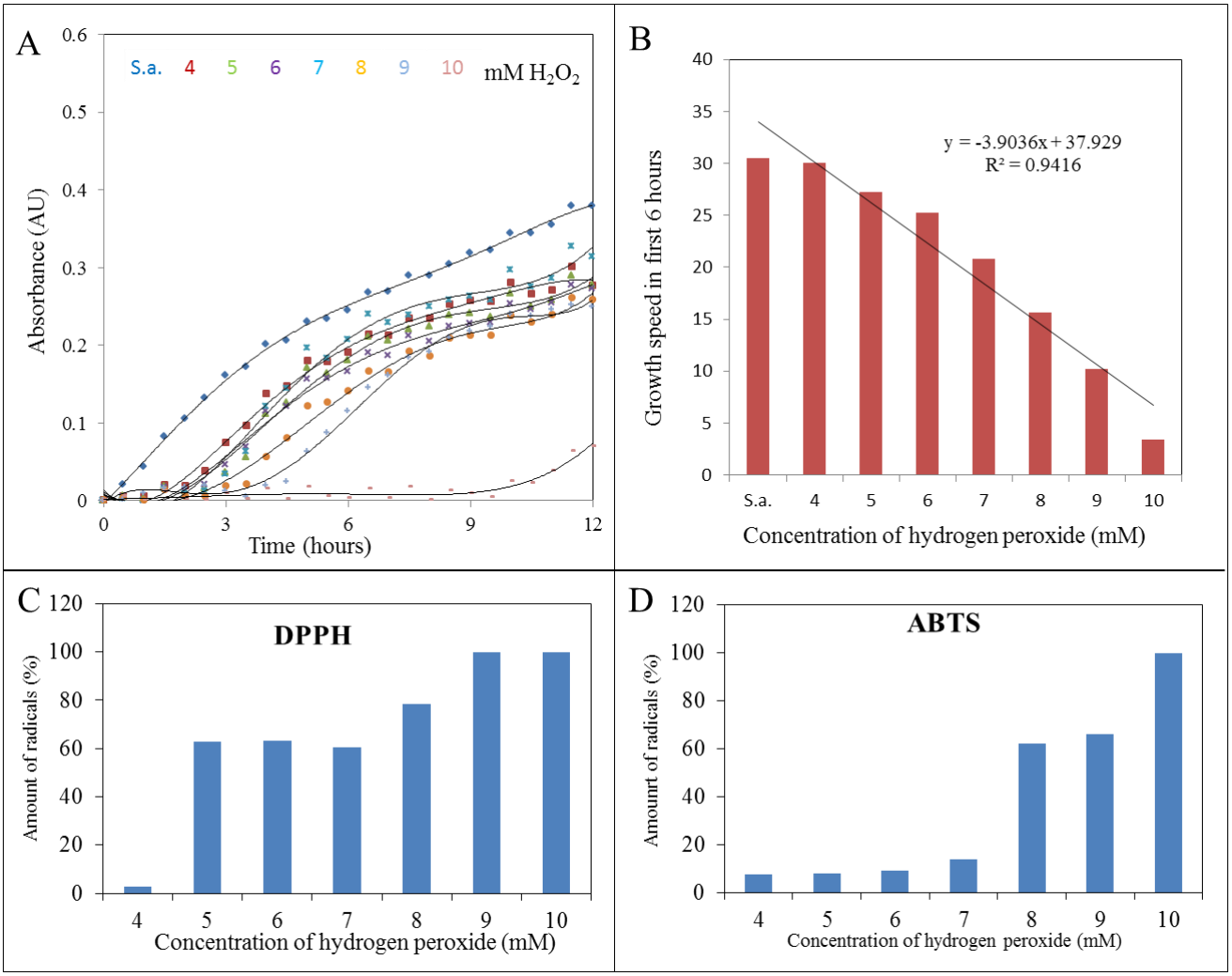

Figure 4. (**A**) Spectrophotometric analysis of the growth of *S. aureus* bacterial culture with concentrations of hydrogen peroxide (0, 4, 5, 6, 7, 8, 9 and 10 mM) in 12 hours. (**B**) Spectrophotometric analysis of the growth speed of *S. aureus* bacterial culture with concentrations of hydrogen peroxide (0, 4, 5, 6, 7, 8, 9 and 10 mM) in 6 hours. Spectrophotometric analysis of *S. aureus* oxidative stress using analytic methods (**C** – DPPH, **D** – ABTS). Dependence of amount of radicals (%) on used hydrogen peroxide concentrations (4, 5, 6, 7, 8, 9 and 10 mM).

Further, combined effect of silver(I) ions and hydrogen peroxide on bacterial culture *S. aureus* was investigated. Seven different concentrations of hydrogen peroxide (4, 5, 6, 7, 8, 9, 10 mM) was added to bacterial cultures containing different concentrations of $AgNO_3$ (10, 25, 50, 75, 150, 225, 300 μM) (Fig. 6). Effect of combination of silver(I) ions with hydrogen peroxide on bacterial culture *S. aureus* was first monitored by growth curves method. The obtained results pointed to a border silver ion concentration of 75 μM, at which there was a significant change in *S. aureus* growth (Fig. 6). Up to

the concentration of 50 µM there was only mild inhibition of bacterial growth, in a concentration of 50 µM there was already partial inhibition and in a concentration of 75 µM and higher there was recorded complete inhibition of *S. aureus* growth.

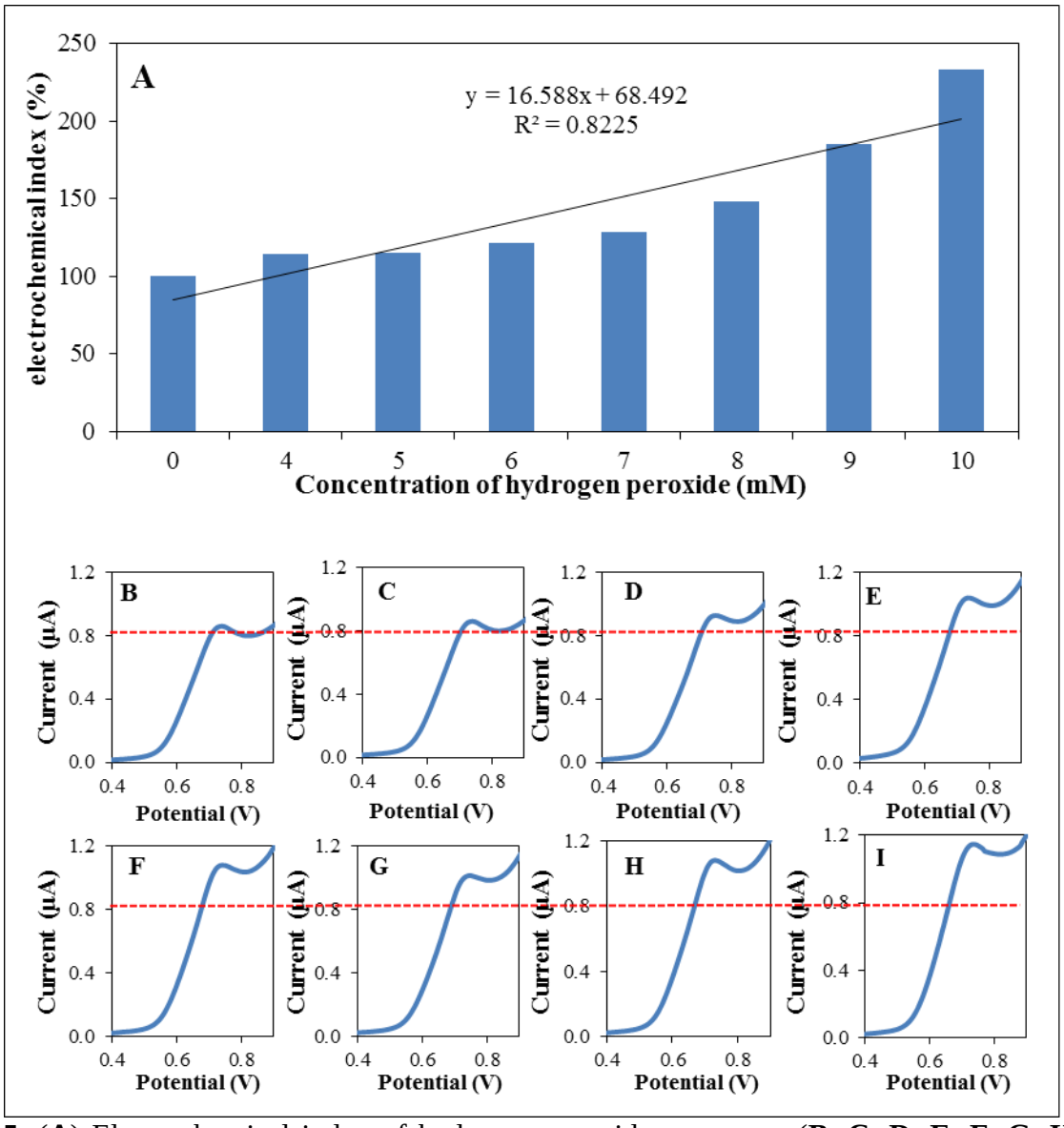

Figure 5. (**A**) Electrochemical index of hydrogen peroxide treatment. (**B, C, D, E, F, G, H and I**) Voltammograms of oxidative changes in signal of *S. aureus* according to the added concentrations of hydrogen peroxide (0, 4, 5, 6, 7, 8, 9 and 10 mM).

The same results were also confirmed by spectrophotometric methods (DPPH, ABTS), when the silver ion(I) concentration of 75 µM again became a border concentration and amount of free radicals at this concentration increased rapidly (Figs. 7A and B).

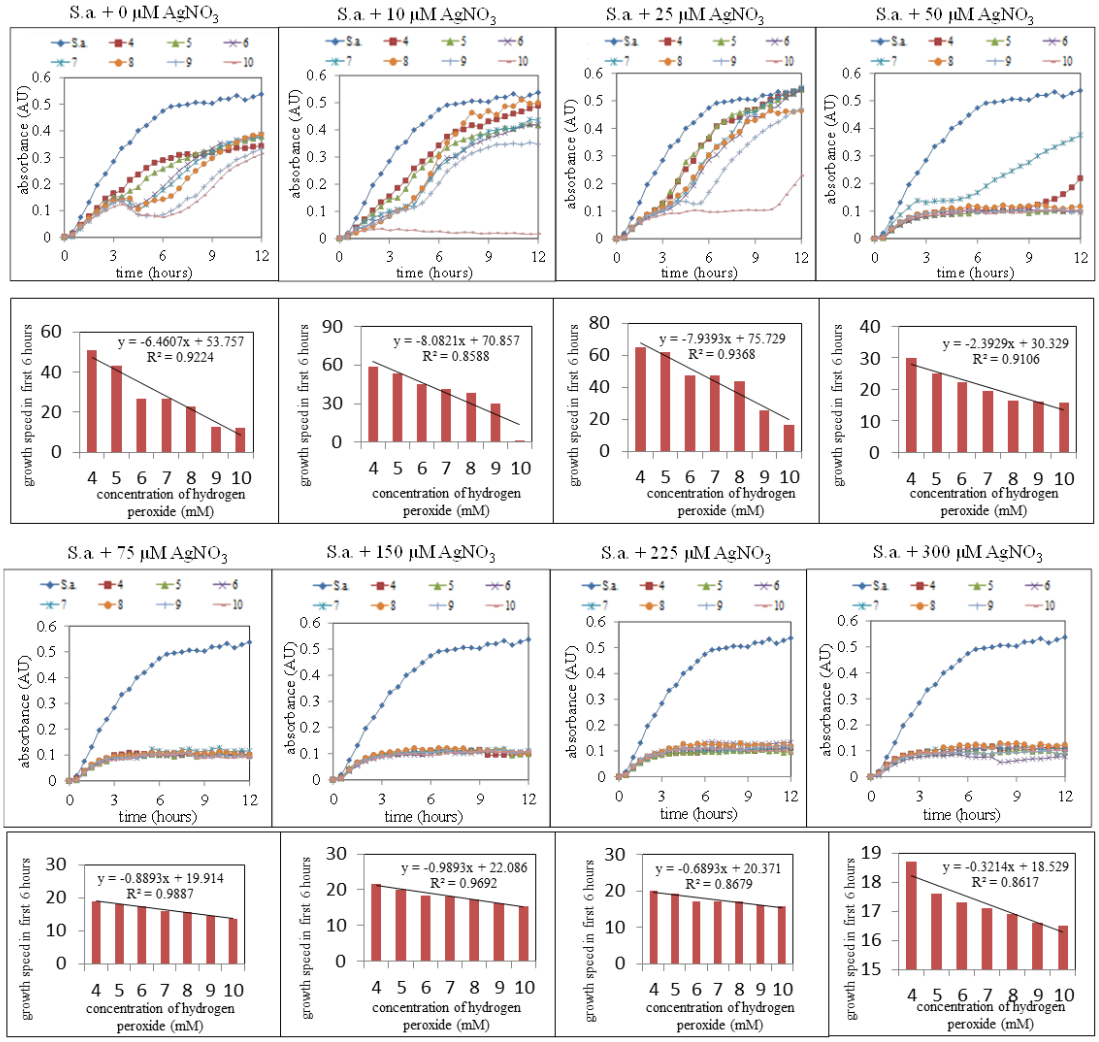

Figure 6. (**A**) Spectrophotometric analysis of the growth of *S. aureus* bacterial culture with concentrations of silver ions (0, 10, 25, 50, 75, 150, 225 and 300 μM) and hydrogen peroxide (0, 4, 5, 6, 7, 8, 9 and 10 mM).

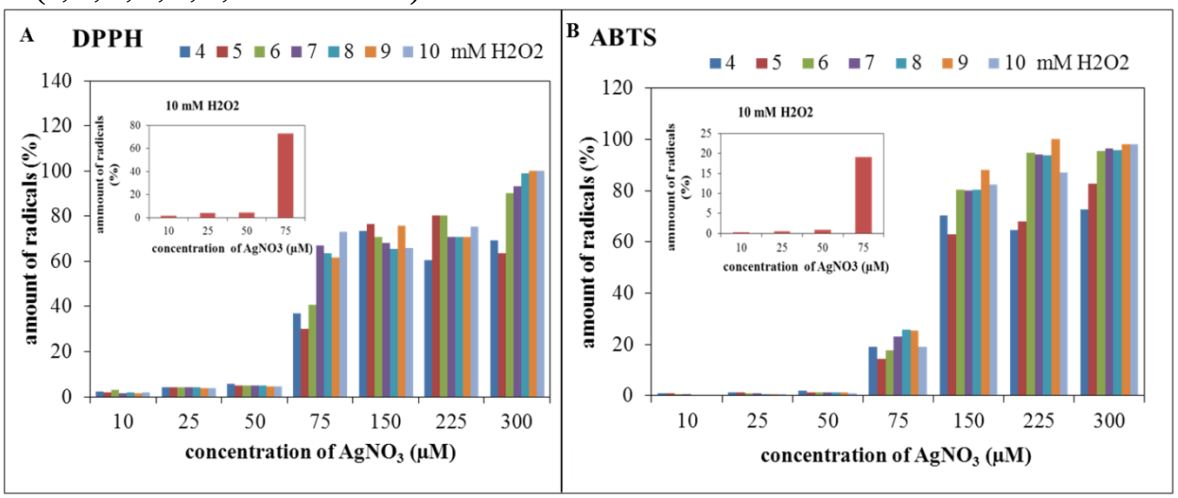

Figure 7. Spectrophotometric analysis of *S. aureus* oxidative stress using analytic methods (**A** – DPPH, **B** – ABTS). Dependence of amount of radicals (%) on used concentrations of silver(I) ions (10, 25, 50, 75, 150, 225 and 300 μM) and concentration of hydrogen peroxide (4, 5, 6, 7, 8, 9 and 10 mM).

3.5 Cyclic voltammetry as a tool for studying of oxidative stress

Cyclic voltammetry (CV) is an elementary electrochemical method implemented in all of electrochemical analyzers. For this reason it is very accessible and easy-to-use for various users [67-70]. Printed electrodes have recently become increasingly popular, especially thanks to its compact dimensions, low analyte consumption, and ease of use for a wide range of applications [33,71-87]. Oxidative stress was also studied by cyclic voltammetry, using flow cell and printed electrodes (Fig. 8). Using this method we were able to demonstrate an increase in oxidative signal in *S. aureus* lysates treated with different concentrations of silver(I) ions and hydrogen peroxide. First, pure *S. aureus* lysates were examined electrochemically in concentration of 3.7×10^7 cells/ml (Fig. 9A). Then *S. aureus* lysates measured were prepared from bacteria, where previously incubated with different concentrations of $AgNO_3$ with subsequent addition of hydrogen peroxide (Fig. 9B and C). Increase in oxidative signal was observed with rising concentrations of silver and hydrogen peroxide, which can be explained by increasing oxidative stress (Fig. 10). Furthermore we observed a shift to higher oxidation potential values with increasing concentrations of $AgNO_3$ and applied hydrogen peroxide (Fig. 9C). For a better understanding of induced oxidative stress, voltammograms were recalculated with growth curves to electrochemical index (Fig. 9B).

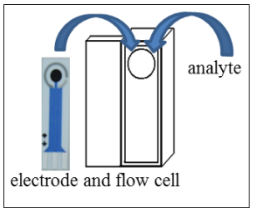

Figure 8. Printed electrode and flow cell.

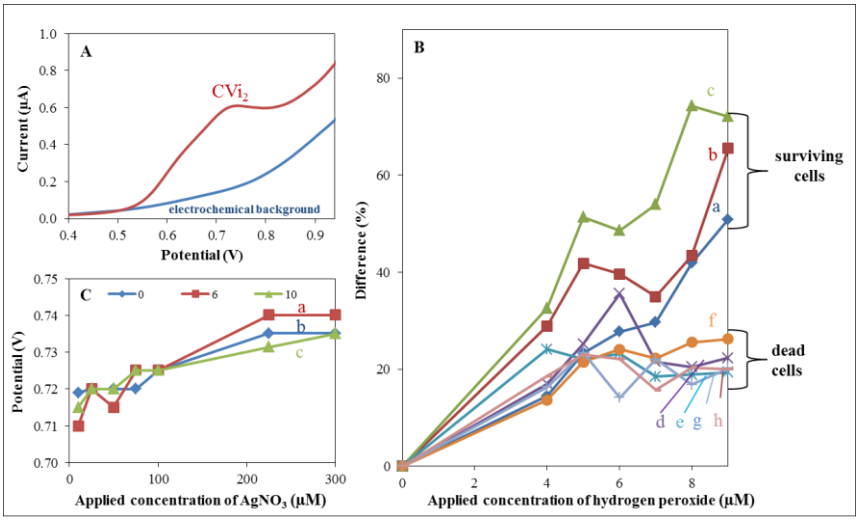

Figure 9. (**A**) Cyclic voltammograms of 0.2 M acetate buffer (pH 5) and oxidative peak of *S. aureus* (concentration of *S. aureus* corresponds 0.1 AU). **(B)** Dependence of oxidative stress of *S. aureus* on applied hydrogen peroxide concentration (0, 6, 10 mM) enriched with $AgNO_3$ (a = 0, b = 10, c = 25, d= 50, e = 75, f = 150, g = 225 and h = 300 µM). (**C**) Dependence values of the potential of oxidative stress *S. aureus* on applied concentration of silver ions and hydrogen peroxide.

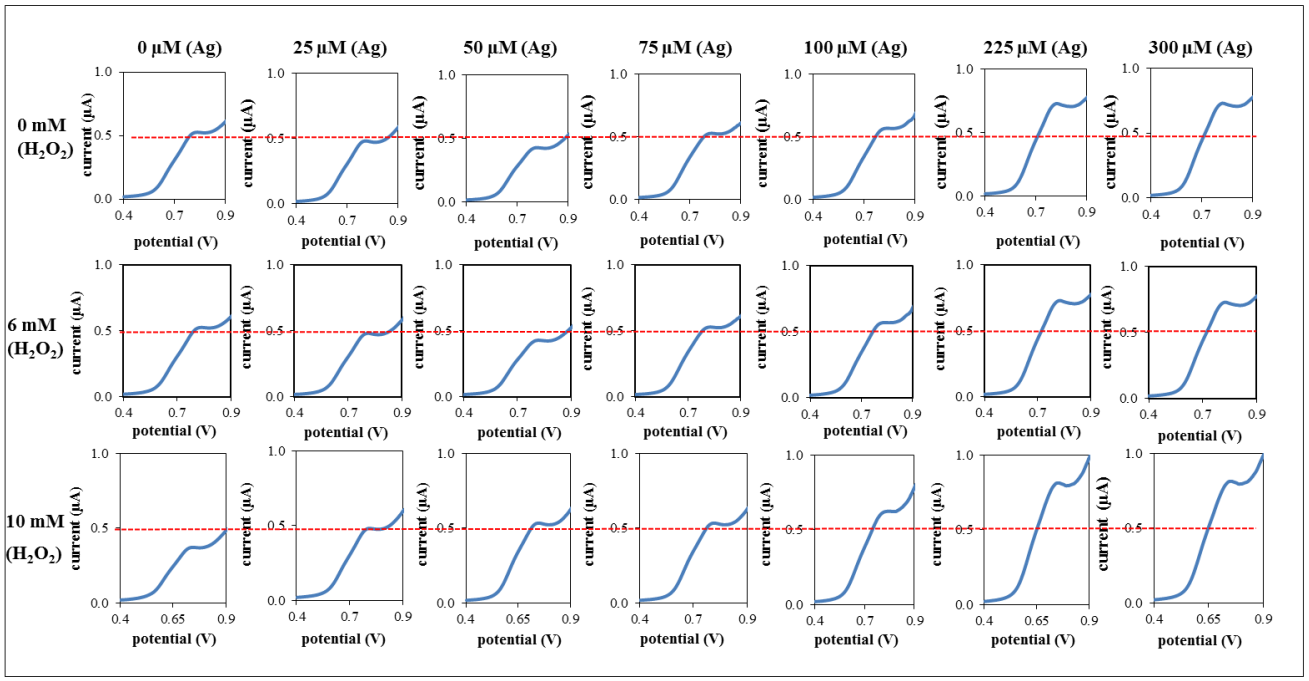

Figure 10. Cyclic voltammograms of detected *S. aureus* oxidative stress, which was developed after addition of $AgNO_3$ (0, 10, 25, 50, 75, 150, 225 and 300 µM) and hydrogen peroxide (0, 6, 10 mM).

Electrochemical index (%) was identified as a direct correlation of peak area and *S. aureus* growth curve absorbance in the sixth hour. It was observed that *S. aureus* resisted to the oxidative stress up to the concentration of 25 µM $AgNO_3$ and 10 mM hydrogen peroxide as is shown in Fig. 9B – a,b,c. Higher concentration of $AgNO_3$ was for *S. aureus* lethal (Fig. 9B – d,e,f,g).

4. CONCLUSION

In recent decades, amount of knowledge about the role of free radicals and their role in oxidative stress in organisms significantly increased. This work was focused on the study of oxidative stress in bacterial culture of *Staphylococcus aureus* induced by silver(I) ions using spectrophotometric and electrochemical methods. It was also done testing antibacterial effects of silver(I) ions on the growth of *S. aureus* bacterial culture using the measuring zones of inhibition and growth curves. Increased concentration of applied silver(I) ions led to the increase in oxidative stress which was enhanced by addition of hydrogen peroxide. Obtained results enabled further elucidation of the role of silver(I) ions in oxidative stress in bacterial culture of *S. aureus*. Our results indicate the possibility of the application of used analytical techniques in microbiology in determination of oxidative stress in bacterial cultures [88-95].

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References

1. H. J. Klasen, *Burns*, 26 (2000) 117.
2. H. J. Park, J. Y. Kim, J. Kim, J. H. Lee, J. S. Hahn, M. B. Gu and J. Yoon, *Water Res.*, 43 (2009) 1027.
3. Y. Yakabe, T. Sano, H. Ushio and T. Yasunaga, *Chem. Lett.* (1980) 373.
4. J. R. Furr, A. D. Russell, T. D. Turner and A. Andrews, *J. Hosp. Infect.*, 27 (1994) 201.
5. W. K. Jung, S. H. Kim, H. C. Koo, S. Shin, J. M. Kim, Y. K. Park, S. Y. Hwang, H. Yang and Y. H. Park, *Mycoses*, 50 (2007) 265.
6. W. R. Li, X. B. Xie, Q. S. Shi, S. S. Duan, Y. S. Ouyang and Y. B. Chen, *Biometals*, 24 (2011) 135.
7. W. L. Low, C. Martin, D. J. Hill and M. A. Kenward, *Int. J. Antimicrob. Agents*, 37 (2011) 162.
8. B. Kwakye-Awuah, C. Williams, M. A. Kenward and I. Radecka, *J. Appl. Microbiol.*, 104 (2008) 1516.
9. H. Y. Xu, F. Qu, H. Xu, W. H. Lai, Y. A. Wang, Z. P. Aguilar and H. Wei, *Biometals*, 25 (2012) 45.
10. C. Marambio-Jones and E. M. V. Hoek, *J. Nanopart. Res.*, 12 (2010) 1531.
11. D. He, J. J. Dorantes-Aranda and T. D. Waite, *Environ. Sci. Technol.*, 46 (2012) 8731.
12. W. K. Jung, H. C. Koo, K. W. Kim, S. Shin, S. H. Kim and Y. H. Park, *Appl. Environ. Microbiol.*, 74 (2008) 2171.
13. V. I. Lushchak, *Biochem.-Moscow*, 66 (2001) 476.
14. C. Calas-Blanchard, M. Cortina-Puig, L. Barthelmebs and T. Noguer, *Curr. Anal. Chem.*, 8 (2012) 428.
15. S. Klayraung and S. Okonogi, *Braz. J. Microbiol.*, 40 (2009) 757.
16. D. Wu, J. Y. Chen, B. Y. Lu, L. N. Xiong, Y. He and Y. Zhang, *Food Chem.*, 135 (2012) 2147.
17. L. K. Shpigun, N. N. Zamyatina, Y. V. Shushenachev and P. M. Kamilova, *J. Anal. Chem.*, 67 (2012) 801.
18. D. Sanna, G. Delogu, M. Mulas, M. Schirra and A. Fadda, *Food Anal. Meth.*, 5 (2012) 759.
19. A. Szydlowska-Czerniak, A. Tulodziecka and E. Szlyk, *Food Anal. Meth.*, 5 (2012) 807.
20. A. Szydlowska-Czerniak, A. Tulodziecka and E. Szlyk, *Analyst*, 137 (2012) 3750.
21. G. A. Garzon, C. E. Narvaez-Cuenca, R. E. Kopec, A. M. Barry, K. M. Riedl and S. J. Schwartz, *J. Agric. Food Chem.*, 60 (2012) 4709.
22. J. F. Arteaga, M. Ruiz-Montoya, A. Palma, G. Alonso-Garrido, S. Pintado and J. M. Rodriguez-Mellado, *Molecules*, 17 (2012) 5126.
23. K. Z. Brainina, D. P. Varzakova and E. L. Gerasimova, *J. Anal. Chem.*, 67 (2012) 364.
24. M. Ozyurek, M. Bener, K. Guclu, A. A. Donmez, S. Suzgec-Selcuk, S. Pirildar, A. H. Mericli and R. Apak, *Rec. Nat. Prod.*, 6 (2012) 263.
25. J. J. Lu, F. J. Tsai, C. M. Ho, Y. C. Liu and C. J. Chen, *Anal. Chem.*, 84 (2012) 5685.
26. X. N. Lu and B. A. Rasco, *Crit. Rev. Food Sci. Nutr.*, 52 (2012) 853.
27. A. Karadag, B. Ozcelik and S. Saner, *Food Anal. Meth.*, 2 (2009) 41.
28. S. Singh and R. P. Singh, *Food Rev. Int.*, 24 (2008) 392.
29. L. K. MacDonald-Wicks, L. G. Wood and M. L. Garg, *J. Sci. Food Agric.*, 86 (2006) 2046.
30. M. Antolovich, P. D. Prenzler, E. Patsalides, S. McDonald and K. Robards, *Analyst*, 127 (2002) 183.
31. L. Parejo, C. Codina, C. Petrakis and P. Kefalas, *J. Pharmacol. Toxicol. Methods*, 44 (2000) 507.
32. J. Sochor, M. Ryvolova, O. Krystofova, P. Salas, J. Hubalek, V. Adam, L. Trnkova, L. Havel, M. Beklova, J. Zehnalek, I. Provaznik and R. Kizek, *Molecules*, 15 (2010) 8618.
33. J. Prasek, L. Trnkova, I. Gablech, P. Businova, J. Drbohlavova, J. Chomoucka, V. Adam, R. Kizek and J. Hubalek, *Int. J. Electrochem. Sci.*, 7 (2012) 1785.
34. J. Prasek, M. Adamek, J. Hubalek, V. Adam, L. Trnkova and R. Kizek, *Sensors*, 6 (2006) 1498.
35. J. Prasek, D. Huska, O. Jasek, L. Zajickova, L. Trnkova, V. Adam, R. Kizek and J. Hubalek, *Nanoscale Res. Lett.*, 6 (2011) 1.

36. P. R. Murray, *J. Mol. Diagn.*, 14 (2012) 419.
37. M. Welker, *Proteomics*, 11 (2011) 3143.
38. E. Jordana-Lluch, E. M. Catala and V. A. Ruiz, *Enferm. Infec. Microbiol. Clin.*, 30 (2012) 635.
39. J. Kok, S. C. A. Chen, D. E. Dwyer and J. R. Iredell, *Pathology*, 45 (2013) 4.
40. R. Ouedraogo, A. Daumas, E. Ghigo, C. Capo, J. L. Mege and J. Textoris, *J. Proteomics*, 75 (2012) 5523.
41. F. Szabados, M. Kaase, A. Anders and S. G. Gatermann, *J. Infect.*, 65 (2012) 400.
42. K. Bohme, S. Morandi, P. Cremonesi, I. C. F. No, J. Barros-Velazquez, B. Castiglioni, M. Brasca, B. Canas and P. Calo-Mata, *Electrophoresis*, 33 (2012) 2355.
43. J. Gagnaire, O. Dauwalder, S. Boisset, D. Khau, A. M. Freydiere, F. Ader, M. Bes, G. Lina, A. Tristan, M. E. Reverdy, A. Marchand, T. Geissmann, Y. Benito, G. Durand, J. P. Charrier, J. Etienne, M. Welker, A. Van Belkum and F. Vandenesch, *Plos One*, 7 (2012) 1.
44. E. M. Charyulu, A. Gnanamani and A. B. Mandal, *Indian J. Microbiol.*, 52 (2012) 337.
45. S. Q. van Veen, E. C. J. Claas and E. J. Kuijper, *J. Clin. Microbiol.*, 48 (2010) 900.
46. F. Szabados, J. Woloszyn, C. Richter, M. Kaase and S. Gatermann, *J. Med. Microbiol.*, 59 (2010) 787.
47. M. C. Radulescu, A. Chira, M. Radulescu, B. Bucur, M. P. Bucur and G. L. Radu, *Sensors*, 10 (2010) 11340.
48. A. A. Peverly and D. G. Peters, *Anal. Chem.*, 84 (2012) 6110.
49. G. P. Yan, Y. H. Wang, X. X. He, K. M. Wang, J. Su, Z. F. Chen and Z. H. Qing, *Talanta*, 94 (2012) 178.
50. F. Bir, H. Khireddine, A. Touati, D. Sidane, S. Yala and H. Oudadesse, *Appl. Surf. Sci.*, 258 (2012) 7021.
51. B. E. F. de Avila, M. Pedrero, S. Campuzano, V. Escamilla-Gomez and J. M. Pingarron, *Anal. Bioanal. Chem.*, 403 (2012) 917.
52. A. Martinez-Abad, G. Sanchez, J. M. Lagaron and M. J. Ocio, *Int. J. Food Microbiol.*, 158 (2012) 147.
53. S. L. Percival, J. Thomas, S. Linton, T. Okel, L. Corum and W. Slone, *Int. Wound J.*, 9 (2012) 488.
54. P. Fernandez-Saiz, C. Soler, J. M. Lagaron and M. J. Ocio, *Int. J. Food Microbiol.*, 137 (2010) 287.
55. D. L. Borneman, S. C. Ingham and C. Ane, *J. Food Prot.*, 72 (2009) 1190.
56. J. A. Rufian-Henares and F. J. Morales, *Food Chem.*, 111 (2008) 1069.
57. B. Mahdavi, W. A. Yaacob, L. B. Din and I. Nazlina, *Sains Malays.*, 41 (2012) 1233.
58. M. B. Joray, M. L. Gonzalez, S. M. Palacios and M. C. Carpinella, *J. Agric. Food Chem.*, 59 (2011) 11534.
59. J. P. Ruparelia, A. K. Chatteriee, S. P. Duttagupta and S. Mukherji, *Acta Biomater.*, 4 (2008) 707.
60. A. A. Tawiah, S. Y. Gbedema, F. Adu, V. E. Boamah and K. Annan, *BMC Microbiol.*, 12 (2012) 1.
61. A. Moussa, D. Noureddine, H. S. Mohamed, M. Abdelmelek and A. Saad, *Asian Pac. J. Trop. Med.*, 5 (2012) 773.
62. N. Lokendrajit, S. Indira, N. Swapana and C. B. Singh, *Asian J. Chem.*, 24 (2012) 4418.
63. C. Barriere, S. Leroy-Setrin and R. Talon, *J. Appl. Microbiol.*, 91 (2001) 514.
64. O. Zitka, S. Skalickova, J. Gumulec, M. Masarik, V. Adam, J. Hubalek, L. Trnkova, J. Kruseova, T. Eckschlager and R. Kizek, *Oncol. Lett.*, 4 (2012) 1247.
65. O. Zitka, M. Kominkova, S. Skalickova, H. Skutkova, I. Provaznik, T. Eckschlager, M. Stiborova, L. Trnkova, V. Adam and R. Kizek, *Int. J. Electrochem. Sci.*, 8 (2013) 2625.
66. M. Pohanka, D. Hynek, A. Kracmarova, J. Kruseova, B. Ruttkay-Nedecky, J. Sochor, V. Adam, J. Hubalek, M. Masarik, T. Eckschlager and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 11978.

67. M. S. Estevao, L. C. Carvalho, L. M. Ferreira, E. Fernandes and M. M. B. Marques, *Tetrahedron Lett.*, 52 (2011) 101.
68. A. Gomes, E. Fernandes, M. B. Q. Garcia, A. M. S. Silva, D. Pinto, C. M. M. Santos, J. A. S. Cavaleiro and J. Lima, *Bioorg. Med. Chem.*, 16 (2008) 7939.
69. A. Nyska and R. Kohen, *Toxicol. Pathol.*, 30 (2002) 620.
70. S. Chevion and M. Chevion, in C.C. Chiueh (Editor), Reactive Oxygen Species: From Radiation to Molecular Biology: A Festschrift in Honor of Daniel L Gilbert, New York Acad Sciences, New York, 2000, p. 308.
71. R. P. Caramit, A. G. D. Andrade, J. B. G. de Souza, T. A. de Araujo, L. H. Viana, M. A. G. Trindade and V. S. Ferreira, *Fuel*, 105 (2013) 306.
72. M. Tomassetti, E. Martini, L. Campanella, G. Favero, L. Carlucci and F. Mazzei, *J. Pharm. Biomed. Anal.*, 73 (2013) 90.
73. T. Shimomura, T. Sumiya, M. Ono, T. Ito and T. Hanaoka, *Anal. Bioanal. Chem.*, 405 (2013) 297.
74. J. S. Caygill, S. D. Collyer, J. L. Holmes, F. Davis and S. P. J. Higson, *Analyst*, 138 (2013) 346.
75. J. Prasek, J. Drbohlavova, J. Chomoucka, J. Hubalek, O. Jasek, V. Adam and R. Kizek, *J. Mater. Chem.*, 21 (2011) 15872.
76. J. Chomoucka, J. Drbohlavova, M. Masarik, M. Ryvolova, D. Huska, J. Prasek, A. Horna, L. Trnkova, I. Provaznik, V. Adam, J. Hubalek and R. Kizek, *Int. J. Nanotechnol.*, 9 (2012) 746.
77. V. Adam, O. Zitka, P. Dolezal, L. Zeman, A. Horna, J. Hubalek, J. Sileny, S. Krizkova, L. Trnkova and R. Kizek, *Sensors*, 8 (2008) 464.
78. D. Huska, J. Hubalek, V. Adam and R. Kizek, *Electrophoresis*, 29 (2008) 4964.
79. L. Trnkova, V. Adam, J. Hubalek, P. Babula and R. Kizek, *Sensors*, 8 (2008) 5619.
80. V. Adam, D. Huska, J. Hubalek and R. Kizek, *Microfluid. Nanofluid.*, 8 (2010) 329.
81. M. Pohanka, J. Fusek, V. Adam and R. Kizek, *Int. J. Electrochem. Sci.*, 8 (2013) 71.
82. V. Shestivska, V. Adam, J. Prasek, T. Macek, M. Mackova, L. Havel, V. Diopan, J. Zehnalek, J. Hubalek and R. Kizek, *Int. J. Electrochem. Sci.*, 6 (2011) 2869.
83. M. Pohanka, M. Hrabinova, J. Fusek, D. Hynek, V. Adam, J. Hubalek and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 50.
84. O. Zitka, S. Krizkova, S. Skalickova, P. Kopel, P. Babula, V. Adam and R. Kizek, *Comb. Chem. High Throughput Screen.*, in press (2013).
85. O. Zitka, S. Krizkova, L. Krejcova, D. Hynek, J. Gumulec, M. Masarik, J. Sochor, V. Adam, J. Hubalek, L. Trnkova and R. Kizek, *Electrophoresis*, 32 (2011) 3207.
86. D. Dospivova, D. Hynek, P. Kopel, A. Bezdekova, J. Sochor, S. Krizkova, V. Adam, L. Trnkova, J. Hubalek, P. Babula, I. Provaznik, R. Vrba and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 6378.
87. P. Sobrova, M. Ryvolova, D. Huska, J. Hubalek, I. Provaznik, V. Adam and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 1.
88. R. Mikelova, J. Baloun, J. Petrlova, V. Adam, L. Havel, J. Petrek, A. Horna and R. Kizek, *Bioelectrochemistry*, 70 (2007) 508.
89. D. Huska, S. Krizkova, J. Hubalek, V. Adam, M. Beklova, L. Trnkova and R. Kizek, *Toxicol. Lett.*, 180 (2008) S236.
90. S. Krizkova, P. Ryant, O. Krystofova, V. Adam, M. Galiova, M. Beklova, P. Babula, J. Kaiser, K. Novotny, J. Novotny, M. Liska, R. Malina, J. Zehnalek, J. Hubalek, L. Havel and R. Kizek, *Sensors*, 8 (2008) 445.
91. S. Krizkova, V. Adam and R. Kizek, *Chem. Listy*, 103 (2009) 559.
92. S. Krizkova, O. Krystofova, L. Trnkova, J. Hubalek, V. Adam, M. Beklova, A. Horna, L. Havel and R. Kizek, *Sensors*, 9 (2009) 6934.
93. S. Krizkova, D. Huska, M. Beklova, J. Hubalek, V. Adam, L. Trnkova and R. Kizek, *Environ. Toxicol. Chem.*, 29 (2010) 492.

94. O. Zitka, D. Huska, V. Adam, A. Horna, M. Beklova, Z. Svobodova and R. Kizek, *Int. J. Electrochem. Sci.*, 5 (2010) 1082.
95. L. Trnkova, I. Fabrik, D. Huska, H. Skutkova, M. Beklova, J. Hubalek, V. Adam, I. Provaznik and R. Kizek, *J. Environ. Monit.*, 13 (2011) 2763.

5.3 Modifikace cévních náhrad komplexy antibakteriálních forem stříbra s polymerními látkami

5.3.1 Vědecký článek III

CHUDOBOVA, D.; NEJDL, L.; GUMULEC, J.; KRYSTOFOVA, O.; MERLOS RODRIGO, M. A.; KYNICKY, J.; RUTTKAY-NEDECKY, B.; KOPEL, P.; BABULA, P.; ADAM, V.; KIZEK, R. Complexes of Silver(I) Ions and Silver Phosphate Nanoparticles with Hyaluronic Acid and/or Chitosan as Promising Antimicrobial Agents for Vascular Grafts. *International Journal of Molecular Sciences*, 2013, roč. 14. č. 7, s. 13592-13614. ISSN 1422-0067.

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Kovy ve formě iontů samotných nejsou jedinou cestou eliminace bakteriálních infekcí. Dalším způsobem, jak zvýšit antimikrobiální vlastnosti kovů, je jejich využití v kombinaci s jinými činidly [138, 139]. Je proto důležité zkoumat účinky kovů ve formě iontů nebo nanočástic s látkami, které jejich antimikrobiální efekt výrazným způsobem podporují. Nejčastěji využívanými látkami pro tyto účely jsou kyselina hyaluronová a chitosan. Obě tyto látky patří do skupiny biopolymerů s prokázanou antimikrobiální aktivitou, které se vyznačují svou biologickou rozložitelností a biokompatibilitou s lidským tělem [220]. Polymery obecně jsou v současné době široce používány jako náhrada různých přírodních materiálů právě s ohledem na jejich příznivé fyzikální a chemické vlastnosti a vzhledem k jejich ekonomické výhodnosti.

Cílem této studie bylo přispět k rozvoji antimikrobiálního polymerního materiálu ideálního pro pokrytí cévních implantátů s pozdějším využitím v transplantační chirurgii. Z tohoto důvodu byly vytvořeny komplexy polymerních látek (kyselina hyaluronová a chitosan) s dusičnanem stříbrným nebo nanočásticemi fosforečnanu stříbrného a byly monitorovány účinky těchto komplexů na G^+ bakteriální kulturu *Staphylococcus aureus*. Jednotlivé fáze tvorby komplexů dusičnanu stříbrného a nanočástic fosforečnanu stříbrného s polymerními sloučeninami byly charakterizovány pomocí elektrochemických a spektrofotometrických metod. Mimo to byla stanovena i antimikrobiální aktivita těchto komplexů s využitím mikrobiologických metod, měření růstových křivek a stanovení velikosti inhibičních zón.

Z výsledků této studie vyplývá zjištění, že komplex chitosanu s nanočásticemi fosforečnanu stříbrného disponují nejvyšším (až o několik řádů vyšším v porovnání s ostatními testovanými komplexy) antibakteriálním účinkem v souvislosti s bakteriální kulturou *S. aureus*. Spektrofotometrické a elektrochemické stanovení ověřilo vzájemné působení jednotlivých komponent a tvorbu komplexu.

Výsledky mohou být využity pro další experimenty s možností uplatnění v cévní chirurgii, zejména z pohledu snížení rizika vzniku a šíření bakteriálních infekcí, které představují vysoké riziko při implantaci umělých cévních náhrad.

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Article

Complexes of Silver(I) Ions and Silver Phosphate Nanoparticles with Hyaluronic Acid and/or Chitosan as Promising Antimicrobial Agents for Vascular Grafts

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Abstract: Polymers are currently widely used to replace a variety of natural materials with respect to their favourable physical and chemical properties, and due to their economic advantage. One of the most important branches of application of polymers is the production of different products for medical use. In this case, it is necessary to face a significant disadvantage of polymer products due to possible and very common colonization of the surface by various microorganisms that can pose a potential danger to the patient. One of the possible solutions is to prepare polymer with antibacterial/antimicrobial properties that is resistant to bacterial colonization. The aim of this study was to contribute to the development of antimicrobial polymeric material ideal for covering vascular implants with subsequent use in transplant surgery. Therefore, the complexes of polymeric substances (hyaluronic acid and chitosan) with silver nitrate or

silver phosphate nanoparticles were created, and their effects on gram-positive bacterial culture of *Staphylococcus aureus* were monitored. Stages of formation of complexes of silver nitrate and silver phosphate nanoparticles with polymeric compounds were characterized using electrochemical and spectrophotometric methods. Furthermore, the antimicrobial activity of complexes was determined using the methods of determination of growth curves and zones of inhibition. The results of this study revealed that the complex of chitosan, with silver phosphate nanoparticles, was the most suitable in order to have an antibacterial effect on bacterial culture of *Staphylococcus aureus*. Formation of this complex was under way at low concentrations of chitosan. The results of electrochemical determination corresponded with the results of spectrophotometric methods and verified good interaction and formation of the complex. The complex has an outstanding antibacterial effect and this effect was of several orders higher compared to other investigated complexes.

Keywords: polymers; antimicrobial activity; silver ions; silver phosphate nanoparticles; hyaluronic acid; chitosan

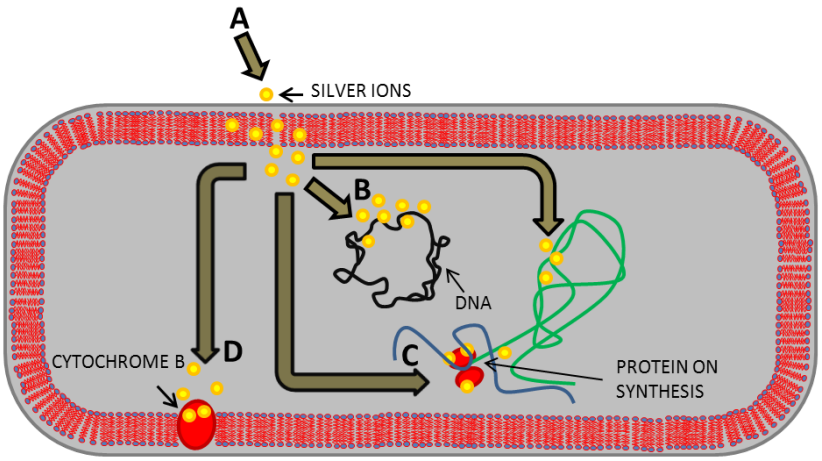
1. Introduction

Bacterial infections represent one of the most serious complications in vascular surgery [1]. This is mainly due to the increase in the using of artificial vascular prostheses [2]. Surgical site infections affect 1%–10% of patients who undergo vascular operations [3–7]. *Staphylococcus aureus* belongs to the most common contagious pathogens associated with clinical infections in vascular surgery [8–11], especially methicillin-resistant *S. aureus* (MRSA), which is responsible for more than 50% of infections in vascular surgery procedures [10,11]. To prevent infections in vascular surgery, substances with antibacterial effects are often used. Binding of antibiotics (rifampin, gentamicin, amikacin, vancomycin, levofloxacin) to vascular prostheses, impregnated with collagen or gelatin, represents one of the possible solutions to the infection issue [12–14].

From experimental studies to animal trials, rifampin bonded to vascular prosthesis Dacron has been determined to be the most effective prosthetic-antibiotic combination to date [13]. However, the use of antibiotics as an antimicrobial agent has one serious problem, the development of bacterial resistance to used antibiotics. Silver belongs to another promising substance with an antibacterial effect, which can reduce the number of infections after vascular surgery [15,16]. It is generally known that silver is highly toxic to microorganisms [17,18]. On the other hand, silver has a low toxicity to human cells, and a far lesser probability to cause bacterial resistance than antibiotics [19]. The mechanism of action of silver(I) ions on bacteria is based on inactivation of membrane proteins, binding with the bacterial DNA and disrupting DNA replication, impairing the ability of ribosomes to transcribe messenger RNA into the vital proteins required by the cell to function, inactivation of the cytochrome b (Figure 1), and consequent bactericidal activity [20,21]. Antimicrobial effects of silver can be increased by its application in the form of nanosilver [22]. Nanoparticles (NPs) with at least one dimension of 100 nm or less have unique physicochemical properties, such as high catalytic capabilities and ability to

generate reactive oxygen species [23]. Silver, in the form of nanoparticles, could therefore be more reactive with its increased catalytic properties, and become more toxic for bacteria than silver(I) ions [24–27]. In searching for a proper antibacterial coating Pallavicini *et al.* prepared monolayers of silver nanoparticles (AgNPs) on glass surfaces [28]. AgNPs in the monolayers remained firmly grafted when the surfaces were exposed to water or phosphate saline buffer. About 15% silver release as silver(I) ions has been found after 15 days when the surfaces are exposed to water. The released silver cations were responsible of an efficient local antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* bacterial strains. In another work, Taglietti *et al.* experiments on glutathione (GSH) coated silver nanoparticles (AgNPs) grafted on glass surface showed that the antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* was reduced in comparison with uncoated AgNPs because of GSH coating and the limitation of the translational freedom of NPs [29].

Figure 1. Mechanisms of action of the silver(I) ions on bacteria. (**A**) Silver(I) ions penetrate the bacterial cell wall and bind to the phospholipid layer of the cytoplasmic membrane; (**B**) Silver(I) ions bind the bacterial DNA with subsequent disrupting of DNA replication; (**C**) Silver(I) ions impair the ability of ribosomes to transcribe messenger RNA; (**D**) Silver(I) ions bind to the sulfhydryl group of the cytochrome b. Adopted and modified according to Ricco and Assadian [14].



Another way to increase the antimicrobial efficiency is to use silver in combination with other agents [30,31]. It is therefore important to investigate the effects of silver(I) ions and silver nanoparticles with compounds that promote their antibacterial effect. Both hyaluronic acid and chitosan belong to biopolymers with antimicrobial activity, which are characterized by biodegradability and biocompatibility with human body [32]. Hyaluronic acid is a high molecular weight glycosaminoglycan, generally regarded as an extracellular matrix component that facilitates cell locomotion and proliferation [33,34]. Hyaluronic acid is present in almost all biological fluids and tissues [34]. Chitosan is a linear polysaccharide composed of randomly distributed β-(1–4)-linked D-glucosamine and *N*-acetyl-D-glucosamine and is the most important derivative of chitin [35]. Chitosan is an effective material for biomedical applications because of its wound healing ability, antimicrobial, and anti-inflammatory activities [36–38]. Chitosan is able to bind metals and create complexes with them [37,39,40]. These interactions proceed through binding on amino groups of

chitosan via chelation or complexation mechanism [35]. Similarly, it has been shown that hyaluronic acid is able to bind silver nanoparticles [41].

A combination of different forms of silver and biopolymer substances to cover the vascular prostheses is therefore a highly suitable solution in the fight against resistant strains of bacteria. The purpose of this study was to investigate the antimicrobial effect of silver(I) ions and silver phosphate nanoparticles (SPNPs), in combination with hyaluronic acid and chitosan.

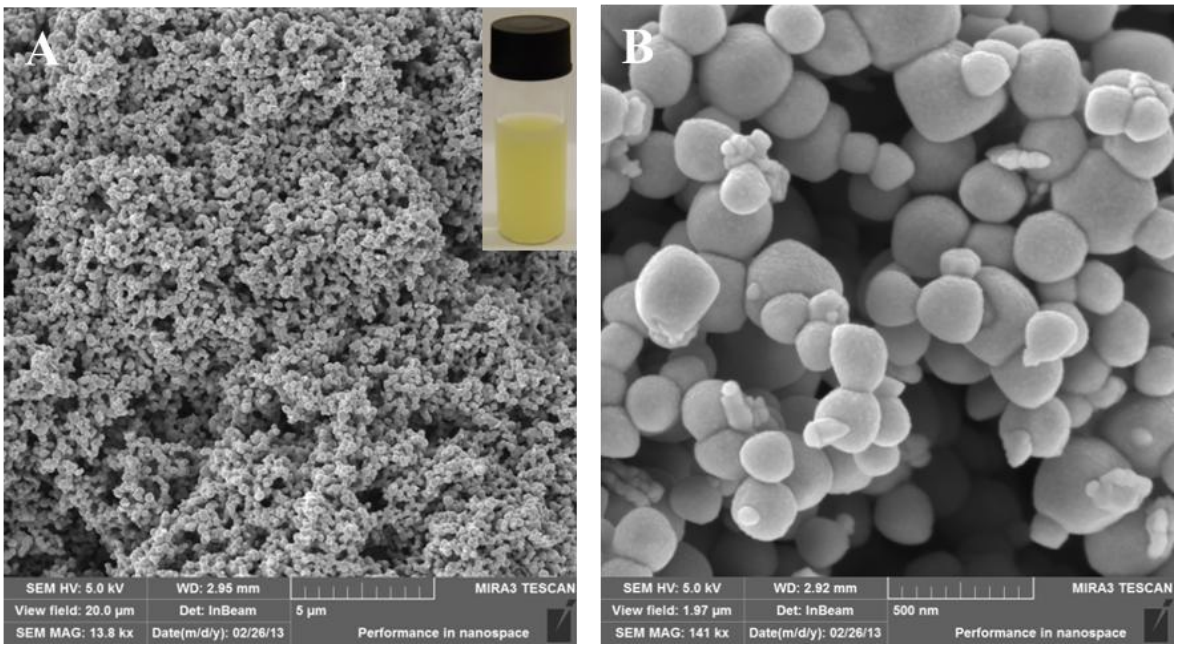
2. Results and Discussion

Resistance to antibiotics represent a significant health issue. Modern biotechnological tools, based on the nanotechnological fundamentals, bring new possibilities and opportunities to overcome resistance. Synthesized nanoparticles and nanoparticles placed in nanotransporters enhance therapeutic possibilities and have sufficient effects against resistance induced by enzymatic pathways [42–44]. Effects of both silver(I) ions and silver-based NPs are sufficiently known; however, their combinations with various substances could improve technological possibilities in the treatment of serious diseases but is not fully understood and explored. In this study, we prepared silver phosphate nanoparticles (SPNPs) by a simple chemical synthesis. Formation of colloidal nano-Ag_3PO_4 was indicated by an immediate change of solution color to yellow, and the solution was opaque and turbid. The mixture was stirred for one hour and stored in the dark at 4 ℃ for further experiments. In addition, nanoparticles were characterized by SEM microscopy.

2.1. Characterization of Silver Phosphate Nanoparticles (SPNPs)

We have used a well-known precipitation reaction to prepare silver phosphate nanoparticles (SPNPs), as it has been shown in previously published papers [44,45]. Diluted solutions were used to prevent formation of bulky precipitate. The formation of SPNPs is very well observed by the turn of colorless solution to yellow, and colloid is formed (Figure 2A). Prepared SPNPs were characterized by SEM microscopy (Figure 2). Arising nanoparticles form a compact structure, which can be followed by formation of aggregates. Nevertheless, significant deep fissures occur in this structure. It may result in the binding of various molecules, eventually bacteria, in this structure (Figure 2A). Detailed SEM records show that SPNPs are spherical in shape and particle size varies in the 80–350 nm range. Minute nanoparticles that grow from bigger spherical SPNP are also visible (Figure 2B). Detailed microscopic study revealed that the majority of nanoparticles were between 200 and 300 nm in diameter (more than 80% of all SPNPs). This variation in nanoparticle size can be explained in terms of diffusion-limited aggregation and/or reaction-limited aggregation [46]. In less concentrated solution, reaction limited aggregation dominates and the cluster-cluster repulsion has to be overcome by a thermal activation process [47]. The nanoparticles were synthesized in the experiment and characterized three times and the results were repeatable.

Figure 2. Silver phosphate nanoparticles (SPNPs) characterized by SEM and visually (**A**) SEM HV: 5 kV, view field: 20 μm, SEM MAF: 13.8 kx, WD: 2.95 mm, det: InBeam. In the upper right corner is a photo of the yellow colloid SPNPs solution; (**B**) SEM HV: 5 kV, view field: 1.97 μm, SEM MAF: 141 kx, WD: 2.92 mm, det: InBeam.



2.2. Formation of Complexes of Silver with Polymeric Compounds

Prepared nanoparticles were used in further experiments for the monitoring and studying of their interactions with polymer compounds. Chitosan complexes with silver nitrate were prepared by addition of different concentrations of $AgNO_3$ to chitosan. In the mild conditions, *i.e.*, room temperature and short reaction time, only coordination of silver ions to electron pairs situated on chitosan nitrogens of amine groups can be expected. Proposed bonding of silver ions is shown in Figure 3. Addition of reducing agents or higher temperatures should lead to formation of silver nanoparticles as was observed by Bin Ahmad *et al.* [48]. Similarly, hyaluronic silver complex was formed after the addition of silver nitrate to the solution of hyaluronic acid. Ionic bond of carboxylic group with silver ion is the most probable mechanism in this case (Figure 3). There is also a possibility of the formation of chelate between two oxygens of carboxylic groups and silver ion, and binding to carboxylic group of second hyaluronic acid molecule. Coordination of silver ions to carboxylic groups is described by Abdel-Mohsen *et al.* [41]. The authors also used hyaluronic acid as a reducing agent and described reduction of silver ions into silver nanoparticles. They found that the reduction was dependent on pH and temperature. As we used only mild conditions, we cannot expect the formation of silver nanoparticles in our study. Formation of silver chitosan and hyaluronic complexes was confirmed by spectrophotometrical and electrochemical studies and was discussed later.

Figure 3. Suggested structures of silver complexes. Upper part of scheme represents complex of silver and chitosan, lower part stands for complex of hyaluronic acid and silver.

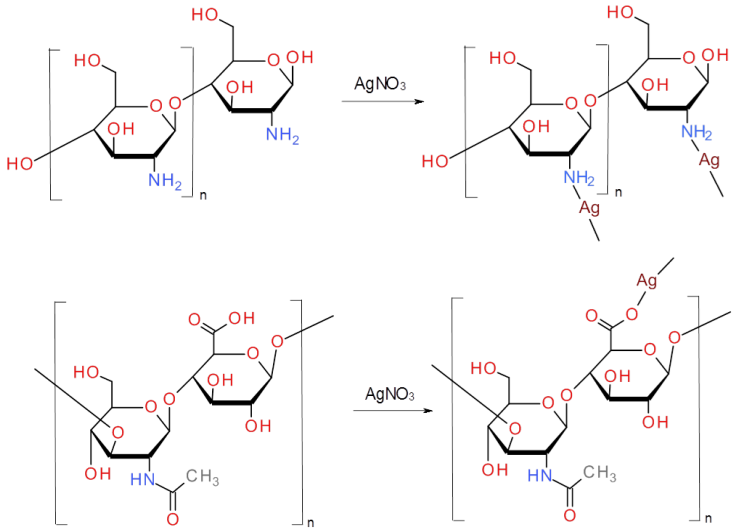
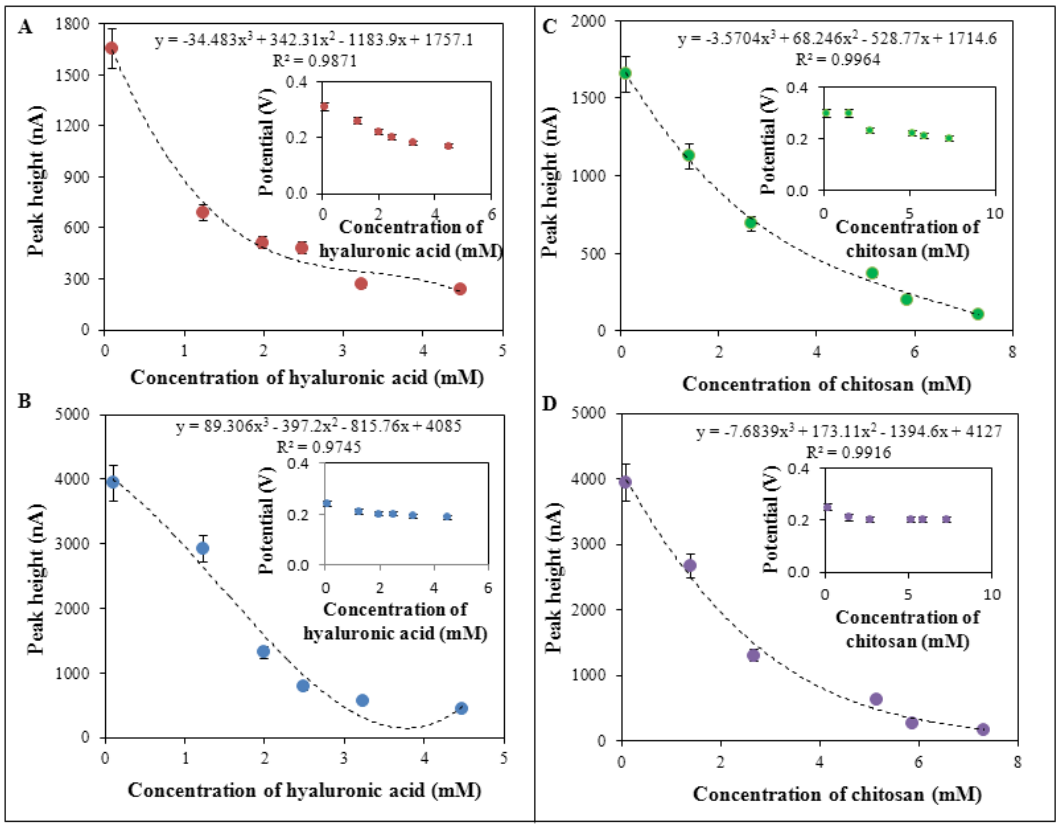


Figure 4. Electrochemical analysis of the interactions of: (**A**) hyaluronic acid (1.24, 1.99, 2.49, 3.23, 4.48 mM) and $AgNO_3$ (100 μM); (**B**) hyaluronic acid (1.24, 1.99, 2.49, 3.23, 4.48 mM) and SPNPs (100 μM); (**C**) chitosan (1.4, 2.66, 5.14, 5.86, 7.32 mM) and silver ions $AgNO_3$ (100 μM) or (**D**) chitosan (1.4, 2.66, 5.14, 5.86, 7.32 mM) and SPNPs (100 μM) using differential pulse voltammetry (DPV). Heights and potentials of silver(I) were determined and are shown. DPV parameters were as follows: start potential −0.2 V, end potential 0.5 V, amplitude 0.05 V, time of accumulation 60 s, acetate buffer pH 5.



2.3. Characterization of Creation of Complex between Silver and Polymeric Compounds Using Electrochemical Methods

The course of creation of complex of silver(I) ions and SPNPs with polymeric compounds was characterized firstly by electrochemical methods, namely differential pulse voltammetry, using the methodologies, which have been published in our previous papers [49–51]. Silver(I) ions (in the form of $AgNO_3$) only provided a signal at the potential of 0.30 V and height of the signal came up to 1700 nA. On the other hand, silver phosphate nanoparticles (SPNPs) provided a signal at the potential of 0.25 V with a signal height of 4000 nA. $AgNO_3$ and SPNPs were used at a concentration of 100 μM. Dependences of the signal potentials and signal heights on the concentrations of the compounds tested in experiments were evaluated based on the obtained results (Figure 4). The figure shows that increasing concentrations of hyaluronic acid or chitosan in combination with silver ions or SPNPs led to a decrease in height of electrochemical signal and it shifted into negative potential values (Figure 4).

In the case of a complex of hyaluronic acid with silver nitrate, the peak potential shifted from the potential of 0.30 V to 0.19 V (Figure 4A). In the case of complex of hyaluronic acid and SPNPs, signal was shifted from the potential of 0.25 V to 0.19 V (Figure 4B). Similar results were observed in the complex of chitosan and silver(I) ions as silver nitrate (shift from the potential of 0.30 V to 0.20 V, Figure 4C), and in complex of chitosan and SPNPs (shift from the potential of 0.25 V to 0.20 V, Figure 4D). The dependences of the height of signal on the concentration of applied compounds (hyaluronic acid and chitosan), in the combination with silver ions and SPNPs, show proportional decrease in the height of the signal with the increasing concentrations of the compounds of interest. These results indicate creation of complexes in all combinations of hyaluronic acid or chitosan with silver nitrate or SPNPs. The complexes of polymeric compounds (hyaluronic acid or chitosan) with silver particles ($AgNO_3$ or SPNPs) were confirmed using different types of electrochemistry such as cyclic voltammetry in other studies [52,53].

2.4. Spectrophotometric Characterization of Course of Creation of Complexes of Silver and Polymeric Compounds

Creation of complexes of silver and polymeric compounds was also studied by UV-VIS spectrophotometry in the wavelength range of 220–420 nm (Figure 5). UV/VIS spectrophotometry represents a simple and valuable method to study interactions and to characterize antimicrobial properties of various compounds [54,55]. This method confirmed results, obtained by the use of electrochemical methods. Firstly, absorption spectra of individual silver compounds were recorded (100 μM $AgNO_3$ and 100 μM SPNPs). These compounds have no absorption maxima in the range 230–420 nm, see Figure 5B-a (100 μM $AgNO_3$) and Figure 5D-a (100 μM SPNPs). The most distinct absorption maximum (at 260 nm) was recorded for 24.9 mM hyaluronic acid (see Figure 5B-b,D-b). Chitosan (29.3 mM) demonstrated absorption maximum at the same wavelength (260 nm). On the other hand, it was significantly less compared to hyaluronic acid (see Figure 5G-b,I-b). In the next step, polymeric compounds were mixed with silver accordingly. 100 μM solution of $AgNO_3$/SPNPs was mixed with stock solution of hyaluronic acid (24.9 mM) to create final concentrations 0.5, 1.0, 1.4, 1.8, 2.3, 2.7, 3.1, 3.4, and 3.8 mM of hyaluronic acid. Absorption spectrum in the range 220–420 nm

was recorded after each addition of hyaluronic acid. The same procedure was used for chitosan (concentrations 0.6, 1.1, 1.7, 2.2, 2.7, 3.1, 3.6, 4.0, and 4.5 mM). Creation of complexes of silver with polymeric compounds was accompanied by a change in absorption at different wavelengths, showed in Figure 5B-c (100 μM $AgNO_3$ and 24.9 mM hyaluronic acid), Figure 5D-c (100 μM SPNPs and 24.9 mM hyaluronic acid), Figure 5G-c (12 mM $AgNO_3$ and 29.3 mM chitosan), and Figure 5I-c (100 μM SPNPs and 29.3 mM chitosan). Titration of polymeric compounds (0.5, 1.0, 1.4, 1.8, 2.3, 2.7, 3.1, 3.4, and 3.8 mM hyaluronic acid or 0.6, 1.1, 1.7, 2.2, 2.7, 3.1, 3.6, 4.0, and 4.5 mM chitosan) with 100 μM $AgNO_3$ or 100 μM SPNPs resulted in an increase in absorbance in created complexes, see Figure 5C (100 μM $AgNO_3$-hyaluronic acid complex, λ = 272 nm), Figure 5D (100 μM SPNPs-hyaluronic acid complex, λ = 280 nm), Figure 5H (100 μM $AgNO_3$-chitosan complex, λ = 260 nm), and Figure 5J (100 μM SPNPs-chitosan complex, λ = 262 nm). Complexes had their absorption maxima at different wavelengths compared to individual reagents. This fact confirms creation of the complexes. Only complex 100 μM $AgNO_3$-chitosan had absorption maximum at the same wavelength (260 nm) as chitosan.

Electrochemical study (chapter 3.4) of this complex showed that creation of complex might also be expected in this case, although spectrophotometric measurement is not supporting this statement. Created complexes also differed in dependence of change of absorbance on the applied polymeric compound at the titration. First complex (100 μM $AgNO_3$-hyaluronic acid) showed logarithmic dependence (R^2 = 0.78), the second one (100 μM SPNPs-hyaluronic acid) logarithmic dependence too (R^2 = 0.98), the third one (100 μM $AgNO_3$-chitosan) linear dependence (R^2 = 0.94), and the last studied complex (100 μM SPNPs-chitosan) exponential dependence (R^2 = 0.97). Low concentrations of silver (0–100 μM, both $AgNO_3$ and SPNPs) provided no signal in the VIS area. Due to this fact, created complexes were studied in the UV area of spectrum (260–280 nm). Nanosilver absorbs radiation in the VIS area (400 nm) in concentrations higher than 100 μM as is shown in the work of Abdel-Mohsen *et al.* [41], who used complex of chitosan and silver nanoparticles for the production of antimicrobial textiles. Nowadays, there are many applications using chitosan and silver as an antibacterial agent [41,54–56]. There are also mentioned complexes of silver and hyaluronic acid [41,48]. Our study provides the characteristics of this complex and presents potential use in clinical practice.

2.5. Mass Spectrometric Characterization of the Used Bacterial Strain

We verified using matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) technique for the presence of the complexes of interest in bacterial culture, which was treated with the abovementioned complexes in the following part of the study. MALDI-TOF is a technique that combines a soft, matrix-assisted, ionization process, and a TOF analyser to separate the generated ions [57,58]. In MALDI-TOF mass spectra, the mixture of biological samples with an energy-absorbing matrix allows the genesis of mostly intact-single-charged biomolecules. This tool is routinely used to identify bacterial species in clinical samples [59–61] and has been extensively used in biology to search biomarkers and to monitor protein post-translational modifications [62]. MALDI-TOF mass spectra were applied also for the classification of *S. aureus* strains. Obtained mass MALDI-TOF spectra of used bacterial culture of *S. aureus* are shown in Figure 6A. MALDI-TOF MS-based identification has been shown to be a fast and accurate technology in the identification of a

variety of *S. aureus* strains [63–66]. Moreover, it has been found that the application of silver(I) ions to the bacterial culture led to a significant change in the mass spectrum of distinct molecular fragments of weight 4227 Da, 6210 Da, and 8455 Da (Figure 6B). The changes in the mass spectrum were also observed by the applications of SPNPs, hyaluronic acid, and chitosan to the bacterial culture (Figure 6C–E, respectively).

Figure 5. (**A**) Dependence of creation of a complex on applied concentration of hyaluronic acid (HA); (**B**) Spectral analysis in the range of 220–420 nm, a = 100 μM $AgNO_3$, b = 24.9 mM HA and c = complex of 24.9 mM HA and 100 μM $AgNO_3$, λ = 272 nm; (**C**) Dependence of creation of a complex (HA + 100 μM $AgNO_3$) on applied concentration of HA, λ = 272 nm; (**D**) Spectral analysis within the range of 220–420 nm, a = 100 μM SPNPs, b = 24.9 mM HA and c = complex of 24.9 mM HA and 100 μM SPNPs, λ = 280 nm; (**E**) Dependence of creation of a complex (HA + 100 μM SPNPs) on applied concentration of HA, λ = 280 nm; (**F**) Dependence of creation of a complex on applied concentration of chitosan (CH); (**G**) Spectral analysis in the range of 220–420 nm, a = 100 μM $AgNO_3$, b = 29.3 mM CH and c = complex of 29.3 mM CH and 100 μM $AgNO_3$, λ = 260 nm; (**H**) Dependence of creation of a complex (CH + 100 μM $AgNO_3$) on applied concentration of CH, λ = 260 nm; (**I**) Spectral analysis in the range of 220–420 nm, a = 100 μM SPNPs, b = 29.3 mM CH and c = complex of 29.3 mM CH and 100 μM SPNPs, λ = 262 nm; (**J**) Dependence of creation of a complex (CH + 100 μM SPNPs) on applied concentration of CH, λ = 262 nm.

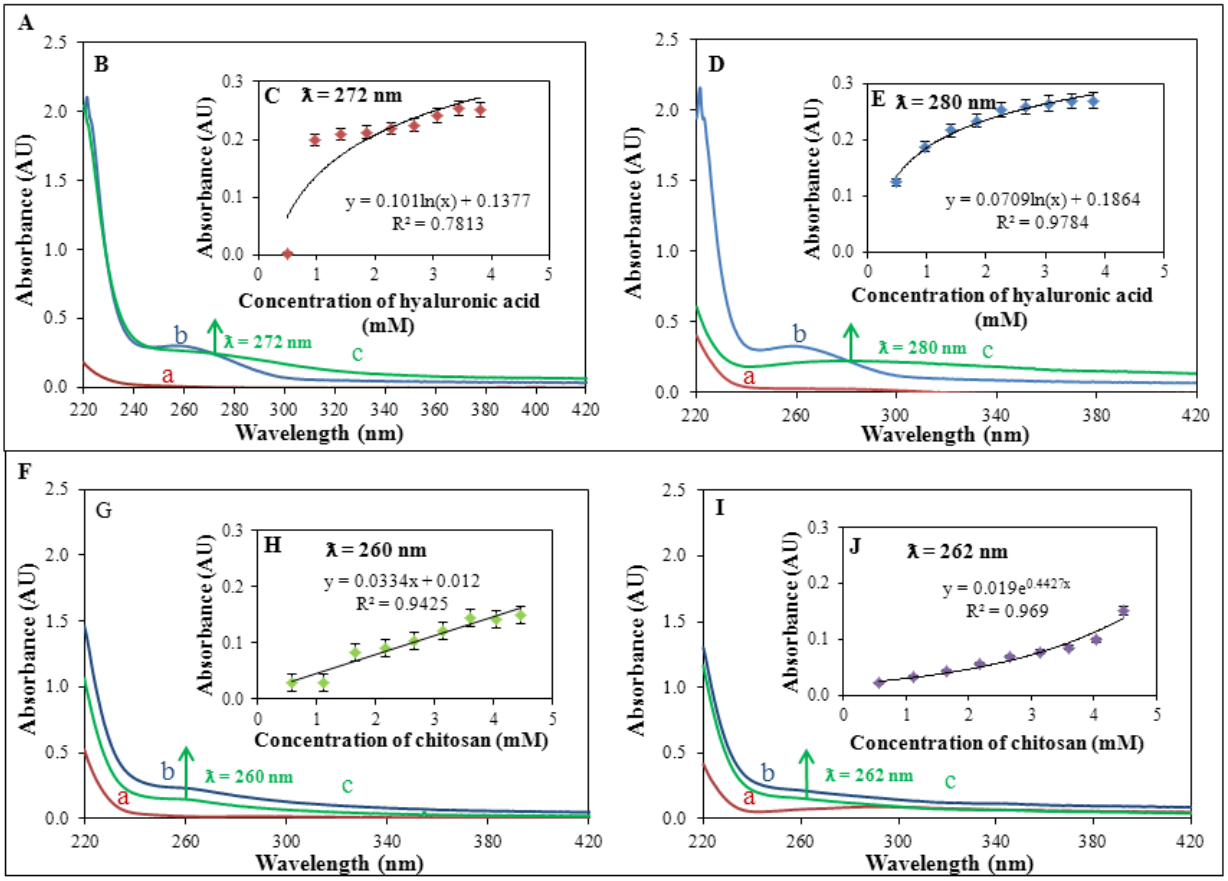
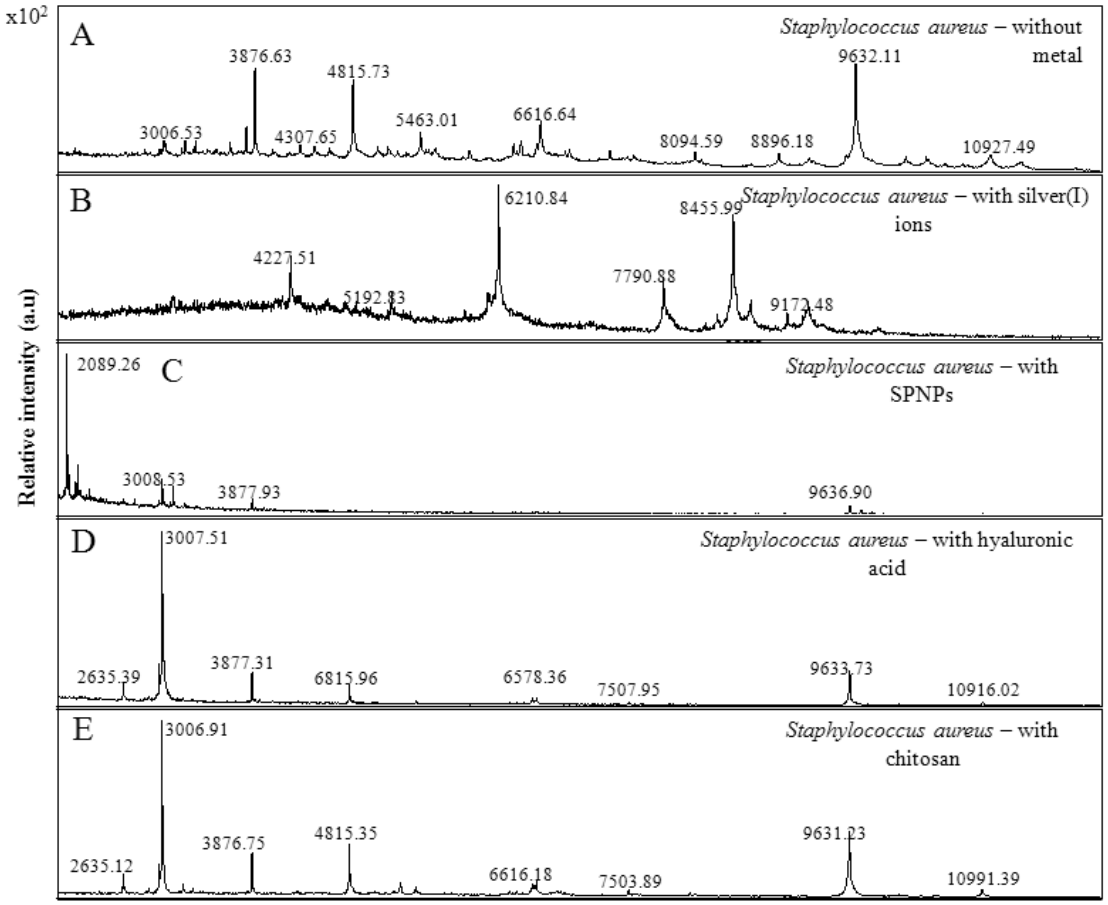


Figure 6. Matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectra protein fingerprints for the identification of *Staphylococus aureus* (**A**) without metal, (**B**) with silver ions, (**C**) with SPNPs, (**D**) with hyaluronic acid and (**E**) with chitosan. Data were collected in the *m*/*z* 2000–20000 range after processing of 1 mL of culture of *S. aureus*.



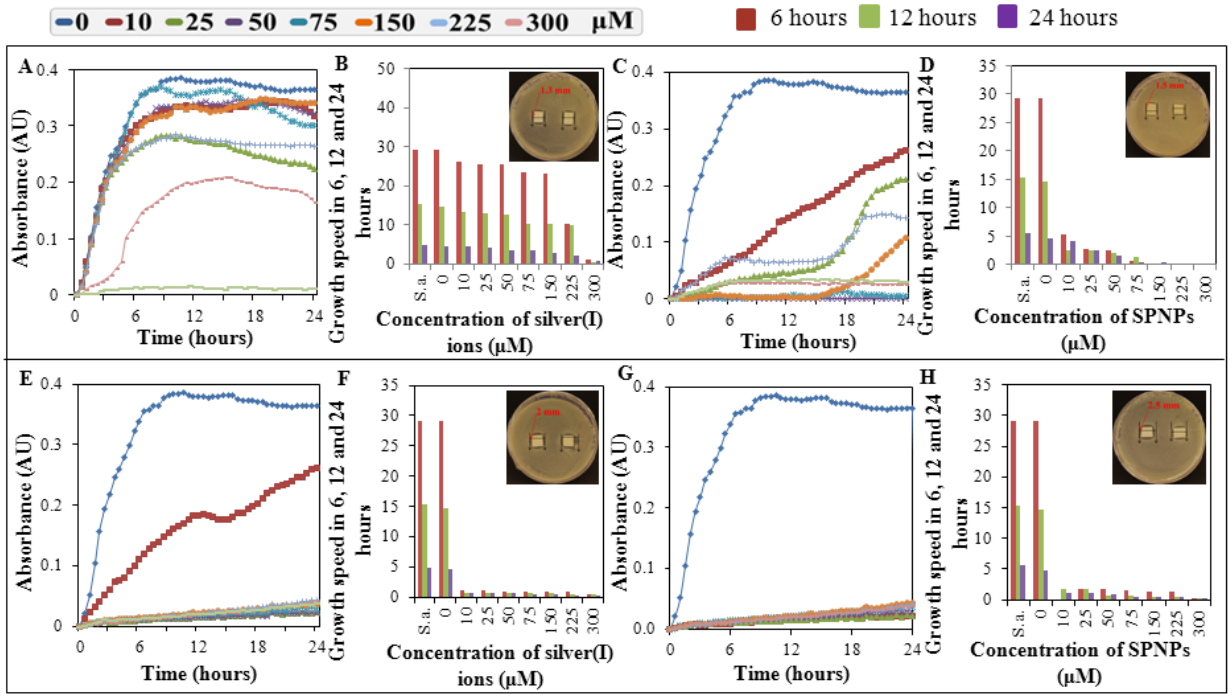
2.6. Determination of Antimicrobial Activity of Complexes of Silver and Polymeric Compounds

The results of the electrochemical and spectrophotometric determination indicate the creation of complexes of silver particles with polymeric compounds. Further, various combinations of polymers and silver particles were tested for their antimicrobial activity, as might be expected based on the previously published works [67–71]. Testing of antimicrobial activity of polymeric compounds (hyaluronic acid and chitosan) in complexes with silver and silver nanoparticles was chosen specifically due to their compatibility with human tissues (biocompatibility) and biodegradability [72–75].

Polymeric compound chitosan was chosen due to its nontoxic, biocompatible, and biodegradable properties used for the targeted therapy, respectively for targeting of toxic compounds to specific cells and tissues in the human body [38]. Polymeric structure of studied compounds provides easy creation of complexes with metal ions [37,39,40]. Surprisingly, these complexes show higher antimicrobial properties than individual compounds. Testing of compounds and complexes was performed by the use of basic microbiological methods, method of determination of growth dependences (“growth curves“) [76–78], where the minimal concentrations of tested compounds inducing inhibition of

growth-minimum inhibitory concentration (MIC) [79] and total inhibitory concentration (TIC) [80] are determined, or method based on the determination of inhibition zones [81–83]. Measuring inhibition zones was the first test to the antimicrobial properties of the compounds of interest. The first results were obtained after a 24 h long cultivation (Figure 7A–H). Only the hyaluronic acid or chitosan demonstrated no inhibitory effect in applied concentrations. Addition of silver ions ($AgNO_3$) or silver phosphate nanoparticles (SPNPs) led to the significant increase in the inhibition of growth of culture of *S. aureus*. The greatest inhibitory effect had the combination of 9.7 mM chitosan with 300 μM silver(I) ions or 300 μM silver phosphate nanoparticles (Figure 7E,G). Size of inhibition zones for complex of chitosan and silver phosphate nanoparticles was about 2.5 mm, in the variant chitosan-silver ions these zones were slightly smaller and reached app. 2 mm. The smallest zones of inhibition were determined for the variants of 8.3 mM hyaluronic acid with 300 μM silver(I) ions and 300 μM silver phosphate nanoparticles (Figure 7A,C). Combination of hyaluronic acid with silver ions induced inhibition zones of app. 1.3 mm, the variant of hyaluronic acid with silver phosphate nanoparticles demonstrated inhibition zones as large as 1.5 mm.

Figure 7. Growth curves, growth rate and inhibition zones of *Staphylococcus aureus* cultures after application of complex of silver ions ($AgNO_3$) or silver phosphate nanoparticles (SPNPs) in various concentrations with hyaluronic acid (8.3 mM) or chitosan (9.7 mM). Growth curves (**A**) and growth rate (**B**) -applied hyaluronic acid with $AgNO_3$, growth curves (**C**) and growth rate (**D**) -applied hyaluronic acid with SPNPs, growth curves (**E**) and growth rate (**F**) -applied chitosan with $AgNO_3$, growth curves (**G**) and growth rate (**H**) -applied chitosan with SPNPs. In the upper right corner of the Figure 7B,D,F,H are photos of inhibition zones of the highest applied concentrations of silver(I) ions or SPNPs in complexes with hyaluronic acid or chitosan.



To compare the results obtained by the method of determination of inhibition zones, growth characterization and determination of growth curves were used. This method clearly showed the highest antibacterial activity of the variants of silver ions and silver phosphate nanoparticles with chitosan (Figure 7E,G). Minimum inhibitory concentration (MIC) of 10 μM was determined for complex of chitosan and silver(I) ions, and total inhibitory concentration (TIC) was 25 μM and higher. In comparison, TIC for silver phosphate nanoparticles was determined as 10 μM. Complexes of silver and hyaluronic acid did not demonstrate significant antimicrobial effect. TIC for complex of silver phosphate nanoparticles and hyaluronic acid was determined as 150 μM, *i.e.*, that this and higher concentrations (namely 150, 225, and 300 μM) led to the total growth inhibition of bacterial culture. On the other hand, complex of silver(I) ions with hyaluronic acid showed only moderate antimicrobial activity with TIC induced by concentration of 225 μM. Lower concentrations showed only slight antimicrobial activity, respectively only slight inhibition of growth of bacterial culture.

In addition, growth was evaluated at three times—6, 12, and 24 h. These results corresponded with previously obtained results and showed inhibition of growth of bacterial culture after application of studied compounds (Figure 7B,D,F,H). To analyse contribution of Ag form, other compounds (chitosan and hyaluronic acid) and time of treatment, a general regression model was used. Using this approach, results provide evidence that both forms of silver, selection of other compound (chitosan and hyaluronic acid) and combination of these affect the toxicity significantly (at $p < 0.001$). Inhibition concentrations are summarized in Table 1. In contrast, variable time does not provide significance. To provide more detailed analysis of obtained data, Turkey's *post hoc* test for homogenous groups was performed (Table 2). Two distinct clusters are evident. First includes chitosan with both silver ions and silver phosphate nanoparticles, and hyaluronic acid with silver phosphate nanoparticles. The second cluster comprises the combination of hyaluronic acid with silver(I) ions. This combination provides distinctly lower cytotoxicity compared to those previously mentioned.

Table 1. Half-maximal inhibition concentrations (IC_{50}).

Type of Ag	Other compound	Time	IC_{50} (μM)
SPNPs	chitosan	6	5.10
SPNPs	chitosan	12	3.72
SPNPs	chitosan	24	1.00
$AgNO_3$	hyaluronic acid	6	209.63
$AgNO_3$	hyaluronic acid	12	246.55
$AgNO_3$	hyaluronic acid	24	188.15
SPNPs	hyaluronic acid	6	1.00
SPNPs	hyaluronic acid	12	1.00
SPNPs	hyaluronic acid	24	29.53
$AgNO_3$	chitosan	6	1.00
$AgNO_3$	chitosan	12	1.00
$AgNO_3$	chitosan	24	1.00

Table 2. Turkey's post hoc test; mean IC_{50} (μM). Asterisks indicate distribution of complexes into two homogenous groups (1 and 2) at $p < 0.05$.

Number	Type of Ag	Other compound	IC_{50} (μM)-Mean	1	2
1	$AgNO_3$	chitosan	1.0000	****	
2	SPNPs	chitosan	3.2704	****	
3	SPNPs	hyaluronic acid	10.5100	****	
4	$AgNO_3$	hyaluronic acid	214.7771		****

2.7. Biochemical Array

Biochemical arrays are used for characterization of bacterial strains of *Staphylococcus*, *Micrococcus* and *Stomatococcus*. These arrays (tests) focus changes in metabolism of chosen sugars in bacteria [84,85]. No significant changes in biochemical parameters, eventually moderate increase in enzymatic activity (for 10%–20%), were observed after application of hyaluronic acid (8.3 mM) to culture of *S. aureus* (0.1 OD). Application of 10 mM SPNPs led to decrease of metabolic activity for about 30%, compared to the control, untreated culture. In the case of the application of chitosan (29.3 mM) and 10 mM SPNPs we observed substantial decrease in metabolic activity of bacterial culture. Only minute changes in enzymatic activities (for 1%–5% of bacterial culture) of urease, phosphatase, β-glucosidase, and in metabolism, maltose and mannose were observed (not shown). Obtained results confirmed significant effect of complex of chitosan and silver phosphate nanoparticles on metabolic activity of bacterial culture of *Staphylococcus aureus*.

3. Experimental Section

3.1. Chemicals

Chemicals used in this study ($AgNO_3$, $Na_2HPO_4 \cdot 7H_2O$, hyaluronic acid, chitosan, tryptone, yeast extract, NaCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity unless noted otherwise. Deionized water was prepared using reverse osmosis equipment Aqual 25 (Brno, Czech Republic). Deionized water was further purified by using a MiliQ Direct QUV apparatus equipped with a UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

3.2. Knitted Vascular Prosthesis

Vascular prostheses are made of polyester fibres, using a knitting technology, and are covered on the marked side with a continuous layer of bovine collagen type I. These vascular prostheses were made by Research Institute of Knitting Technologies in Brno (Brno, Czech Republic) of different lengths and thicknesses, usually marked as Ra 1v K. Vascular prostheses are solid, flexible, do not fray, and are provided with a color guide line. Water permeability through the wall of the vascular prosthesis is 0–5 mL cm^{-2} min^{-1}. Vascular prostheses are sterilized by irradiation and are not designed for resterilization.

3.3. Preparation of Silver Phosphate Nanoparticles (SPNPs)

SPNPs were prepared by the method of Khan *et al.* [47]. Heptahydrate of disodium hydrogen phosphate (0.134 g) was dissolved in ACS water (25 mL). A solution of $AgNO_3$ (0.085 g) in 25 mL of ACS water was quickly added to a stirred solution of disodium hydrogen phosphate. There was an immediate change of solution colour to yellow with formation of colloidal nanoform of Ag_3PO_4. The mixture was stirred for 1 h. SPNPs were stored in the dark at 4 ℃. One millilitre of mixture contains 1.08 mg of Ag. To create complexes, stock solutions of silver(I) ions ($AgNO_3$—12 mM), silver phosphate nanoparticles (SPNPs—10 mM), and a pair of polymer substances, hyaluronic acid (24.9 mM) and chitosan (29.3 mM) were used.

3.4. Cultivation of Staphylococcus aureus

Staphylococcus aureus (NCTC 8511) was obtained from the Czech Collection of Microorganisms, Faculty of Science, Masaryk University, Brno, Czech Republic. Strains were stored in the form of a spore suspension in 20% (*v*/*v*) glycerol at −20 ℃. Prior to use, the strains were thawed and the glycerol was removed by washing with distilled water in this study. The composition of cultivation medium was as it follows: meat peptone 5 g L^{-1}, NaCl 5 g L^{-1}, bovine extract 1.5 g L^{-1}, yeast extract 1.5 g L^{-1} (HIMEDIA, Mumbai, India), sterilized MilliQ water with 18 MΩ. pH of the cultivation medium was adjusted at 7.4 before sterilization. Sterilization of media was carried out at 121 ℃ for 30 min in sterilizer (Tuttnauer 2450EL, Beit Shemesh, Israel). The prepared cultivation media were inoculated with bacterial culture into 25 ml Erlenmeyer flasks. After the inoculation, bacterial cultures were cultivated for 24 h on a shaker at 600 rpm and 37 ℃. Bacterial culture cultivated under these conditions was diluted by cultivation medium to OD600 = 0.1 and used in the following experiments.

3.5. Determination of Antibacterial Properties

To determine the antimicrobial effect of silver(I) ions and silver phosphate nanoparticles (SPNPs) in complexes with hyaluronic acid and chitosan, the measurement of the inhibition zones was performed. Agar surface in Petri dish was covered with a mixture of 100 mL of 24 h culture of *S. aureus* in the exponential phase of growth, and 3 mL of LB medium (Luria Bertani medium). Excess volume of the mixture of the Petri dishes was aspirated. Squares of size of 1 × 1 cm were cut out from the knitted vascular prosthesis. These squares were mixed with combinations of hyaluronic acid or chitosan with different concentration of silver(I) ions or silver phosphate nanoparticles in Eppendorf tubes. Soaked squares were then laid crosswise on a Petri dish, two squares per dish. Petri dishes were insulated against possible external contamination and placed in a thermostat (Tuttnauer 2450EL, Beit Shemesh, Israel) set at 37 ℃ for 24 h. After 24 h of incubation, the inhibition zones were measured and photographed in each Petri dish.

3.6. Determination of Growth Curves

The second procedure for the evaluation of an antimicrobial effect of tested compounds and their combinations was based on the measurement of an absorbance of these compounds in combination with a bacterial culture of *Staphylococcus aureus*. An apparatus Multiskan EX (Thermo Fisher

Scientific, Bremen, Germany) via Ascent Software for Multiskan, and subsequent analysis in the form of growth curves, was used. The same culture as in the measurement of inhibition zones was diluted with LB medium to absorbance of 0.1 measured using a Specord spectrophotometer 210 (Analytik Jena, Jena, Germany) at a wavelength of 600 nm. The diluted culture was pipetted into a microplate (total volume of 300 μL) alone as a control variant, or with various concentrations of tested substances (silver(I) ions or SPNPs). The concentration of silver(I) ions and SPNPs were 0, 10, 25, 50, 75, 150, 225, and 300 μM, the concentration of hyaluronic acid (8.3 mM) and chitosan (9.7 mM) were always the same. Measurements were carried out at time 0, then each half-hour for 24 h at 37 °C, at a wavelength of 620 nm. The measured absorbance values were analysed in graphic form as growth curves for each experimental group individually.

3.7. Biochemical Tests

Bacterial culture was treated with individual substances deposited on the bottom of the microplate wells. The substances were as follows: urease, arginine, ornithine, β-galactosidase, β-glucuronidase, β-glucosidase, phosphatase, esculin, *N*-acetyl-β-D-glucosamine, sucrose, mannitol, xylose, galactose, trehalose, maltose, mannose, lactose, sorbitol, ribose, fructose, cellobiose, arabinose, xylitol, and raffinose. All substances were purchased from Sigma-Aldrich. Bacterial culture of *S. aureus* of the concentration about 8.0.107 KTJ/1 mL was pipetted into the individual wells. This culture was diluted to an absorbance of 0.1 measured using a Specord spectrophotometer set at a wavelength of 600 nm. Silver nanoparticles at a concentration of 10 μM and hyaluronic acid (24.9 mM) or chitosan (29.3 mM) were subsequently added to this culture. Complexes were measured for 24 h using a Multiskan EX apparatus (Thermo Fisher Scientific, Germany) via Ascent Software for Multiskan.

3.8. Determination of Protein Matrix-Assisted Laser Desorption-Ionization Time-of-Flight (MALDI) Mass Spectra

A sample of 500 μL *S. aureus* (0.1 OD) culture, cultivated overnight, was centrifuged at 14,000 × *g* for 2 min. After this, supernatant was discarded and the pellet was suspended in 300 μL of deionized water. Then, 900 μL of ethanol was added. After centrifugation at 14,000 × *g* for 2 min, supernatant was discarded and the obtained pellet was air-dried. The pellet was then dissolved in 25 μL of 70% formic acid (*v*/*v*) and 25 μL of acetonitrile and mixed. The samples were centrifuged at 14,000 × *g* for 2 min and 1 μL of the clear supernatant was spotted in duplicate onto the MALDI target (MTP 384 target polished steel plate; Bruker Daltonics, Bremen, Germany) and air-dried at room temperature. Then, each spot was overlaid with 1 μL of α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution saturated with organic solvent (50% acetonitrile and 2.5% trifluoroacetic acid, both *v*/*v*) and air-dried completely prior to matrix-assisted laser desorption-ionization time-of-flight mass spectrometric (MALDI-TOF MS) measurement. Spectra were taken in the *m*/*z* range of 2000 Da to 20,000 Da, and each was a result of the accumulation of at least 1000 laser shots obtained from ten different regions of the same sample spot. Spectra were analysed with the Flex Analysis software (Version 3.4). Prior to analysis, the mass spectrometer was externally calibrated with a peptide mix of bombesin, angiotensin I, glu-fibrinopeptide B, adrenocorticotropic hormone (ACTH) (18-39), ubiquitine, and cytochrome c.

Spectra with peaks outside the allowed average were not considered. Modified spectra were loaded into the MALDI BioTyper™ 3.1 Version (Bruker Daltonik GmbH, Bremen, Germany).

3.9. Electrochemical Determination of Silver(I) Ions, and SPNPs in Complexes with Hyaluronic Acid and Chitosan Using Sensor Array

Differential pulse voltammetric (DPV) measurements [86–88] were performed using a PalmSens (PalmSens, The Netherlands) potentiostat connected with sensor array (Brno University of Technology, Brno, Czech Republic) through a control box (Brno University of Technology). For smoothing and baseline correction the PalmSens software supplied was employed. As the supporting electrolyte, acetate buffer (0.2 M CH_3COOH; 0.2 M CH_3COONa) was used. Applied volume of samples was 50 μL. DPV conditions were as follows: start potential −0.2 V, end potential 0.5 V, amplitude 0.05 V, time of accumulation 60 s.

3.10. Spectrophotometric Determination of Silver(I) Ions, and SPNPs in Complexes with Hyaluronic Acid and Chitosan

Absorption spectra were recorded by a SPECORD 210 spectrophotometer (Analytic Jena, Jena, Germany) in the wavelength range λ = 200–700 nm using the WinAspect 2.2.7.0. (Analytic Jena, Jena, Germany). Quartz cuvettes of 1 cm optical path (Hellma, Essex, Great Britain) were used. The analyzed sample was made up of a combination of tested substances (12 mM silver(I) ions or 10 mM SPNPs with 24.9 mM hyaluronic acid or 29.3 mM chitosan). Primarily, we registered absorption spectra (200–700 nm) of each of reagents ($AgNO_3$ and SPNPs). Then, a polymer substance of 24.9 mM hyaluronic acid or 29.3 mM chitosan in 20 μL volume was pipetted into the sample of 12 mM $AgNO_3$ or 10 mM SPNPs. The samples were incubated for 5 min at 25 °C under vigorous stirring. Then, absorption spectra were recorded. This procedure was repeated eight times. ACS water was used as a solvent. Final concentrations of polymer substances were 0.5, 1.0, 1.4, 1.8, 2.3, 2.7, 3.1, 3.4, and 3.8 mM for hyaluronic acid and 0.6, 1.1, 1.7, 2.2, 2.7, 3.1, 3.6, 4.0, and 4.5 mM for chitosan.

3.11. Scanning Electron Microscope

Structures were characterized by scanning electron microscopy. For documentation of the selected nanomaterials, a FEG-SEM MIRA XMU (Tescan, Brno, Czech Republic) was used. This model is equipped with a high brightness Schottky field emitter for low noise imaging at fast scanning rates. SEM was fitted with Everhart-Thronley type of SE detector, high speed YAG scintillator based BSE detector, panchromatic CL Detector, and EDX spectrometer. The MIRA 3 XMU system is based on a large specimen chamber with motorized stage movements 130 × 130 mm. Samples were coated by 10 nm of carbon (Sigma-Aldrich, St. Louis, MO, USA) to prevent a sample charging. A carbon coater K950X (Quorum Technologies, Grinstead, UK) was used. For automated acquisition of selected areas, a TESCAN proprietary software tool Image Snapper was used. The software enables automatic acquisition of selected areas with defined resolution. Different conditions were optimized in order to reach either minimum analysis time or maximum detail during overnight, automated analysis. An

accelerating voltage of 15 kV and beam currents of about 1 nA gives satisfactory results regarding maximum throughput.

3.12. Statistical Analyses

Software STATISTICA (data analysis software system), version 10 .0 (Tulsa, OK, USA) was used for data processing. Half-maximal concentrations (IC_{50}) were calculated from logarithmic regression of sigmoideal dose-response curve. General regression model was used to analyse differences between the combinations of compounds. To reveal differences between cell lines, Turkey's *post hoc* test within homogenous groups was employed. Unless noted otherwise, $p < 0.05$ was considered significant.

4. Conclusions

Testing of antibacterial properties of silver complexes ($AgNO_3$ or SPNPs) with the body's own substances (hyaluronic acid or chitosan) was carried out on the bacterial culture of *Staphylococcus aureus*, by measuring the inhibition zones and determination of growth characteristics. Our results indicate the highest antimicrobial properties of complexes of silver(I) ions and silver nanoparticles with chitosan. The results can be used for further experiments with possible application in vascular surgery, particularly to reduce bacterial infections, which represent a high risk in the implantation of artificial vascular grafts.

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Conflict of Interest

The authors declare no conflict of interest.

References

1. Perl, T.M.; Cullen, J.J.; Wenzel, R.P.; Zimmerman, M.B.; Pfaller, M.A.; Sheppard, D.; Twombley, J.; French, P.P.; Herwaldt, L.A.; the Mupirocin and the Risk of *Staphylococcus aureus* Study Team. Intranasal mupirocin to prevent postoperative *Staphylococcus aureus* infections. *N. Engl. J. Med.* **2002**, *346*, 1871–1877.
2. Ginalska, G.; Osinska, M.; Uryniak, A.; Urbanik-Sypniewska, T.; Belcarz, A.; Rzeski, W.; Wolski, A. Antibacterial activity of gentamicin-bonded gelatin-sealed polyethylene terephthalate vascular prostheses. *Eur. J. Vasc. Endovasc. Surg.* **2005**, *29*, 419–424.
3. Bandyk, D.F. Vascular surgical site infection: Risk factors and preventive measures. *Semin. Vasc. Surg.* **2008**, *21*, 119–123.
4. Teebken, O.E.; Bisdas, T.; Assadian, O.; Ricco, J.B. Recommendations for reporting treatment of aortic graft infections. *Eur. J. Vasc. Endovasc. Surg.* **2012**, *43*, 174–181.
5. Homer-Vanniasinkam, S. Surgical site and vascular infections: Treatment and prophylaxis. *Int. J. Infect. Dis.* **2007**, *11*, S17–S22.

6. O'Brien, R.; Pocock, N.; Torella, F. Wound infection after reconstructive arterial surgery of the lower limbs: Risk factors and consequences. *Surg. J. R. Coll. Surg. Edinb. Irel.* **2011**, *9*, 245–248.
7. Tatterton, M.R.; Homer-Vanniasinkam, S. Infections in vascular surgery. *Injury-Int. J. Care Inj.* **2011**, *42*, S35–S41.
8. Nasim, A.; Thompson, M.M.; Naylor, A.R.; Bell, P.R.F.; London, N.J.M. The impact of MRSA on vascular surgery. *Eur. J. Vasc. Endovasc. Surg.* **2001**, *22*, 211–214.
9. Young, M.H.; Upchurch, G.R.; Malani, P.N. Vascular graft infections. *Infect. Dis. Clin. N. Am.* **2012**, *26*, 41–56.
10. Anderson, D.J.; Sexton, D.J.; Kanafani, Z.A.; Auten, G.; Kaye, K.S. Severe surgical site infection in community hospitals: Epidemiology, key procedures, and the changing prevalence of methicillin-resistant staphylococcus aureus. *Infect. Control Hosp. Epidemiol.* **2007**, *28*, 1047–1053.
11. Earnshaw, J.J. Methicillin-resistant *Staphylococcus aureus*: Vascular surgeons should fight back. *Eur. J. Vasc. Endovasc. Surg.* **2002**, *24*, 283–286.
12. Driscoll, A.J.; Bhat, N.; Karron, R.A.; O'Brien, K.L.; Murdoch, D.R. Disk diffusion bioassays for the detection of antibiotic activity in body fluids: Applications for the pneumonia etiology research for child health project. *Clin. Infect. Dis.* **2012**, *54*, S159–S164.
13. Lew, W.; Moore, W. Antibiotic-impregnated grafts for aortic reconstruction. *Semin. Vasc. Surg.* **2011**, *24*, 211–219.
14. Ricco, J.B.; Assadian, O. Antimicrobial silver grafts for prevention and treatment of vascular graft infection. *Semin. Vasc. Surg.* **2011**, *24*, 234–241.
15. Green, J.B.D.; Fulghum, T.; Nordhaus, M.A. Review of immobilized antimicrobial agents and methods for testing. *Biointerphases* **2011**, *6*, MR13–MR28.
16. Osinska-Jaroszuk, M.; Ginalska, G.; Belcarz, A.; Uryniak, A., Vascular prostheses with covalently bound gentamicin and amikacin reveal superior antibacterial properties than silver-impregnated ones—An *in vitro* study. *Eur. J. Vasc. Endovasc. Surg.* **2009**, *38*, 697–706.
17. Rai, M.K.; Deshmukh, S.D.; Ingle, A.P.; Gade, A.K. Silver nanoparticles: The powerful nanoweapon against multidrug-resistant bacteria. *J. Appl. Microbiol.* **2012**, *112*, 841–852.
18. Unger, C.; Luck, C. Inhibitory effects of silver ions on *Legionella pneumophila* grown on agar, intracellular in *Acanthamoeba castellanii* and in artificial biofilms. *J. Appl. Microbiol.* **2012**, *112*, 1212–1219.
19. Xu, H.Y.; Qu, F.; Xu, H.; Lai, W.H.; Wang, Y.A.; Aguilar, Z.P.; Wei, H. Role of reactive oxygen species in the antibacterial mechanism of silver nanoparticles on *Escherichia coli* O157:H7. *Biometals* **2012**, *25*, 45–53.
20. Park, H.J.; Kim, J.Y.; Kim, J.; Lee, J.H.; Hahn, J.S.; Gu, M.B.; Yoon, J. Silver-ion-mediated reactive oxygen species generation affecting bactericidal activity. *Water Res.* **2009**, *43*, 1027–1032.
21. Kwakye-Awuah, B.; Williams, C.; Kenward, M.A.; Radecka, I. Antimicrobial action and efficiency of silver-loaded zeolite X. *J. Appl. Microbiol.* **2008**, *104*, 1516–1524.
22. Li, W.R.; Xie, X.B.; Shi, Q.S.; Duan, S.S.; Ouyang, Y.S.; Chen, Y.B. Antibacterial effect of silver nanoparticles on *Staphylococcus aureus*. *Biometals* **2011**, *24*, 135–141.
23. Limbach, L.K.; Wick, P.; Manser, P.; Grass, R.N.; Bruinink, A.; Stark, W.J. Exposure of engineered nanoparticles to human lung epithelial cells: Influence of chemical composition and catalytic activity on oxidative stress. *Environ. Sci. Technol.* **2007**, *41*, 4158–4163.

24. Choi, O.; Deng, K.K.; Kim, N.J.; Ross, L.; Surampalli, R.Y.; Hu, Z.Q. The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth. *Water Res.* **2008**, *42*, 3066–3074.
25. Chen, Y.G.; Chen, H.; Zheng, X.; Mu, H. The impacts of silver nanoparticles and silver ions on wastewater biological phosphorous removal and the mechanisms. *J. Hazard. Mater.* **2012**, *239*, 88–94.
26. Jo, H.J.; Choi, J.W.; Lee, S.H.; Hong, S.W. Acute toxicity of Ag and CuO nanoparticle suspensions against Daphnia magna: The importance of their dissolved fraction varying with preparation methods. *J. Hazard. Mater.* **2012**, *227*, 301–308.
27. Joshi, N.; Ngwenya, B.T.; French, C.E. Enhanced resistance to nanoparticle toxicity is conferred by overproduction of extracellular polymeric substances. *J. Hazard. Mater.* **2012**, *241*, 363–370.
28. Pallavicini, P.; Taglietti, A.; Dacarro, G.; Diaz-Fernandez, Y.A.; Galli, M.; Grisoli, P.; Patrini, M.; de Magistris, G.S.; Zanoni, R. Self-assembled monolayers of silver nanoparticles firmly grafted on glass surfaces: Low Ag^+ release for an efficient antibacterial activity. *J. Colloid Interface Sci.* **2010**, *350*, 110–116.
29. Taglietti, A.; Fernandez, Y.A.D.; Amato, E.; Cucca, L.; Dacarro, G.; Grisoli, P.; Necchi, V.; Pallavicini, P.; Pasotti, L.; Patrini, M. Antibacterial activity of glutathione-coated silver nanoparticles against gram positive and gram negative bacteria. *Langmuir* **2012**, *28*, 8140–8148.
30. Dowling, D.P.; Betts, A.J.; Pope, C.; McConnell, M.L.; Eloy, R.; Arnaud, M.N. Anti-bacterial silver coatings exhibiting enhanced activity through the addition of platinum. *Surf. Coat. Technol.* **2003**, *163*, 637–640.
31. Ruden, S.; Hilpert, K.; Berditsch, M.; Wadhwani, P.; Ulrich, A.S. Synergistic interaction between silver nanoparticles and membrane-permeabilizing antimicrobial peptides. *Antimicrob. Agents Chemother.* **2009**, *53*, 3538–3540.
32. Zitka, O.; Sobrova, P.; Adam, V.; Hubalek, J.; Provaznik, I.; Zizkova, V.; Kizek, R. Nanotechnology for more efficient blood vessel replacements. *Chem. Listy* **2013**, *107*, 24–29.
33. Evanko, S.P.; Wight, T.N. Intracellular localization of hyaluronan in proliferating cells. *J. Histochem. Cytochem.* **1999**, *47*, 1331–1341.
34. Kogan, G.; Soltes, L.; Stern, R.; Gemeiner, P. Hyaluronic acid: A natural biopolymer with a broad range of biomedical and industrial applications. *Biotechnol. Lett.* **2007**, *29*, 17–25.
35. Guibal, E. Interactions of metal ions with chitosan-based sorbents: A review. *Sep. Purif. Technol.* **2004**, *38*, 43–74.
36. Pires, N.R.; Cunha, P.L.R.; Maciel, J.S.; Angelim, A.L.; Melo, V.M.M.; de Paula, R.C.M.; Feitosa, J.P.A. Sulfated chitosan as tear substitute with no antimicrobial activity. *Carbohydr. Polym.* **2013**, *91*, 92–99.
37. Madhumathi, K.; Kumar, P.T.S.; Abhilash, S.; Sreeja, V.; Tamura, H.; Manzoor, K.; Nair, S.V.; Jayakumar, R. Development of novel chitin/nanosilver composite scaffolds for wound dressing applications. *J. Mater. Sci.* **2010**, *21*, 807–813.
38. Jayakumar, R.; Menon, D.; Manzoor, K.; Nair, S.V.; Tamura, H. Biomedical applications of chitin and chitosan based nanomaterials—A short review. *Carbohydr. Polym.* **2010**, *82*, 227–232.
39. Huang, G.Q.; Sun, Y.T.; Xiao, J.X.; Yang, J. Complex coacervation of soybean protein isolate and chitosan. *Food Chem.* **2012**, *135*, 534–539.

40. Lee, S.B.; Kim, Y.H.; Chong, M.S.; Lee, Y.M. Preparation and characteristics of hybrid scaffolds composed of beta-chitin and collagen. *Biomaterials* **2004**, *25*, 2309–2317.
41. Abdel-Mohsen, A.M.; Hrdina, R.; Burgert, L.; Krylova, G.; Abdel-Rahman, R.M.; Krejcova, A.; Steinhart, M.; Benes, L. Green synthesis of hyaluronan fibers with silver nanoparticles. *Carbohydr. Polym.* **2012**, *89*, 411–422.
42. Wan, Y.; Guo, Z.R.; Jiang, X.L.; Fang, K.; Lu, X.; Zhang, Y.; Gu, N. Quasi-spherical silver nanoparticles: Aqueous synthesis and size control by the seed-mediated Lee-Meisel method. *J. Colloid Interface Sci.* **2013**, *394*, 263–268.
43. Jiang, J.; Chae, B.; Jeong, S.K.; Min, B.K.; Kim, S.H.; Piao, L.; Yoon, S. Assembling Ag nanoparticles into morphology controlled secondary structures on loosely packed self-assembled monolayers. *J. Colloid Interface Sci.* **2013**, *394*, 639–642.
44. Boomi, P.; Prabu, H.G.; Mathiyarasu, J. Synthesis and characterization of polyaniline/Ag-Pt nanocomposite for improved antibacterial activity. *Colloid Surf. B* **2013**, *103*, 9–14.
45. Li, G.Y.; Wen, Q.W.; Zhang, T.; Ju, Y.Y. Synthesis and properties of silver nanoparticles in chitosan-based thermosensitive semi-interpenetrating hydrogels. *J. Appl. Polym. Sci.* **2013**, *127*, 2690–2697.
46. Li, X.; Lenhart, J.J. Aggregation and dissolution of silver nanoparticles in natural surface water. *Environ. Sci. Technol.* **2012**, *46*, 5378–5386.
47. Khan, A.; Qamar, M.; Muneer, M. Synthesis of highly active visible-light-driven colloidal silver orthophosphate. *Chem. Phys. Lett.* **2012**, *519–520*, 54–58.
48. Bin Ahmad, M.; Lim, J.J.; Shameli, K.; Ibrahim, N.A.; Tay, M.Y., Synthesis of silver nanoparticles in chitosan, gelatin and chitosan/gelatin bionanocomposites by a chemical reducing agent and their characterization. *Molecules* **2011**, *16*, 7237–7248.
49. Dospivova, D.; Hynek, D.; Kopel, P.; Bezdekova, A.; Sochor, J.; Krizkova, S.; Adam, V.; Trnkova, L.; Hubalek, J.; Babula, P., *et al.* Electrochemical behaviour of apoferritin encapsulating of silver(i) ions and its application for treatment of *Staphylococcus aureus*. *Int. J. Electrochem. Sci.* **2012**, *7*, 6378–6395.
50. Trnkova, L.; Krizkova, S.; Adam, V.; Hubalek, J.; Kizek, R. Immobilization of metallothionein to carbon paste electrode surface via anti-MT antibodies and its use for biosensing of silver. *Biosens. Bioelectron.* **2011**, *26*, 2201–2207.
51. Zitka, O.; Huska, D.; Adam, V.; Horna, A.; Beklova, M.; Svobodova, Z.; Kizek, R. CoulArray detector as a tool for estimation of acute toxicity of silver(I) ions. *Int. J. Electrochem. Sci.* **2010**, *5*, 1082–1089.
52. Chekin, F.; Raoof, J.B.; Bagheri, S.; Abd Hamid, S.B. The porous chitosan-sodium dodecyl sulfate-carbon nanotube nanocomposite: Direct electrochemistry and electrocatalysis of hemoglobin. *Anal. Methods* **2012**, *4*, 2977–2981.
53. Ma, L.P.; Yuan, R.; Chai, Y.Q.; Chen, S.H. Amperometric hydrogen peroxide biosensor based on the immobilization of HRP on DNA-silver nanohybrids and PDDA-protected gold nanoparticles. *J. Mol. Catal. B* **2009**, *56*, 215–220.
54. An, J.; Luo, Q.Z.; Yuan, X.Y.; Wang, D.S.; Li, X.Y. Preparation and characterization of silver-chitosan nanocomposite particles with antimicrobial activity. *J. Appl. Polym. Sci.* **2011**, *120*, 3180–3189.

55. Sharma, S.; Sanpui, P.; Chattopadhyay, A.; Ghosh, S.S. Fabrication of antibacterial silver nanoparticle-sodium alginate-chitosan composite films. *RSC Adv.* **2012**, *2*, 5837–5843.
56. Rodriguez-Arguelles, M.C.; Sieiro, C.; Cao, R.; Nasi, L. Chitosan and silver nanoparticles as pudding with raisins with antimicrobial properties. *J. Colloid Interface Sci.* **2011**, *364*, 80–84.
57. Murray, P.R. What is new in clinical microbiology microbial identification by MALDI-TOF mass spectrometry a paper from the 2011 William Beaumont Hospital Symposium on molecular pathology. *J. Mol. Diagn.* **2012**, *14*, 419–423.
58. Welker, M. Proteomics for routine identification of microorganisms. *Proteomics* **2011**, *11*, 3143–3153.
59. Jordana-Lluch, E.; Catala, E.M.; Ruiz, V.A. Mass spectrometry in the clinical microbiology laboratory. *Enferm. Infec. Microbiol. Clin.* **2012**, *30*, 635–644.
60. Kok, J.; Chen, S.C.A.; Dwyer, D.E.; Iredell, J.R. Current status of matrix-assisted laser desorption ionisation-time of flight mass spectrometry in the clinical microbiology laboratory. *Pathology* **2013**, *45*, 4–17.
61. Lu, J.J.; Tsai, F.J.; Ho, C.M.; Liu, Y.C.; Chen, C.J. Peptide biomarker discovery for identification of methicillin-resistant and vancomycin-intermediate *Staphylococcus aureus* strains by MALDI-TOF. *Anal. Chem.* **2012**, *84*, 5685–5692.
62. Ouedraogo, R.; Daumas, A.; Ghigo, E.; Capo, C.; Mege, J.L.; Textoris, J. Whole-cell MALDI-TOF MS: A new tool to assess the multifaceted activation of macrophages. *J. Proteomics* **2012**, *75*, 5523–5532.
63. Van Veen, S.Q.; Claas, E.C.J.; Kuijper, E.J., High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization-time of flight mass spectrometry in conventional medical microbiology laboratories. *J. Clin. Microbiol.* **2010**, *48*, 900–907.
64. Szabados, F.; Woloszyn, J.; Richter, C.; Kaase, M.; Gatermann, S. Identification of molecularly defined *Staphylococcus aureus* strains using matrix-assisted laser desorption/ionization time of flight mass spectrometry and the Biotyper 2.0 database. *J. Med. Microbiol.* **2010**, *59*, 787–790.
65. Charyulu, E.M.; Gnanamani, A.; Mandal, A.B., Identification and Discrimination of methicillin resistant *Staphylococcus aureus* strains isolated from burn wound sites using pcr and authentication with MALDI-TOF-MS. *Indian J. Microbiol.* **2012**, *52*, 337–345.
66. Bohme, K.; Morandi, S.; Cremonesi, P.; No, I.C.F.; Barros-Velazquez, J.; Castiglioni, B.; Brasca, M.; Canas, B.; Calo-Mata, P. Characterization of *Staphylococcus aureus* strains isolated from Italian dairy products by MALDI-TOF mass fingerprinting. *Electrophoresis* **2012**, *33*, 2355–2364.
67. Verma, V.C.; Kharwar, R.N.; Gange, A.C., Biosynthesis of antimicrobial silver nanoparticles by the endophytic fungus *Aspergillus clavatus*. *Nanomedicine* **2010**, *5*, 33–40.
68. Dar, M.A.; Ingle, A.; Rai, M. Enhanced antimicrobial activity of silver nanoparticles synthesized by *Cryphonectria* sp. evaluated singly and in combination with antibiotics. *Nanomedicine* **2013**, *9*, 105–110.
69. Mohanty, S.; Mishra, S.; Jena, P.; Jacob, B.; Sarkar, B.; Sonawane, A. An investigation on the antibacterial, cytotoxic, and antibiofilm efficacy of starch-stabilized silver nanoparticles. *Nanomedicine* **2012**, *8*, 916–924.

70. Martinez-Gutierrez, F.; Thi, E.P.; Silverman, J.M.; de Oliveira, C.C.; Svensson, S.L.; Hoek, A.V.; Sanchez, E.M.; Reiner, N.E.; Gaynor, E.C.; Pryzdial, E.L.G.; *et al.* Antibacterial activity, inflammatory response, coagulation and cytotoxicity effects of silver nanoparticles. *Nanomedicine* **2012**, *8*, 328–336.
71. Martinez-Gutierrez, F.; Olive, P.L.; Banuelos, A.; Orrantia, E.; Nino, N.; Sanchez, E.M.; Ruiz, F.; Bach, H.; Av-Gay, Y. Synthesis, characterization, and evaluation of antimicrobial and cytotoxic effect of silver and titanium nanoparticles. *Nanomedicine* **2010**, *6*, 681–688.
72. Hrabarova, E.; Valachova, K.; Rychly, J.; Rapta, P.; Sasinkova, V.; Malikova, M.; Soltes, L. High-molar-mass hyaluronan degradation by Weissberger's system: Pro- and anti-oxidative effects of some thiol compounds. *Polym. Degrad. Stabil.* **2009**, *94*, 1867–1875.
73. Sintzel, M.B.; Bernatchez, S.F.; Tabatabay, C.; Gurny, R. Biomaterials in ophthalmic drug delivery. *Eur. J. Pharm. Biopharm.* **1996**, *42*, 358–374.
74. Stern, R. Devising a pathway for hyaluronan catabolism: Are we there yet? *Glycobiology* **2003**, *13*, 105R–115R.
75. Teh, B.M.; Shen, Y.; Friedland, P.L.; Atlas, M.D.; Marano, R.J. A review on the use of hyaluronic acid in tympanic membrane wound healing. *Expert Opin. Biol. Ther.* **2012**, *12*, 23–36.
76. Fernandez-Saiz, P.; Soler, C.; Lagaron, J.M.; Ocio, M.J. Effects of chitosan films on the growth of *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella* spp. in laboratory media and in fish soup. *Int. J. Food Microbiol.* **2010**, *137*, 287–294.
77. Borneman, D.L.; Ingham, S.C.; Ane, C. Mathematical approaches to estimating lag-phase duration and growth rate for predicting growth of *Salmonella serovar*s, *Escherichia coli* O157:H7, and *Staphylococcus aureus* in raw beef, bratwurst, and poultry. *J. Food Prot.* **2009**, *72*, 1190–1200.
78. Rufian-Henares, J.A.; Morales, F.J. Microtiter plate-based assay for screening antimicrobial activity of melanoidins against *E-coli* and *S-aureus*. *Food Chem.* **2008**, *111*, 1069–1074.
79. Mahdavi, B.; Yaacob, W.A.; Din, L.B.; Nazlina, I. Antimicrobial activity of consecutive extracts of *Etlingera brevilabrum*. *Sains Malays.* **2012**, *41*, 1233–1237.
80. Joray, M.B.; Gonzalez, M.L.; Palacios, S.M.; Carpinella, M.C. Antibacterial activity of the plant-derived compounds 23-Methyl-6-*O*-desmethylauricepyrone and (*Z*,*Z*)-5-(Trideca-4,7-dienyl)resorcinol and their synergy with antibiotics against methicillin-susceptible and -resistant *Staphylococcus aureus*. *J. Agric. Food Chem.* **2011**, *59*, 11534–11542.
81. Tawiah, A.A.; Gbedema, S.Y.; Adu, F.; Boamah, V.E.; Annan, K. Antibiotic producing microorganisms from River Wiwi, Lake Bosomtwe and the Gulf of Guinea at Doakor Sea Beach, Ghana. *BMC Microbiol.* **2012**, *12*, 1–8.
82. Moussa, A.; Noureddine, D.; Mohamed, H.S.; Abdelmelek, M.; Saad, A. Antibacterial activity of various honey types of Algeria against *Staphylococcus aureus* and *Streptococcus pyogenes*. *Asian Pac. J. Trop. Med.* **2012**, *5*, 773–776.
83. Lokendrajit, N.; Indira, S.; Swapana, N.; Singh, C.B. Antioxidant and antimicrobial activity of *Croton caudatus* Geisel. *Asian J. Chem.* **2012**, *24*, 4418–4420.
84. Zaremba, M.; Borowski, J.; Polejczuk, E.; Jakubicz, P. *Evaluation of Staphytest, A New System for Identification of* Staphylococci; Gustav Fischer Verlag: Stuttgart, Germany, 1991; Volume 21, p. 87.

85. Sedlacek, I.; Kocur, M. Identification of *Staphylococcus* and *Micrococcus* species with the Staphytest system. *Folia Microbiol.* **1991**, *36*, 401–405.
86. Radulescu, M.C.; Chira, A.; Radulescu, M.; Bucur, B.; Bucur, M.P.; Radu, G.L. Determination of silver(i) by differential pulse voltammetry using a glassy carbon electrode modified with synthesized *N*-(2-Aminoethyl)-4,4'-Bipyridine. *Sensors* **2010**, *10*, 11340–11351.
87. Peverly, A.A.; Peters, D.G. Electrochemical determination of trihalomethanes in water by means of stripping analysis. *Anal. Chem.* **2012**, *84*, 6110–6115.
88. Yan, G.P.; Wang, Y.H.; He, X.X.; Wang, K.M.; Su, J.; Chen, Z.F.; Qing, Z.H. A highly sensitive electrochemical assay for silver ion detection based on un-labeled C-rich ssDNA probe and controlled assembly of MWCNTs. *Talanta* **2012**, *94*, 178–183.

5.4 Studium efektu nanostruktur různých typů nanočástic na bakteriální kmeny

5.4.1 Vědecký článek IV

CHUDOBOVA, D.; CIHALOVA, K.; DOSTALOVA, S.; RUTTKAY-NEDECKY, B.; MERLOS RODRIGO, M. A.; KOPEL, P.; NEJDL, L.; KUDR, J.; GUMULEC, J.; KRIZKOVA, S.; KYNICKY, J.; ADAM, V.; KIZEK, R. The effect of silver phosphate nanoparticles (SPNPs) and selenium nanoparticles (SeNPs). *FEMS Microbiology Letters*, 2014, roč. 351. č. 2, s. 195-201. ISSN 0378-1097.

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Jak již bylo řečeno, velmi efektivní zbraní při potlačování vzniku a šíření obávaných bakteriálních infekcí je použití kovů nebo i polokovů, zejména ve formě jejich nanočástic [221-223]. Jak již dlouhodobě známé a probádané syntetizované nanočástice stříbra, tak i novější nanočástice selenu byly shledány jako látky s velmi dobrou baktericidní aktivitou a prokázány jako dobrá alternativa pro rozvoj antimikrobiálních látek [136, 223, 224]. Kovové (polokovové) nanočástice interagují s buněčnými složkami (DNA, RNA a ribozomy), deaktivují a účinně zastavují buněčné procesy [134]. Bylo zjištěno, že nanočástice stříbra, respektive v našem případě fosforečnanu stříbrného (SPNPs), pronikají buněčnou membránou do cytosolu díky pomalému rozpouštění a schopnosti uvolňování Ag^+ iontů. Pro dosažení antimikrobiální aktivity nanočástic je důležitá jejich „velikost“, která brání agregaci nanočástic do větších celků [142, 225].

Naším hlavním cílem bylo studium interakce námi syntetizovaných nanočástic fosforečnanu stříbrného (SPNPs) a selenových nanočástic (SeNPs) s bakteriální kulturou *Staphylococcus aureus* na buněčné, molekulární a proteinové úrovni. Mikrobiologickými studiemi byl potvrzen výrazný antimikrobiální účinek obou typů nanočástic, zejména pak přídavkem SeNPs, kdy byla způsobena totální inhibice růstu bakteriální kultury *S. aureus* v porovnání se stejnou aplikovanou koncentrací SPNPs, která zapříčinila inhibici pouze z 37,5 %. Pozorovaný účinek může být vysvětlen interakcí nanočástic s DNA a proteiny. Z tohoto důvodu byla studována interakce nanočástic s amplifikovaným fragmentem *zntR* genu, která způsobila pokles teploty tání komplexů nanočástic s genem *zntR* v porovnání s kontrolou. Aplikace SeNPs zároveň

způsobovala pokles hladiny bakteriálního metalothioneinu po aplikaci obou typů nanočástic.

Studií byl pozorován výrazný antimikrobiální účinek typů nanočástic na růst bakteriálních kultur a integritu DNA. Tyto nanočástice tak mohou být použity ke snížení rizika bakteriálních infekcí, které se stále více stávají nekontrolovatelnými z hlediska vzniku rezistence vůči konvenční léčbě antibiotickými léčivy.

RESEARCH LETTER

Comparison of the effects of silver phosphate and selenium nanoparticles on *Staphylococcus aureus* growth reveals potential for selenium particles to prevent infection

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Abstract

Interactions of silver phosphate nanoparticles (SPNPs) and selenium nanoparticles (SeNPs) with *Staphylococcus aureus* cultures have been studied at the cellular, molecular and protein level. Significant antibacterial effects of both SPNPs and SeNPs on *S. aureus* were observed. At a concentration of 300 μM, SPNPs caused 37.5% inhibition of bacterial growth and SeNPs totally inhibited bacterial growth. As these effects might have been performed due to the interactions of nanoparticles with DNA and proteins, the interaction of SPNPs or SeNPs with the amplified *zntR* gene was studied. The presence of nanoparticles decreased the melting temperatures of the nanoparticle complexes with the *zntR* gene by 23% for SeNPs and by 12% for SPNPs in comparison with the control value. The concentration of bacterial metallothionein was 87% lower in bacteria after application of SPNPs (6.3 μg mg^{-1} protein) but was increased by 29% after addition of SeNPs (63 μg mg^{-1} protein) compared with the *S. aureus* control (49 μg mg^{-1} protein). Significant antimicrobial effects of the nanoparticles on bacterial growth and DNA integrity provide a promising approach to reducing the risk of bacterial infections that cannot be controlled by the usual antibiotic treatments.

Introduction

Staphylococcus aureus is often a cause of infection in vascular grafts. Such infection may treated with appropriate antibiotics, but most *S. aureus* strains have developed resistance to available antibiotics, and resistance even to new antibiotics is steadily growing (van Hal & Fowler, 2013). The use of metal-based drugs and nanoparticles is one of the highly promising means to suppress infections caused by antibiotic-resistant bacteria (Rai *et al.*, 2009). Synthesized silver and selenium nanoparticles (SeNPs) have been found to be bactericidal and have been proved to be good alternatives for the development of antimicrobial agents (Tran & Webster, 2011; Wang & Webster, 2013). Metallic nanoparticles interacting with cellular components (DNA, RNA and ribosomes) deactivate and effectively alter cellular processes (Grigor'eva *et al.*, 2013). It is assumed that silver nanoparticles can penetrate the cell membrane to reach the cytosol due to their ability to dissolve slowly while releasing Ag^+ ions, but the exact mechanism of the silver nanoparticles antimicrobial action remains unclear.

Selenium has been studied for various medical applications and as a potential material for orthopaedic implants (Wang & Webster, 2012). There also are studies indicating an ability of selenium compounds to inhibit the growth of bacteria and the formation of bacterial biofilms (Wang & Webster, 2012). Among numerous selenium compounds, 2,4,6-tri-para-methoxyphenylselenopyrylium chloride, 9-para-chlorophenyloctahydroselenoxanthene, and perhydroselenoxanthene have been demonstrated to have antibacterial activity *in vitro*, especially against *S. aureus*.

However, the precise effect of elemental SeNPs remains largely unknown (Tran & Webster, 2011; Ramos *et al.*, 2012).

In the past decade, study of the toxicological properties of nanomaterials and/or nanoparticles has opened up a new research field known as nanotoxicology (Yan *et al.*, 2013). Research on nanotoxicity is of extremely high scientific, social and economic value (Pumera, 2011). Nanomaterial-induced reactive oxygen species play a key role in cellular and tissue toxicity (Yan *et al.*, 2013). The toxicity of silver nanoparticles is enhanced in the presence of H_2O_2, highlighting the biological relevance of investigating the oxidative dissolution of these nanoparticles (Ho *et al.*, 2010). In addition, it has been shown that SeNPs can inhibit proliferation and induce apoptosis in promastigote forms of the protozoa *Leishmania major* (Beheshti *et al.*, 2013).

The aims of this study were to investigate the effects of SeNPs and silver phosphate nanoparticles (SPNPs) on inhibiting growth in *S. aureus*, morphologically altering the bacteria cell structure and changing its biochemical parameters, and thereby to identify a suitable substance with antibacterial properties to reduce the risk of bacterial (staphylococcal) infections. Another focus was to observe effects of both SeNPs and SPNPs on bacterial metallothionein concentration and to study the interactions of these nanoparticles with the amplified *zntR* gene.

Materials and methods

Cultivating *S. aureus*

Staphylococcus aureus (NCTC 8511) was obtained from the Czech Collection of Microorganisms, Masaryk University, CZ. *Staphylococcus aureus* was inoculated into lysogeny broth medium for 24 h on a shaker at 40 ***g*** and 37 °C. The bacterial culture was diluted to $OD_{600\ nm} = 0.1$ for all experiments. Petri dishes were each covered with 3 mL of the *S. aureus* culture in lysogeny broth that had been grown for 24 h. Discs 1 cm in diameter applied to Petri dishes with bacteria were cut out from vascular grafts produced by the VUP Medical (Czech Republic) and were mixed with SPNPs or SeNPs in various concentrations. Dishes were incubated in a growth chamber at 37 °C for 24 h. Growth was measured by absorbance using a Multiskan EX apparatus (Thermo Fisher Scientific, Germany). For measurement purposes, *S. aureus* culture was mixed in the microplate with various concentrations of SPNPs and SeNPs. As a control, the culture was also grown without nanoparticles. The concentrations of SPNPs or SeNPs were 0, 10, 25, 50, 75, 150, 225 and 300 μM. Total volume in the microplate wells was always 300 μL (Chudobova *et al.*, 2013a, b).

Analysing biochemical parameters of *S. aureus*

The effect of nanoparticles on *S. aureus* was tested using STAPHYtest 24 biochemical detection tests (Erba Lachema, Czech Republic) for the following substances: urease, arginine, ornithine, β-galactosidase, β-glucuronidase, β-glucosidase, phosphatase, esculin, *N*-acetyl-β-D-glucosamine, sucrose, mannitol, xylose, galactose, trehalose, maltose, mannose, lactose, sorbitol, ribose, fructose, cellobiose, arabinose, xylitol and raffinose. Substances were mixed with *S. aureus* bacterial culture with or without added nanoparticles (300 μM). Complexes were measured for 24 h using the Multiskan EX apparatus via ASCENT Software for Multiskan.

Preparing and characterizing SPNPs and SeNPs

SPNPs were prepared by dissolving (di)sodium hydrogen phosphate heptahydrate in water purified to the American Chemical Society (ACS) standard and then adding a solution of SPNPs nitrate in ACS-standard water according to Khan *et al.* (2012). The reaction proceeded immediately with the formation of yellow colloidal nanoparticles (200–300 nm in diameter; Chudobova *et al.*, 2013a, b). To the prepared SeNPs, sodium selenite pentahydrate $Na_2SeO_3{\cdot}5H_2O$ (26.3 mg) was dissolved in 50 mL ACS-standard water, and 3-mercaptopropionic acid (40 μL) was then slowly added to the solution under stirring. Afterwards, pH was adjusted to eight using 1 M NaOH. The reaction mixture was stirred for 2 h. SeNPs were stored in darkness at 4 °C. One millilitre of the solution contains 158 μg of Se nanoparticles (50–100 nm in diameter). Chemicals used in this study were purchased from Sigma-Aldrich in ACS purity unless noted otherwise.

SPNPs and SeNPs were measured on a Spectro Xepos spectrometer (Spectro Analytical Instruments, Germany). The samples were measured on a powder diffraction anode X-ray tube at 47.63 kV and 0.5 mA current and were detected with a Barkla scatter aluminium oxide. The sample was measured through the polyethylene bottle side wall 20 mm above the bottom. The SPECTRO XEPOS software and TurboQuant method were applied for data analysis.

Cell microscopy

An Olympus IX 71 inverted system microscope (Japan) was used for imaging of the cells. Total magnification was 1600×. The parameters included an exposure time of 32 ms and ISO was set to 200. Structures were also characterized using an FEG-SEM MIRA XMU electron microscope (Tescan, Czech Republic). Samples were coated with 10 nm of carbon using a K950X carbon coater (Quorum Technologies, UK). For automated acquisition

of selected areas, the TESCAN IMAGE SNAPPER proprietary software tool was used.

Determining total proteins

Pyrogallol red protein assay (Skalab, Czech Republic) is based upon the formation of a blue protein-dye complex in the presence of molybdate under acidic conditions (pH 2.5). 150 μL of the reagent mixture (50 mM succinic acid, 3.47 mM sodium benzoate, 0.06 mM sodium molybdate, 1.05 mM sodium oxalate, and 0.07 mM pyrogallol red) was pipetted into a plastic cuvette. Then, 8 μL of sample was added. Absorbance was measured at $\lambda = 605$ nm using a BS-400 chemistry analyser (Mindray, China) after 6 min of incubation. The resulting value was calculated from the absorbance values of the pure reagent mixture and that of the sample after 6 min of incubation.

Differential pulse voltammetric determination of metallothionein

Electrochemical measurements were performed on a 747 VA Stand instrument connected to a 746 VA Trace Analyzer and 695 Autosampler (Switzerland), using a standard cell with three electrodes and cooled sample holder (4 °C). As a supporting electrolyte, Brdicka solution containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonium buffer [$NH_3(aq)$ and NH_4Cl, pH 9.6] was used (Adam *et al.*, 2008).

Interaction of *zntR* gene from DNA with SPNPs and SeNPs

The *zntR* gene was amplified from *S. aureus* according to Singh *et al.* (1999). Spectra were recorded at 200–400 nm after 120 min of interaction using 1-cm quartz cuvettes (Hellma, UK) on a SPECORD 210 spectrophotometer (Analytik Jena, Germany) at 25 °C. Denaturation of a complex of amplified *zntR* gene with SPNPs and SeNPs in eight concentrations (0, 10, 25, 50, 75, 150, 225 and 300 μM) was monitored spectrophotometrically using a SPECORD S600 spectrophotometer with a diode detector (Analytik Jena). The sample was incubated for 3 min at increasing temperatures at 25–99 °C and the absorbance was measured at 200–800 nm.

Determining protein mass spectra by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF)

After cultivation overnight, 500 μL culture ($0.1 = OD_{600\ nm}$) was centrifuged at 14 000 ***g*** for 2 min. Supernatant was discarded and the pellet was suspended in 300 μL of deionized water. Then, 900 μL of ethanol was added. After centrifugation at 14 000 ***g*** for 2 min, the supernatant was discarded and the obtained pellet was air-dried. The pellet was then dissolved in 25 μL of 70% formic acid (v/v) and 25 μL of acetonitrile and mixed. The samples were centrifuged at 14 000 ***g*** for 2 min and 1 μL of the clear supernatant was spotted in duplicate onto an MTP 384 target (Bruker Daltonics) and air-dried at room temperature. Each spot was overlaid with 1 μL of α-cyano-4-hydroxycinnamic acid matrix solution. Spectra were measured on a Bruker MALDI-TOF/TOF apparatus in the *m*/*z* range of 2–20 kDa.

Results

Metal nanoparticles caused significant growth inhibition of *S. aureus*, with application of SeNPs inducing twice as much growth inhibition as SPNPs (7.0 ± 0.5 and 3.0 ± 0.5 mm, respectively). There were also significant morphological changes in the cell structure, these being most evident in the nucleoid (Fig. 1A).

In addition, our synthesized nanoparticles (SPNPs or SeNPs) were characterized with a scanning electron microscope (Fig. 1B). Figure 1(B-d) shows the structure of *S. aureus* culture without the addition of nanoparticles. In the case of SPNPs, the formations were square or spherical in character (Fig. 1B-e) and in the case of SeNPs only small spherical particles (Fig. 1B-f) were observed, which confirmed our previous hypothesis.

After *S. aureus* had been exposed for 18 h to SPNPs, inhibition of bacterial growth was observed and absorbance values at 600 nm had decreased from 0.4 to 0.25 a.u. (Fig. 2A-a). In applying the SPNP concentration with the weakest antibacterial activity (10 μM) to the bacterial culture, absorbance values comparable to those of the control group (0.4) were obtained. The greatest decrease in absorbance values (by 38%) in comparison with the control was achieved by applying 300 μM SPNPs (Fig. 2A-a). By contrast, in the case of SeNPs, the minimum inhibitory concentration was achieved when 10 μM SeNPs was applied and the total inhibitory concentration occurred when 300 μM SeNPs was applied (Fig. 2A-b). Half maximal inhibitory concentration values confirmed the obtained results, as IC_{50} was 268.2 for SPNPs and 4.4 μM for SeNPs.

We also investigated changes in the biochemical parameters of *S. aureus* after the addition of nanoparticles. Twenty-four hours after application of SPNPs (300 μM), changes in 11 biochemical tests (of 24 tests in total) were observed (Fig. 2B-b). After application of SeNPs, the results of only seven tests were different from those of the control group (Fig. 2B-c).

Because we observed changes in the morphology of the bacterial cell nucleoid after exposure of *S. aureus* to

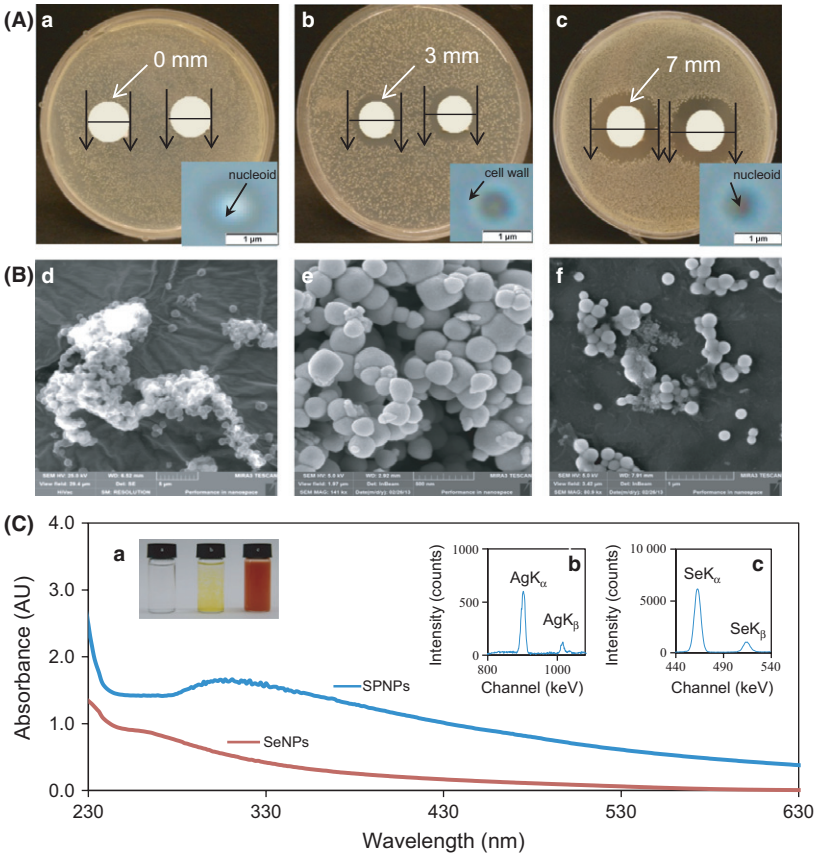

Fig. 1. (A) Growth inhibition zones in bacterial culture after application of 300 μM of nanoparticles and a microscopic view of cells: (a) *Staphylococcus aureus*, (b) after application of SPNPs, (c) after application of SeNPs. (B) Bacterial culture and nanoparticles under electron microscopy: (d) *S. aureus*, (e) SPNPs, (f) SeNPs. Images are in different scales, with 1 unit of the scale corresponding to 5 μm in B-d, 500 nm in B-e, and 1 μm in B-f. (C) Absorption spectra of 300 μM of SPNPs and SeNPs: (a) visualization in ambient light: MilliQ water, SPNPs, SeNPs; X-ray view of content elements: (b) SPNPs; (c) SeNPs.

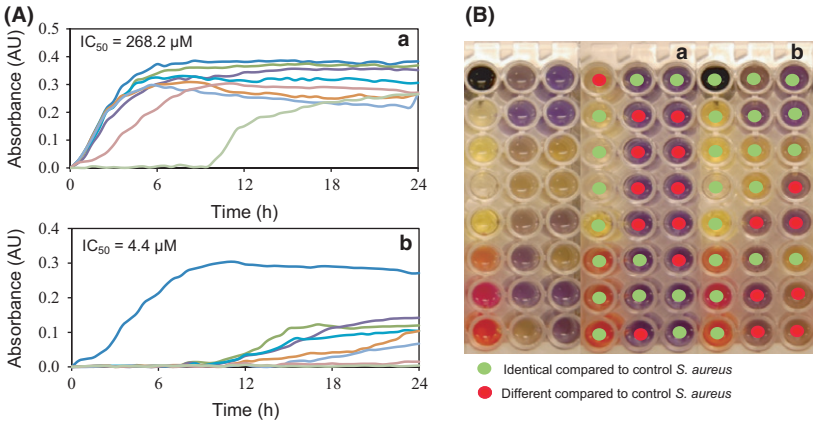

Fig. 2. (A) Spectrophotometric analysis of the growth of *S. aureus* bacterial culture with (a) SPNPs and (b) SeNPs in concentrations 0, 10, 25, 50, 75, 150, 225 and 300 μM. (B) Biochemical parameters of (a) *S. aureus*, (b) *S. aureus* with 300 μM SPNPs, and (c) *S. aureus* with 300 μM SeNPs.

SPNPs or SeNPs, we investigated the interaction of both nanoparticle types with DNA using amplified *zntR* gene. After application of the various concentrations of SeNPs (0, 10, 25, 50, 75, 150, 225 and 300 μM) there was a marked increase in absorbance signal at 260 nm with increasing concentrations of SeNPs (Fig. 3A-b) and composition of nanoparticles, but this shift was not observed when SPNPs were applied (Fig. 3A-a). Statistically significant changes were observed in the melting temperatures of the amplified *zntR* gene in complexes with SPNPs or SeNPs at the highest concentration of 300 μM (Fig. 3B). Differential changes in the melting temperature of amplified *zntR* gene probably can be caused by different affinity of nanoparticles to the bacterial DNA. A greater decrease in melting temperature in comparison with the control was observed in the interaction of SPNPs and amplified *zntR* gene, than in the interaction of SeNPs with amplified *zntR* gene (23% vs. 12%, respectively). SPNPs may

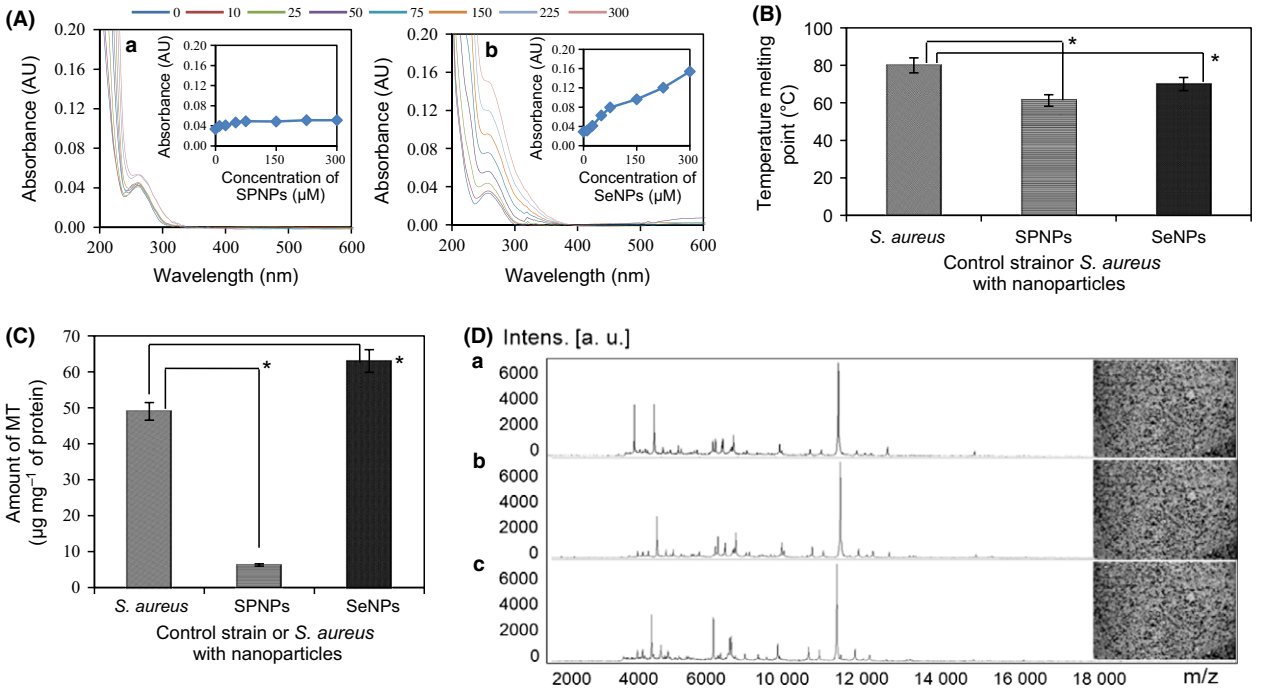

Fig. 3. (A) Spectrophotometric determination of amplified *zntR* gene after application of different concentrations (0, 10, 25, 50, 75, 150, 225 and 300 μM) of nanoparticles: (a) SPNPs, (b) SeNPs. (B) Changes of bacterial DNA melting temperature after application of SPNPs and SeNPs. (C) Determination of metallothionein in control strain and after application of silver and SeNPs. (B and C) Data represent the mean ± SD from three measurements, $*P < 0.05$. (D) MALDI-TOF mass spectra protein fingerprints for the identification of (a) *Staphylococcus aureus* and *S. aureus* after application of (b) SPNPs or (c) SeNPs.

have a greater affinity for the amplified *zntR* gene than SeNPs.

The concentration of bacterial metallothionein was significantly lower (by about 87%) in bacterial cells after application of SPNPs (6.3 μg mg^{-1} protein) and a higher (about 29%) concentration of metallothionein occurred in bacterial cells after addition of SeNPs (63 μg mg^{-1} protein) as compared with the *S. aureus* control (49 μg mg^{-1} protein; Fig. 3C). The presence of the *zntR* gene, which encodes metallothionein formation, was confirmed by polymerase chain reaction (results not shown). The effect of nanoparticles probably caused a change in the protein composition of the *S. aureus* culture, as was demonstrated by MALDI-TOF mass spectrometry, although the change was not statistically significant (Fig. 3D).

Discussion

Advances in nanotechnology, and particularly in the preparation of metal nanoparticles, can be considered to constitute one of the keys to developing new vascular grafts, because silver ions can lead to the prevention of bacterial infection (Ho *et al.*, 2013). In addition, SeNPs have been found to have high antibacterial activity and they can be easily prepared (Ramos *et al.*, 2012). The antibacterial activity of SeNPs was greater than that of the SPNPs used in this study, and it was comparable to that of the smaller SPNPs (18–21 nm) used by Prabakar *et al.* (2013). This probably was because we prepared SeNPs that were smaller in size (50–100 nm) than the SPNPs (200–300 nm). The present study showed that the silver nanoparticles exhibited antibacterial activity even at nanoparticle sizes equal to hundreds of nm. When comparing our findings on inhibition of *S. aureus* growth in the presence of selenium to those from other scientists, we found that our results were similar to those of Naik *et al.* (2009). However, our colonies were smaller than those of Naik *et al.*, probably because they had used selenium in a complex with 2-selenobenzo[*h*]quinoline-3-carbaldehyde.

The effect of 40–60 nm SeNPs on *S. aureus* was studied by Tran & Webster (2011). In that work, bacterial growth was inhibited by SeNPs in the concentration range 7.8–31 μg mL^{-1} to approximately 1.7% of that seen in the controls and the SeNPs killed approximately 40% of *S. aureus* cells. Antimicrobial activity of SeNPs was also confirmed in the work of Hariharan *et al.* (2012).

In our study the highest used concentration of SeNPs (300 μM, 23.7 μg mL^{-1}) caused total growth inhibition

within 24 h. Similar results for a 24-h inhibition period in relation to *S. aureus* growth on selenium nanoparticle-coated polyvinyl chloride are presented in the work of Ramos *et al.* (2012). The most interesting finding of our study is that the total inhibition concentration of SeNPs should be at least 5 μg mL^{-1}, which corresponds with results published by Tran & Webster (2011). Biochemical parameters showed greater similarity between *S. aureus* with the addition of SeNPs and the control, but greater differences with the addition of SPNPs.

Our study also demonstrated the interaction of DNA with SeNPs, whereby these particles probably impair the DNA structure of the *zntR* gene amplified *in vitro*. This statement is supported by the study of Beheshti *et al.* (2013), which reported increasing concentrations of SeNPs biosynthesized by *Bacillus* sp. MSH-1-induced cytotoxic effects and apoptosis in promastigote forms of the protozoa *L. major*. Apoptosis was demonstrated by DNA fragmentation in the concentration range 1–150 μg mL^{-1} of SeNPs. A similar toxicity effect of SeNPs on genomic DNA had been reported by Chen *et al.* (2008) for human melanoma cells treated with chemically synthesized SeNPs. Treatment of A375 human melanoma cells with SeNPs resulted in dose-dependent cell apoptosis, as indicated by DNA fragmentation and phosphatidylserine translocation (Chen *et al.*, 2008).

In our study, the results regarding concentration of metallothionein were comparable to those of Dar *et al.* (2013). Their group was interested in the production of metallothionein in various microorganisms in response to cadmium and they showed an increased accumulation of cadmium in cells expressing the protein metallothionein in comparison with control cells. Our results show a distinct decrease in metallothionein synthesis after addition of SPNPs to *S. aureus*. In contrast, only a slight increase was observed after the addition of SeNPs. This may be associated with a similarity in the biochemical parameters of *S. aureus* with and without the addition of SeNPs. After the addition of SPNPs, *S. aureus* showed more differences in biochemical parameters. After application of both SPNPs and SeNPs, on the other hand, *S. aureus* bacterial culture showed only minor differences in MALDI-TOF mass spectra protein fingerprints in comparison with the control. This leads to the conclusion that smaller nanoparticles (SeNPs) have a greater inhibitory effect compared with larger nanoparticles (SPNPs). Our results suggest an effective way to prevent *S. aureus* infections using nanostructured selenium.

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References

Adam V, Baloun J, Fabrik I, Trnkova L & Kizek R (2008) An electrochemical detection of metallothioneins at the zeptomole level in nanolitre volumes. *Sensors* **8**: 2293–2305.

Beheshti N, Soflaei S, Shakibaie M, Yazdi MH, Ghaffarifar F, Dalimi A & Shahverdi AR (2013) Efficacy of biogenic selenium nanoparticles against *Leishmania major*. *In vitro* and *in vivo* studies. *J Trace Elem Med Biol* **27**: 203–207.

Chen T, Wong YS, Zheng W, Bai Y & Huang L (2008) Selenium nanoparticles fabricated in *Undaria pinnatifida* polysaccharide solutions induce mitochondria-mediated apoptosis in A375 human melanoma cells. *Colloids Surf B Biointerfaces* **67**: 26–31.

Chudobova D, Dobes J, Nejdl L *et al.* (2013a) Oxidative stress in *Staphylococcus aureus* treated with silver(I) ions revealed by spectrometric and voltammetric assays. *Int J Electrochem Sci* **8**: 4422–4440.

Chudobova D, Nejdl L, Gumulec J *et al.* (2013b) Complexes of silver(I) ions and silver phosphate nanoparticles with hyaluronic acid and/or chitosan as promising antimicrobial agents for vascular grafts. *Int J Mol Sci* **14**: 13592–13614.

Dar S, Shuja RN & Shakoori AR (2013) A synthetic cadmium metallothionein gene (PMCd1syn) of *Paramecium* species: expression, purification and characteristics of metallothionein protein. *Mol Biol Rep* **40**: 983–997.

Grigor'eva A, Saranina I, Tikunova N, Safonov A, Timoshenko N, Rebrov A & Ryabchikova E (2013) Fine mechanisms of the interaction of silver nanoparticles with the cells of *Salmonella typhimurium* and *Staphylococcus aureus*. *Biometals* **26**: 479–488.

Hariharan H, Al-Dhabi NA, Karuppiah P & Rajaram SK (2012) Microbial synthesis of selinium nanocomposite using *Saccharomyces cerevisiae* and its antimicrobial activity against pathogens causing nosocomial infection. *Chalcogenide Lett.* **9**: 509–515.

Ho CM, Yau SKW, Lok CN, So MH & Che CM (2010) Oxidative dissolution of silver nanoparticles by biologically relevant oxidants: a kinetic and mechanistic study. *Chem Asian J* **5**: 285–293.

Ho CH, Odermatt EK, Berndt I & Tiller JC (2013) Long-term active antimicrobial coatings for surgical sutures based on silver nanoparticles and hyperbranched polylysine. *J Biomater Sci Polym Ed* **24**: 1589–1600.

Khan A, Qamar M & Muneer M (2012) Synthesis of highly active visible-light-driven colloidal silver orthophosphate. *Chem Phys Lett* **519–20**: 54–58.

Naik HRP, Naik HSB, Naik TRR, Naika HR, Gouthamchandra K, Mahmood R & Ahamed BMK (2009) Synthesis of novel benzo(*h*)quinolines: Wound healing, antibacterial, DNA binding and *in vitro* antioxidant activity. *Eur J Med Chem* **44**: 981–989.

Prabakar K, Sivalingam P, Rabeek SIM *et al.* (2013) Evaluation of antibacterial efficacy of phyto fabricated silver nanoparticles using *Mukia scabrella* (Musumusukkai) against

drug resistance nosocomial gram negative bacterial pathogens. *Colloid Surf B Biointerfaces* **104**: 282–288.

Pumera M (2011) Nanotoxicology: the molecular science point of view. *Chem Asian J* **6**: 340–348.

Rai M, Yadav A & Gade A (2009) Silver nanoparticles as a new generation of antimicrobials. *Biotechnol Adv* **27**: 76–83.

Ramos JF, Tran PA & Webster TJ (2012) *Selenium Nanoparticles for the Prevention of PVC-related Medical Infections*. Bioengineering Conference (NEBEC), 38th Annual Northeast March 2012.

Singh VK, Xiong AM, Usgaard TR, Chakrabarti S, Deora R, Misra TK & Jayaswal RK (1999) ZntR is an autoregulatory protein and negatively regulates the chromosomal zinc resistance operon znt of *Staphylococcus aureus*. *Mol Microbiol* **33**: 200–207.

Tran PA & Webster TJ (2011) Selenium nanoparticles inhibit *Staphylococcus aureus* growth. *Int J Nanomed* **6**: 1553–1558.

van Hal SJ & Fowler VG (2013) Is it time to replace vancomycin in the treatment of methicillin-resistant *Staphylococcus aureus* infections? *Clin Infect Dis* **56**: 1779–1788.

Wang Q & Webster TJ (2012) Nanostructured selenium for preventing biofilm formation on polycarbonate medical devices. *J. Biomed. Mater. Res. A* **100A**: 3205–3210.

Wang Q & Webster TJ (2013) Short communication: inhibiting biofilm formation on paper towels through the use of selenium nanoparticles coatings. *Int J Nanomed* **8**: 407–411.

Yan L, Gu ZJ & Zhao YL (2013) Chemical mechanisms of the toxicological properties of nanomaterials: generation of intracellular reactive oxygen species. *Chem Asian J* **8**: 2342–2353.

5.5 Vliv dlouhodobého působení iontů kovů na vznik rezistence nebo multirezistence s antibiotickými léčivy

5.5.1 Vědecký článek V

CHUDOBOVA, D.; DOSTALOVA, S.; BLAZKOVA, I.; MICHALEK, P.; RUTTKAY-NEDECKY, B.; SKLENAR, M.; NEJDL, L.; KUDR, J.; GUMULEC, J.; TMEJOVA, K.; KONECNA, M.; VACULOVICOVA, M.; HYNEK, D.; MASARIK, M.; KYNICKY, J.; KIZEK, R.; ADAM, V. Effect of Ampicillin, Streptomycin, Penicillin and Tetracycline on Metal Resistant and Non-Resistant *Staphylococcus aureus*. *International Journal of Environmental Research and Public Health*, 2013, roč. 11. č. 3, s. 3233-3255. ISSN 1660-4601.

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Závažnost bakteriální rezistence je potvrzována počtem úmrtí v souvislosti s bakteriálními infekcemi způsobenými rezistentními kmeny bakterií po celém světě [226]. Antimikrobiální rezistence (AMR) je rezistence mikroorganismů na antimikrobiální léky, ke kterým byli původně citlivé. Vytvoření rezistence je závislé na genech nacházejících se na plasmidech, které se mohou přenášet mezi bakteriemi, tudíž se rezistence rychle šíří. Rezistentní mikroorganismy jsou schopné odolávat účinku antimikrobiálních léků, takže standardní léčba začíná být čím dál více neúčinnou a přetrvávající infekce tak jsou vysokým rizikem z hlediska šíření na další hostitele. Rezistence vznikající ve vnějším prostředí je součástí přirozeného vývoje každého živého systému. Výskyt rezistentních mikroorganismů se sleduje především v nemocnicích, ale mnohem větším rizikem mohou být jejich přítomnost v půdě a vodě [227]. Úmrtnost pacientů s vážnými infekcemi, kteří jsou ošetřeni v nemocnicích je asi dvakrát vyšší ve srovnání s pacienty trpícími infekcemi způsobenými nerezistentními typy bakterií [228].

Cílem této studie bylo porovnání účinku antibiotických léčiv na nerezistentní kulturu *Staphylococcus aureus* a totožnou kulturu rezistentní vůči působení iontů kadmia a olova. Kmeny rezistentní vůči působení iontů kovu byly vytvořeny postupným přídavkem 2 mM zásobních roztoků iontů kovu (kadmia a olova) k bakteriální kultuře *S. aureus*. Za použití metody růstových křivek byl pozorován vzrůstající antimikrobiální efekt ampicilinu, streptomycinu, penicilinu a tetracyklinu (0, 10, 25, 50, 75, 150, 225 a

300 μM) na kmeny rezistentní vůči iontům těžkých kovů. Výrazná inhibice růstu (v porovnání s kontrolou) byla pozorována u rezistentního kmene ke kadmiu v přítomnosti všech čtyř typů antibiotických léčiv. Oproti tomu přídavek streptomycinu a ampicilinu nezpůsobil inhibici růstu u bakteriálního kmene rezistentního k iontům olova. Ostatní antibiotika však pro kmen toxická byla. Zároveň byly pozorovány výrazné morfologické změny buněčné stěny bakteriálních buněk, kdy se po aplikaci těžkých kovů objevila v bakteriálních buňkách tzv. vnitřní příčná stěna („septal midline“).

Naše studie poukazovala zejména na to, že přítomnost kovových iontů v prostředí může výraznou měrou přispět k rozvoji rezistence bakteriálních kmenů vůči celé řadě látek, včetně antibiotických léčiv, což by mohlo mít výrazný vliv na veřejné zdraví.

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Article

Effect of Ampicillin, Streptomycin, Penicillin and Tetracycline on Metal Resistant and Non-Resistant *Staphylococcus aureus*

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Abstract: There is an arising and concerning issue in the field of bacterial resistance, which is confirmed by the number of deaths associated with drug-resistant bacterial infections. The aim of this study was to compare the effects of antibiotics on *Staphylococcus aureus* non-resistant strain and strains resistant to cadmium or lead ions. Metal resistant strains were created by the gradual addition of 2 mM solution of metal ions (cadmium or lead) to the *S. aureus* culture. An increasing antimicrobial effect of ampicillin, streptomycin, penicillin and tetracycline (0, 10, 25, 50, 75, 150, 225 and 300 μM) on the

resistant strains was observed using a method of growth curves. A significant growth inhibition (compared to control) of cadmium resistant cells was observed in the presence of all the four different antibiotics. On the other hand, the addition of streptomycin and ampicillin did not inhibit the growth of lead resistant strain. Other antibiotics were still toxic to the bacterial cells. Significant differences in the morphology of cell walls were indicated by changes in the cell shape. Our data show that the presence of metal ions in the urban environment may contribute to the development of bacterial strain resistance to other substances including antibiotics, which would have an impact on public health.

Keywords: *S. aureus*; antimicrobial resistance; antibiotics; metal resistance; cross resistance; growth curves; inhibition concentrations; spectrophotometry

1. Introduction

The seriousness of the problem of bacterial resistance is confirmed by the number of deaths associated with drug-resistant bacterial infections—only in the EU it affects 25,000 people a year [1]. Recently, with the discovery of multi-resistant strains in the broader community, public health officials have begun to realize the potential danger of the spread of these antibiotic resistant bacteria [2]. Antimicrobial resistance (AMR) is a resistance of microorganism to an antimicrobial medicine to which it was originally sensitive. Creation of a resistance effect is dependent on the genes located in plasmids that are infectious matter transferred to other cells, so the resistance between bacteria spreads rapidly. Resistant microorganisms are able to withstand an attack of antimicrobial medicines, so that standard treatments become ineffective and infections persist increasing the risk of spreading to others. The evolution of resistant strains is natural phenomenon that happens when microorganisms are exposed to antimicrobial drugs and resistant genes can be distributed between certain types of bacteria. The misuse of antimicrobial medicines accelerates this phenomenon.

The incidence of resistant microorganisms is monitored primarily in hospitals, but much higher risk is present in the soil and waters [3]. Resistance, generated in the external environment, is a natural development of every live system. Infections caused by resistant microorganisms often fail to respond to the standard treatment, resulting in prolonged illness and greater risk of death [4–7]. The death rate of patients with serious infections treated in hospitals is about twice as high compared to the patients with infections caused by non-resistant bacteria [8].

A high percentage of hospital-acquired infections are caused by highly resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) or multidrug-resistant enterococci Gram-negative bacteria. The general mechanisms of resistance are: (1) the limited penetration of antibiotics into the bacterial cell; (2) the change of the target structure (receptor); (3) metabolic changes within the bacterial cell, which prevents the effect of antibiotics on the target structures; and (4) enzymatic inhibition/inactivation of antibiotics [9–14]. Metal resistance of microbes is accomplished by intra- and extracellular mechanisms. Metals can be excreted via efflux transport systems, sequestering compounds of the cytosol can bind and detoxify metals inside the cell. The release of chelators into the extracellular milieu fixes the bound metals. The structure of the cell envelope is prone to bind large

amounts of metals by sorption, thus preventing influx [15]. Newly discovered resistance mechanisms, such as enzymes produced by the bacteria that destroy last generation antibiotics, have emerged among several Gram-negative bacilli and have rapidly spread in many countries. This can render ineffective powerful antibiotics, which are often the last defence against multi-resistant strains of bacteria. This new resistance mechanism is associated with ordinary human pathogens (e.g., *Escherichia coli*) which cause common infections such as urinary tract infection.

The use of antibiotics and toxic metals should be considered carefully with the attention paid to the environmental impacts [16–18]. Heavy metals are toxic and can be harmful to organisms. For this reason, a number of organisms including bacteria develop processes which are able to withstand the effects of these pollutants [15]. Toxic metals in the environment can enrich the antibiotic multi-resistance property of bacteria [19]. Resende *et al.* [20] evaluated medically relevant bacteria in an aquaculture system and their susceptibility to antibiotics and toxic metals. Multidrug-resistant bacteria were also found to be tolerant to nickel, zinc, chromium and copper. In another study by Ji *et al.* [21], eight antibiotic resistance genes (ARG), seven heavy metals and six antibiotics were quantified in manures and soils collected from multiple feedlots in Shanghai (China). Overall, sulfonamide ARGs were more abundant than tetracycline ARGs. The significant positive correlations were found between some ARGs and typical heavy metals such as Cu, Zn and Hg. Similarly, in the study by Malik *et al.* [22], majority of the *Pseudomonas* isolates from water and soil exhibited resistance to multiple metals (Hg, Cd, Pb, Cu, Zn, Ni) and antibiotics (water—tetracycline, polymyxin B; soil— sulphadiazine, ampicillin and erythromycin) was presented [22].

With the appearance of antibiotic-resistant bacteria, increasing numbers of infections are causing huge losses to both economic concerns and social resources over recent decades, and this has become a global problem [2]. This study aimed on testing of non-resistant strain of bacterial culture wild type *S. aureus* and *S. aureus* strains resistant to heavy metals ions (cadmium = RCd or lead = RPb) exposed to different concentrations of four various antibiotics (ampicillin, streptomycin, penicillin and tetracycline). The antimicrobial activity of antibiotics on *S. aureus* bacterial culture was tested by the growth curves and the results were statistically evaluated.

2. Experimental Section

2.1. Cultivation of S. aureus

S. aureus (NCTC 8511) was obtained from the Czech Collection of Microorganisms, Faculty of Science, Masaryk University, Brno, Czech Republic. The strains were stored as a spore suspension in 20% (*v*/*v*) glycerol at −20 ℃. Prior to use in this study, the strains were thawed and the glycerol was removed by washing with distilled water. The composition of cultivation medium was as follows: meat peptone 5 g/L, NaCl 5 g/L, bovine extract 1.5 g/L, yeast extract 1.5 g/L (HIMEDIA, Mumbai, India), sterilized MilliQ water with 18 MΩ. pH of the cultivation medium was adjusted at 7.4 before sterilization. The sterilization of the media was carried out at 121 ℃ for 30 min. in sterilizer (Tuttnauer 2450EL, Beit Shemesh, Israel). The prepared cultivation media were inoculated with bacterial culture into 25 mL Erlenmeyer flasks. After inoculation, the bacterial cultures were cultivated

for 24 h on a shaker at 600 rpm and 37 ℃. The bacterial culture, cultivated under these conditions, was diluted by cultivation medium to OD600 = 0.1 and used in the following experiments.

2.2. Preparation of Resistant Strains of S. aureus

The resistant strains of *S. aureus* have been developed in the laboratory. 2 mM basic solutions of heavy metals ions (Cd^{2+} and Pb^{2+}) was added to non-resistant bacterial culture of *S. aureus*, cultivated in Luria Bertani medium. Low concentration (50 μM) of the metal ions in a medium was inoculated into the bacterial culture, and then the concentration of heavy metal ions was always increased by the concentration of 50 μM to the maximum possible dose for regeneration of *S. aureus*.

2.3. Chemicals, Preparation of Deionised Water and pH Measurement

Chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity unless noted otherwise. The deionised water was prepared using Aqual 25 reverse osmosis equipment (Aqual, Brno, Czech Republic). The deionised water was further purified by using a Milli-Q Direct QUV apparatus equipped with a UV lamp. The resistance was 18 MΩ. The pH was measured using a WTW inoLab pH meter (Weilheim, Germany).

2.4. Heavy Metals Ions Preparation

Heavy metals used for the preparation of heavy metals ions have always been in the form of nitrates of these metals ($Cd(NO_3)_2 \cdot 4H_2O$ and $Pb(NO_3)_2$) dissolved in 100 mL Milli-Q water and final concentration of these ions were always 2 mM.

2.5. The Microscopy of the Cells in Ambient Light

The inverted system microscope Olympus IX 71 (Tokyo, Japan) was used for the imaging of the cells. The cells in cultivation medium were pipetted (5 μL) on the microscope slide and covered by cover slip. The cover slip was placed on the sample and the immersion oil was used. The objective (PlanFLN; Mag. 100×; NA 1, 3; F.N. 26.5) and the magnification lens 1.6× were used, the total magnification was 1600×. The images were captured by Camera Olympus DP73 and processed by Stream Basic 1.7 Software, the images resolution was 4800 × 3600 pixels. The parameters were as follows: exposure time: 32 ms and ISO 200.

2.6. Determination of Growth Curves

The procedure for the evaluation of the antimicrobial effect of tested compounds and their combinations consisted in measuring of the absorbance using the apparatus Multiskan EX (Thermo Fisher Scientific, Bremen, Germany) and subsequent analysis in the form of growth curves. Non-resistant bacterial culture of *S. aureus* or resistant strain *S. aureus* to cadmium and lead ions were cultivated in LB medium for 24 h with shaking and was diluted with LB medium using Specord spectrophotometer 210 (Analytik, Jena, Germany) at a wavelength of 600 nm to absorbance 0.1 AU. On the microplate, these cultures were mixed with various concentrations of four types of antibiotics (ampicillin, streptomycin, penicillin and tetracycline) or *S. aureus* alone as a control for measurements.

The concentrations of antibiotics were 0, 10, 25, 50, 75, 150, 225 and 300 μM. Total volume in the microplate wells was always 300 μL. The measurements were carried out at time 0, then each half-an hour for 24 h at 37 °C and a wavelength of 620 nm. The obtained values were analyzed in graphical form as growth curves for each variant individually.

2.7. Determination of Cadmium and Lead Ions by Atomic Absorption Spectrometry

The determination of cadmium and lead ions was carried out using 240FS Agilent Technologies atomic absorption spectrometer (Agilent, Santa Clara, CA, USA) with flame atomization. Cadmium was measured on the wavelength 228.8 nm with spectral bandwidth of 0.5 nm and lead it was 217.0 nm with spectral bandwidth of 1.0 nm. The mixture of air and acetylene was used for the flame atomization. Deuterium background correction was used and the signal was measured in integration mode for 3 s.

2.8. Interaction of Bacterial DNA Fragment of zntR and 16S Gene with Cadmium and Lead Ions

DNA fragments of zntR and 16S gene (16 μg/mL) were incubated with different concentrations of $Cd(NO_3)_2$ and $Pb(NO_3)_2$ in 1:1 ratio. The stock solutions of DNA fragment of zntR gene (16 μg/mL) were incubated with 0, 0.06, 0.24 and 0.95 mM Cd(II) and Cd(II) in water (ACS purity, Sigma-Aldrich). The same procedure was carried out for the DNA fragment of 16S gene. The samples were incubated for 60 min at 25 °C. After incubation, unbound cadmium and lead ions were removed using Amicon Ultra 3K centrifugal filter device (Millipore Corp., Billerica, MA, USA). After centrifugation at 14,000 rpm for 10 min at 25 °C (5417R Eppendorf, Hauppauge, NY, USA), the sample was complemented with water to the original volume (200 μL).

The spectra were recorded within the range from 220 to 420 nm using quartz cuvettes (1 cm, Hellma, Essex, UK) on a SPECORD 210 spectrophotometer (Analytik Jena, Germany) at 25 °C (Julabo, Sellbach, Germany). The spectra were recorded after 60 min of interaction. The denaturation of the complex of DNA with Cd(II) and Pb(II) was monitored spectrophotometrically using a SPECORD S600 spectrophotometer with a diode array detector (Analytik Jena). The sample was incubated for 3 min at increasing temperatures in a range from 25 to 90 °C and the absorbance was measured at 260 nm. The changes in absorbance spectra were recorded during denaturation. The absorption spectra were evaluated in the WinASPECT 2.2.7.0.

2.9. Determination of Metallothionein

The electrochemical detection was carried out using the method of differential pulse voltammetry (three electrodes involvement, working mercury drop electrode (HMDE), reference silver chloride electrode Ag/AgCl/3M KCl, and auxiliary carbon electrode) [23]. The analysed samples were deoxidized by argon bubbling for 120 s. As a supporting electrolyte, Brdicka solution (containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammoniacal buffer ($NH_3(aq)$ + NH_4Cl, pH = 9.6)) was used. The supporting electrolyte was changed after each analysis of the sample. The parameters for the measurements were following: initial potential of −0.7 V, final potential of −1.75 V, the time interval 0.2 s, step potential 2 mV, amplitude −250 mV [24–27].

2.10. Expression of ZntR Gene and 16S Gene

2.10.1. Isolation of RNA

Bacterial cultures (1×10^6 of cells) were centrifuged at 300 rcf and 20 ℃ for 10 min and the pellets were resuspended in 100 μL of PBS buffer and 0.2 μL of RNase inhibitors. Thus the prepared solution was mixed with 100 μL of Tissue Lysis Buffer. The entire volume (200 μL) were pipetted into the sample tube, which is the component of the isolation kit MagNA Pure Compact RNA Isolation Kit (Roche, Basel, Schweiz), and inserted with other instruments on the appropriate place in the machine. In the second row of the machine, the vials with 20 μL of DNAase were inserted. Next steps were carried out according to the manufacturer's instructions ("RNA Cell" protocol MagNA).

2.10.2. Amplification of mRNA

The mRNA were converted to cDNA using PrimeScript One Step RT-PCR Kit Ver. 2 (TaKaRa, Mountain View, CA, USA). The reaction profile was as follows: initial denaturation at 94 ℃ for 2 min, 30 cycles of 94 ℃ for 30 s, 50 ℃ for 30 s and 72 ℃ for 1.5 min.

2.10.3. Amplification of cDNA for zntR Gene

The *zntR* gene was amplified using polymerase chain reaction (PCR). The sequences of forward and reverse primers were 5'-GGATCCATGTCAGAACAATATTCAGA-3', and 5'-AAGCTTTTATAACCCACTTTCTTTAG-3' respectively. The final volume of the PCR reaction mixture was 25 μL containing 15.875 μL of sterile water, 2.5 μL of 1× *Taq* reaction buffer, 0.5 μL of 100 mM dNTP, 0.5 μL of forward primer, 0.5 μL of reverse primer and 0.125 μL of *Taq* DNA polymerase. The reaction profile was as follows: 30 cycles of 94 ℃ for 3 min, 50 ℃ for 1 min and 72 ℃ for 1 min and a final extension at 72 ℃ for 4 min. The amplification was carried out using Mastercycler ep realplex^4 S (Eppendorf AG, Hamburg, Germany) and a 333 bp fragment was obtained.

2.10.4. Amplification of cDNA for 16S Gene

The *16S* gene was amplified using polymerase chain reaction (PCR). The sequences of forward and reverse primers were 5'-GAGTTTGATCCTGGCTCAG-3' and 5'-GGTTACCTTGTTACGACTT-3' respectively. The final volume of the PCR reaction mixture was 25 μL containing 14.42 μL of sterile water, 2.5 μL of 1× *Taq* reaction buffer, 0.5 μL of 100 mM dNTP, 1.25 μL of forward primer, 1.25 μL of reverse primer and 0.085 μL of *Taq* DNA polymerase. The reaction profile was as follows: initial denaturation at 94 ℃ for 4 min, 30 cycles of 94 ℃ for 30 s, 52 ℃ for 30 s and 72 ℃ for 1.5 min and a final extension at 72 ℃ for 10 min. Finally, a 1500 bp fragment was obtained.

2.11. Statistical Analyses

The STATISTICA data analysis software system, version 10.0 (StatSoft, Tulsa, OK, USA) was used for data processing. The half-maximal concentrations (IC_{50}) were calculated from logarithmic regression of sigmoidal dose-response curve. The general regression model was used to analyse differences between the combinations of compounds. To reveal differences between the cell lines,

Turkey's *post hoc* test within homogenous groups was employed. Unless noted otherwise, $p < 0.05$ was considered significant.

Besides applied antibiotics, the general regression model statistics were also used to analyse the effect of individual *S. aureus* strains. The data were natural logarithm-transformed and adjusted by the time variable using regression model. Consequently, Bonferroni post hoc test was employed to reveal the differences between *S. aureus* strains. Kruskal-Wallis and multiple comparisons of mean ranks test were used to compare the IC_{50} values based on MTT assay. Unless noted otherwise, *p*-level < 0.05 was considered significant.

3. Results and Discussion

In the last decade, the number of infections caused by Gram-positive bacteria, resistant to formerly effective antibiotics, has increased significantly around the World. The phenomenon of multi-resistance considerably complicates the choice of antibiotics for infection treatment, which usually involves the combination of antibiotics in order to increase the efficiency [13]. Antibiotic drugs in particular groups are characterized by the same mechanism of action and very similar mechanism of resistance. The target of the antibiotics are mostly ribosomes where the antibiotics as an inhibitor of protein translocation are bound to the 50S subunit of the ribosome, stimulating the dislocation of peptide-tRNA from the ribosome during the elongation phase and induce rapid collapse of the polyribosomes [28].

Antimicrobial agents, their effects and resistance formation on various bacterial strains had very important impact on human and animal medicine. On the other hand, in view of global evolution theory, the resistance formation is only the next step at assimilation of live organism to the environment. The connection of effects from various antimicrobial agents leads to the formation of cross resistance. Concretely in our case, metal contamination functions as a selective agent in the proliferation of antibiotic resistance. This study is focused just on the investigation of metal (cadmium and lead) and antibiotic (PNC—penicillin, STR—streptomycin, AMP—ampicillin, TTC—tetracycline) resistance on bacteria *S. aureus* just because of one important fact that most antibiotics are readily degraded in the environment, but metals are not, and so can represent a long-term selective pressure.

3.1. Characterization on the Cellular Level

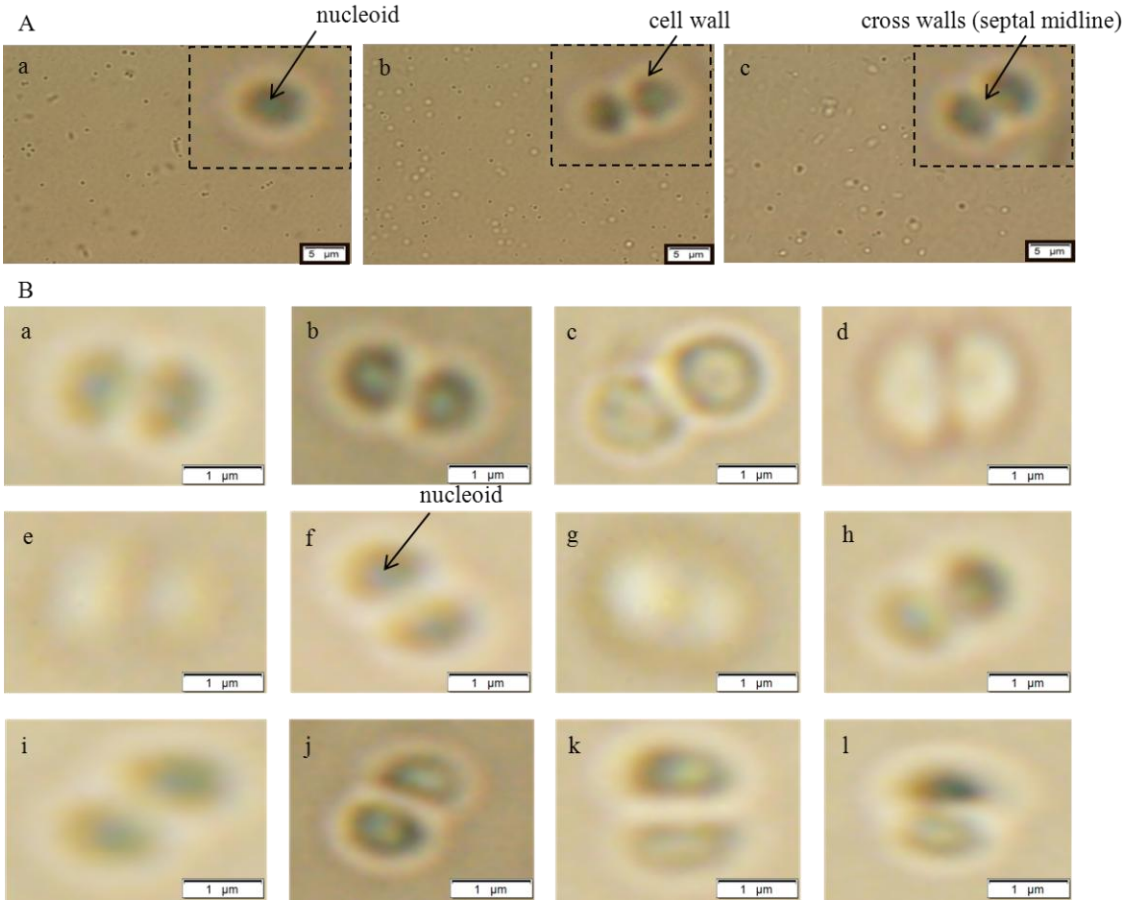
The testing was performed using bacterial culture *S. aureus* NCTC 8511 without the metal ions and two strains *S. aureus* NCTC 8511—RCd or RPb. The highest concentration of heavy metal ions in 24-h growing cell culture was 950 μM. For this experiment, the 950 μM resistant strains were recultivated for 24 h in clean medium. Selected antibiotics (penicillin, streptomycin, ampicillin and tetracycline) at various concentrations were applied to the tested bacterial strains.

3.1.1. Morphological Characterization

Significant morphological changes were observed in the cells in terms of cell shapes. The presence of so called cross walls (septal midline) was observed (Figure 1). These inner transverse walls were formed due to the development of the resistance. Similar phenomenon is commonly observed in case of MRSA strains [29–31]. The above mentioned morphological changes were observed in both, metal

resistant strains and control strain, after application of antibiotics. The detected phenomenon was the evidence of developing multi-resistance due to the fact that characteristic morphological hallmarks were present after addition of metal ions as well as antibiotics. Cell morphology was not influenced by the antibiotic administration due to the fact that the morphology was changed already by application of metal ions.

Figure 1. Images of *S. aureus* cells using microscopy in ambient light: (**A**) Micrographs of cells with resistance to metal ions (**a**) non-resistant strain of *S. aureus*; (**b**) RCd; (**c**) RPb. Parameters were as follows: Device: Microscopy; Volume: 5 µL; Zoom: 1600×; Ambient light; Exp.t.: 32.05 ms; ISO 200; Resolution: 4800 × 3600. (**B**) Micrographs of cells with resistance to metal ions after the application of antibiotics (50 µM): (**a**) penicillin; (**b**) streptomycin; (**c**) ampicillin; (**d**) tetracycline on non-resistant strain of *S. aureus*; (**e**) penicillin (**f**) streptomycin (**g**) ampicillin (**h**) tetracycline on RCd; or (**i**) penicillin (**j**) streptomycin (**k**) ampicillin (**l**) tetracycline on RPb.



3.1.2. Determination of Antimicrobial Activity

The mechanism of metal toxicity in the cell is determined by the interaction of the specific metals with a specific biological species [32]. Some metals like cobalt, copper, nickel and zinc are essential for many cellular processes in bacteria. However, higher concentrations of these can be cytotoxic. Other heavy metals, including lead, cadmium, mercury, silver and chromium have unknown beneficial effects on bacterial cells and are toxic even at low concentrations [33]. Metals enter into the cells in two ways [34]. The first way is mediated through non-specific transporters. The second way, substrate specific transport, is slower and often uses ATP hydrolysis as the energy source [35]. The heavy metal resistance in bacteria is connected with various possible mechanisms as follows: (a) exclusion of the metal by a permeability barrier; (b) exclusion by active export of the metal from the cell; (c) intracellular physical sequestration of metal binding proteins or other ligands to prevent it from damaging the metal-sensitive cellular targets; (d) extracellular sequestration and (e) transformation and detoxification [36].

Cadmium resistance is supported mainly by efflux mechanisms. *S. aureus* had cadmium resistance through an efflux mechanism consisting of the P-type ATPase transport system [34]. P-type ATPases are a metal transporting group of proteins involved in the transport of heavy metals across biological membranes. P-type ATPase consists of a single, large catalytic monomer of 70–200 kDa. The energy released by the removal of the γ-phosphate from ATP is coupled to the translocation of an ion across biological membranes. The direction of transport of P-type ATPases is mostly to the periplasm without further transport from the periplasm to the outside. These metal transporters prevent the over accumulation of highly toxic and reactive ions. The substrates *in vivo* are likely metal-thiolate complexes rather than the free metals. P-type ATPases can be divided into two subgroups: (I) Cu(I)/Ag(I)-translocating ATPases, e.g., *copA* in *Enterococcus hirae*, *Helicobacter pylori*, *Escherichia coli* and (II) Zn(II)/Cd(II)/Pb(II)-translocating ATPases, e.g., *zntA* in *E. coli* and *cadA* in *S. aureus* plasmid, pI258 [34,37].

Resistance to lead in *S. aureus* is probably mainly caused by the same *cadA* gene which encodes multipurpose P-type ATPase [38,39], but in addition to the membrane transport pumps (that remove metal ions from the bacterial cell), binding factors (detoxifying metals by sequestration) are also involved in tolerance to heavy metal ions in some bacteria. Intracellular bioaccumulation is usually connected with metallothioneins which play an important role in immobilization of toxic heavy metals, thereby protecting bacterial metabolic processes catalysed by enzymes [35]. Sequestration is also a detoxification mechanism for lead ions. *S. aureus* uses intra- and extracellular binding of lead to avoid toxicity of free lead ions by precipitating this element as a phosphate salt [40].

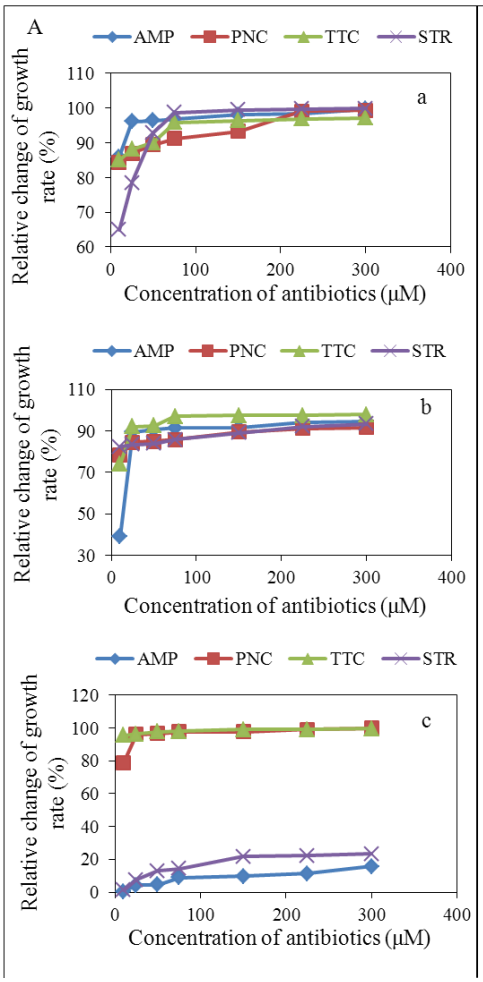
The synergic effect of antibiotics on RCd, RPb and control strain was investigated in this study. Antibiotics are one of the most commonly used drugs causing the resistance of the organism. Significant impact of the drug on the bacterial cell was observed after exposition of the cells with the existing heavy metal ions, resistance to the antibiotics. It can be expected that the mechanism of antibiotic action differs from metal ions and they do not overlap [10,41].

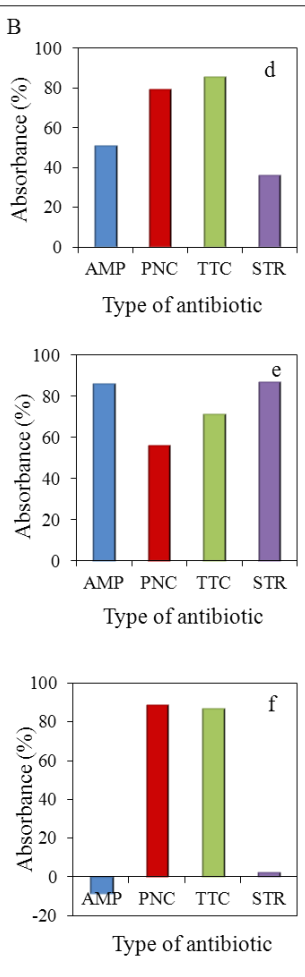
In the case of penicillin and tetracycline, the effect was manifested by growth inhibition in first 6 h of measurement in all tested strains, control strain (Figure 2Aa), RCd (Figure 2Ab) and RPb (Figure 2Ac). The decrease of growth in comparison to the control group was observed already after

addition of 10 µM of penicillin and tetracycline. Further, the increase of penicillin and tetracycline concentrations influenced the final effect only slightly (Figure 2A).

After addition of ampicillin and streptomycin, decline in RPb growth was not observed even after application of 300 µM (Figure 2Ac). The results were also confirmed by deduction of the absorbance in 24 h of measurement (Figure 2B). Growth inhibition was not observed only in the strain resistant to the action of lead ions (RPb) after application of concentration range of streptomycin and ampicillin (Figure 2Ac,Bf).

Figure 2. Spectrophotometric analysis of wild type *S. aureus* or *S. aureus* strains resistant to 950 µM concentration of heavy metal ions after application of various concentration of antibiotics (0, 10, 25, 50, 75, 150, 225 and 300 µM): (**A**) Relative change of growth rate as the relative difference between *S. aureus* strain without antibiotic and individual dose of antibiotic (related to the value obtained without antibiotic) in first 6 h of measurement in strains of *S. aureus* (%): (**a**) non-resistant *S. aureus*; (**b**) RCd; (**c**) RPb. (**B**) The cumulative effect of different types of antibiotics (%) on: (**d**) non-resistant *S. aureus*; (**e**) RCd; (**f**) RPb. All values were deducted from the non-resistant *S. aureus* (100%). (**C**) Inhibitory concentrations of tested antibiotics. AMP—ampicillin, PNC—penicillin, TTC—tetracycline, STR—streptomycin.





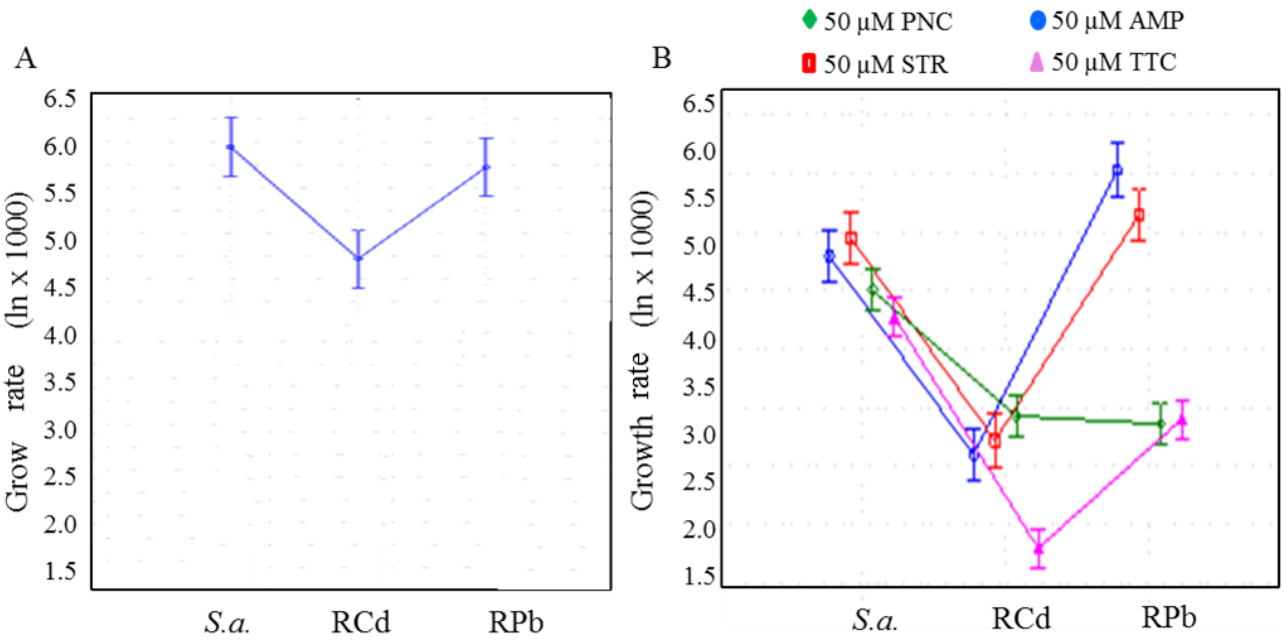
C

ATB	Time of growth	*S.aureus*	RCd	RPb
AMP	6 h	5.5	3.8	346.8
	12 h	12.2	2.6	125.9
	18 h	25.3	3.1	124.2
	24 h	59.5	1.8	135.4
PNC	6 h	2.2	6.6	2.4
	12 h	2.7	2.3	2.7
	18 h	1.8	1.1	2.0
	24 h	2.2	1.0	2.2
TTC	6 h	4.8	3.8	2.2
	12 h	5.2	2.3	2.4
	18 h	5.4	2.4	2.3
	24 h	4.8	1.6	2.3
STR	6 h	8.5	2.3	565.5
	12 h	24.1	1.8	295.9
	18 h	61.4	2.3	137.4
	24 h	2.3	5.0	20.1

The inhibition, expressed as inhibitory concentrations (IC_{50}) values after first 6 h, confirmed the effects of tested antibiotics (Figure 2C). Figure 2C presents statistically evaluated values of IC_{50}. This value is expressed as a concentration that causes 50% growth inhibition of test strain, in our case, non-resistant and resistant strains of *S. aureus*. The table shows the lowest IC_{50} values, determined in all strains tested after the application of penicillin and tetracycline. A small increase in values of IC_{50} was determined for the control strain and RCd after the application of ampicillin and streptomycin. The highest IC_{50} values were recorded for RPb after the application of ampicillin and streptomycin when diametrically diverging values was achieved. These indicate multi-resistance of RPb against the effect of these antibiotics.

After adjustment of the effect of time, growth rate was analysed on natural logarithm-transformed data. Significant differences between the resistant and sensitive group was determined, $F(8, 274) = 24.33$, $p < 0.001$ (Figure 3). To reveal the differences between the groups, Bonferroni *post hoc* test was employed: RCd expressed at lower growth rate and RPb expressed at higher growth rate were compared to sensitive strain (AMP, STR) and lower growth rate (PNC, TTC) at p-level < 0.05 (Table 1).

Figure 3. (**A**) Statistical evaluation of the cell culture behaviour at zero concentration of antibiotics, ANOVA $F(8, 274) = 24.333$, $p < 0.001$. (**B**) Comparison of growth rate at 50 µM concentration in each group for all antibiotics. ANOVA $F(8, 270) = 65.860$, $p < 0.001$. Data displayed as mean natural logarithm of residuals and 95 % confidence intervals. *S. a.*—*S. aureus* non-resistant strain, RCd, RPb. PNC—penicillin, STR—streptomycin, AMP—ampicillin, TTC—tetracycline.



For penicillin, RCd strain expressed at lower growth rate and RPb cells expressed even at lower growth rate compared to sensitive strain at p-level < 0.05. For streptomycin, RCd strain expressed at lower growth rate and RPb cells expressed at higher growth rate compared to sensitive strain at p-level < 0.05. For ampicillin, RCd strain showed lower growth rate and RPb cells showed higher growth rate compared to sensitive strain at p-level < 0.05. For tetracycline, RPb strain expressed lower at growth rate and RCd cells expressed even at lower growth rate compared to sensitive strain at

p-level < 0.05. Strain resistant to cadmium ions are generally characterized by lower growth rate after all tested antibiotics individually.

Table 1. Growth rate after application of 50 μM concentration of antibiotic in *S. aureus* non-resistant strain and strains resistant to cadmium and lead. Stars in columns indicate difference at *p*-level < 0.05 using Bonferroni *post hoc* test for homogenous groups.

Antibiotics	*S. aureus* strains	N	Absorbance (mean ± S.D × 10^3)	Group different at $p < 0.05$
Penicillin (50 μM)				
	RPb	48	26.01 ± 0.76	****
	RCd	46	28.78 ± 0.9	****
	S. a.	48	90.81 ± 0.93	****
Streptomycin (50 μM)				
	RCd	46	21.83 ± 0.22	****
	S. a.	48	121.62 ± 1.26	****
	RPb	48	254.09 ± 0.83	****
Ampicillin (50 μM)				
	RCd	46	23.33 ± 3.21	****
	S. a.	48	175.22 ± 4.95	****
	RPb	48	323.05 ± 5.15	****
Tetracycline (50 μM)				
	RCd	46	9.86 ± 0.83	****
	RPb	48	30.04 ± 0.35	****
	S. a.	48	71.91 ± 0.58	****

The cross resistance is caused by the common presence of metal ions and antibiotics and represents a great problem. It is assumed that the presence of metal ions affects the antibiotic resistance through the two basic mechanisms as follows: (I) co-resistance (different resistance determinants present on the same genetic element) and (II) cross-resistance (the same genetic determinant responsible for resistance to antibiotics and metals) [42]. Co-resistance occurs when the genes, specifying resistant phenotypes, are located together on the same genetic element such as a plasmid. In contrast, the cross resistance occurs when different antimicrobial agents attack the same target. The result is development of resistance to one antibacterial agent accompanied by resistance to another. Considering the fact that structurally dissimilar compounds can use the same efflux mechanism, one may suggest that this can serve as an example of cross-resistance [42,43].

Resistance to antibiotics is usually achieved using four main strategies: (i) reduction of membrane permeability to antibiotics; (ii) drug inactivation; (iii) rapid efflux of the antibiotic; and (iv) mutation of cellular target(s) [42]. These mechanisms are predicted by chromosomal or mobile genetic elements (plasmids).

Penicillin and ampicillin belong to the group of β-lactam antibiotics where ampicillin is in the aminopenicillins subgroup. These antibiotics produce a bactericidal effect by inhibiting the membrane-bound enzymes responsible for catalysing vital stages in the biosynthesis of the cell wall. Such inhibition is the direct result of the covalent binding of the antibiotic to one or more penicillin-sensitive enzymes, termed penicillin-binding proteins (PBPs). The penicillin resistant

bacteria produce an extracellular β-lactamase which inactivates antibiotics through hydrolysis of the β-lactam ring [44,45].

The β-lactamase structural gene (*blaZ*) is present on Tn*552*-like transposons [46]. These elements are located on β-lactamase plasmids which exhibit resistance to other antimicrobial agents, in particular heavy metal ions. There are four subgroups of these lactamase. The most famous and well described plasmids types are pI524 (encodes resistance to inorganic ions and organomercurials in addition to β-lactamase production), pI258 (except metal ion resistance carries the erythromycin resistance transposon Tn*551*) [46], and pSK23 which encodes metal ion resistance too. Tn*552*-like elements have been found at various sites in naturally occurring plasmids such as pSK4 and pSK1. The other locations of Tn*552*-like transposons are chromosomes [45].

Tetracycline inhibits protein synthesis by binding to the 30S ribosomal subunit and preventing association of aminoacyl-tRNA with its acceptor site [47]. Two mechanisms of tetracycline resistance have been identified in *S. aureus*; active efflux via *tetA*(*K*) and *tetA*(*L*) and ribosomal protection via *tetA*(*M*) [45,47]. The closely related TetA(*K*) and TetA(*L*) efflux proteins belong to the major facilitator superfamily. In most cases, tetracycline efflux in *S. aureus* strains is mediated by *tetA*(*K*), which is commonly carried by plasmid pT181. This plasmid could be integrated into Type III SCC*mec* elements of chromosome and thus chromosomally encoded resistance. Resistance to tetracycline can also be mediated by mutations that cause increased expression of various chromosomally encoded efflux pumps, such as Tet38 [45,47].

Streptomycin exhibited resistance due to chromosomal mutations, affecting ribosome affinity [48]. Low-level resistance was usually indicative of small plasmids, such as pS194, which carries streptomycin adenyltransferase-encoding gene *str*. Chromosomal segment Tn*5405* is responsible for streptomycin resistance through gene aphC [45,49].

From the presented summary of individual antibiotics resistance mechanisms, it is obvious that these mechanisms are predicted by the chromosomal and mobile genetic elements (plasmids, transposons). The above presented results show that the lead resistant *S. aureus* is resistant to ampicillin and streptomycin also. On the other hand, the cadmium resistant *S. aureus* shows no resistance to ampicillin and streptomycin. When these results were compared to above descripted mechanisms of antibiotic resistance, it seems that especially the role of transposons would be critical. The resistance of penicillin and tetracycline is related, among other things, to the transposons. In contrast, the streptomycin resistance is not connected with transposons. Similar results were published elsewhere [44]. The linkage of the metal ions (cadmium and lead) and antibiotic resistance is obvious at β-lactam antibiotics where the creation of co-resistance is offered due to the same location of genes related to various antimicrobial agents. The genes responsible for metal and antibiotic resistance located on plasmid pI258 are probably the main reason of cross resistance formation. But the behaviour of ampicillin is unknown. Its resistance, according to our theory, should not be related with transposons. But the general theory in literature for β-lactam antibiotics claims the contrary. No specific study according to the ampicillin resistance in *S. aureus* has been published yet. Comparison of the resulted data from different studies of toxic metal tolerance among bacteria is very complicated because of lack of technical standards in these experimental designs.

3.2. Characterization on the Molecular Level

The resistance to metal ions in *S. aureus* can be formed due to various mechanisms as follows: extracellular accumulation, sequestration by metallothioneins, intracellular physical sequestration, or efflux-based [36]. The efflux based mechanism is mainly supported by the occurrence of the *cadC* system present on a plasmid and the *znt* system present on the chromosome [50–52]. It was reported that *zntA* gene encodes a transmembrane structural protein, responsible for the efflux of zinc and cobalt ions in *S. aureus* [36]. In *E. coli* Znt efflux protein can also be activated by cadmium, lead and silver, but not by copper [36,53]. A chromosomally encoded *znt* operon in *S. aureus* consists of two consecutive putative genes designated as *zntR* and *zntA*. The structural gene *zntA* encodes a transmembrane protein that facilitates extrusion of zinc and cobalt ions, whereas the regulatory gene *zntR* encodes a putative regulatory protein that controls the expression of *znt* operon.

ZntR protein, encoded by *zntR* gene, regulates *zntA* and belongs to the family of regulators which introduce changes in the DNA conformation, which apparently make the promoter a better substrate for RNA polymerase [50]. It acts as a direct Zn sensor and catalyses transcriptional activation of zinc efflux gene.

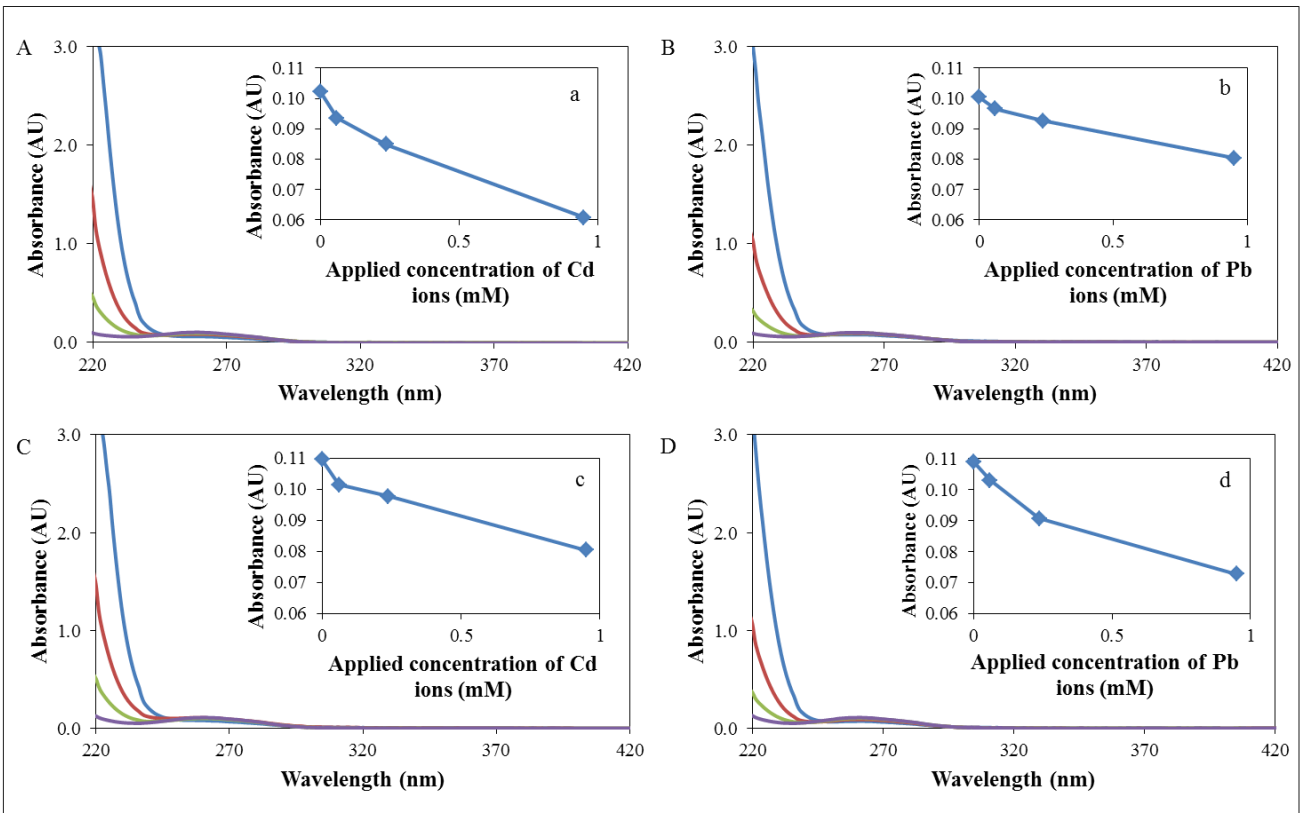
One of the components of small subunit of prokaryotic ribosomes is 16S ribosomal RNA, conferred by 16S rDNA. This gene is about 1500 bp in length in *S. aureus* and it is often used in phylogenetic studies due to its hypervariable regions, useful for identification of bacteria (species or genera) [54].

To confirm the simplified penetration of heavy metal ions into the cellular DNA, the interaction of cadmium and lead ions with a bacterial DNA fragment *zntR* and 16S was studied. This phenomenon was studied by spectrometric methods, particularly by absorbance measurement (Figure 4). The presence of heavy metal ions in DNA was manifested by a significant decrease of records for each concentration in comparison with a control sample. These results confirm the easy interaction of cadmium and lead ions with bacterial DNA.

The family of genes of the *S. aureus* bacterial strain is represented by *zntA* and *zntR* genes. These genes form open reading frames in the chromosomal fragment with the length of 3.2 kb, which confers resistance to heavy metal ions [55]. The genes *zntA* and *zntR* share the same promoter and are transcribed together [45]. In this work, we used the reverse transcription and amplification of fragment of the length of 333 bp [50] in the control strain of *S. aureus* NCTC 8511 and RCd or RPb and also in these strains after application of 50 μM concentration of antibiotics (Figure 5A).

The fluorescence intensity of the amplified fragment varies in dependence on the antibiotic type. In the RPb strains, the expression of *zntR* gene was observed higher than that of control *S. aureus*. In contrast, in the RCd, the expression of *zntR* gene was similar to the control *S. aureus* strain.

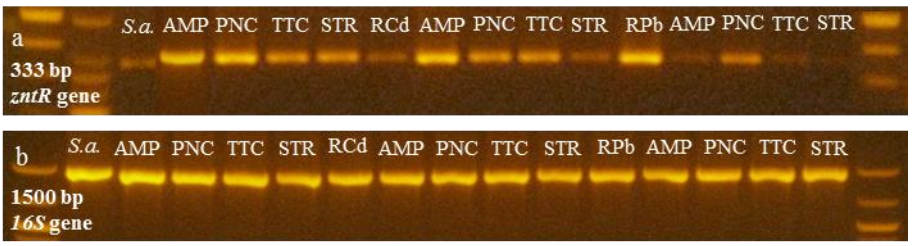
Figure 4. Interaction of bacterial DNA fragment with different concentrations of heavy metal ions (• 0, • 60, • 240 and • 950 μM): (**A**) Interaction of 16S gene with cadmium ions, (**a**) decrease of absorbance of 16S gene with cadmium ions in 260 nm. (**B**) Interaction of 16S gene with lead ions, (**b**) decrease of absorbance of 16S gene with lead ions in 260 nm. (**C**) Interaction of *zntR* gene with cadmium ions, (**c**) decrease of absorbance of *zntR* gene with cadmium ions in 260 nm. (**D**) Interaction of *zntR* gene with lead ions, (**d**) decrease of absorbance of *zntR* gene with lead ions in 260 nm.



In all variants of RCd strain after addition of antibiotics, the expression of *zntR* gene was significantly increased compared to the RCd without the antibiotics. The most significant increase was observed after the application of ampicillin (Figure 5Aa) too. The highest expression of *zntR* gene was observed in the RPb strain without any addition of antibiotics. After the addition of antibiotics the intensity of the expression was reduced (Figure 5Aa). The expression of 16S gene (always present in bacteria) confirmed the presence of bacteria strain in the samples. This presence was independent both on applied metal and antibiotics, therefore the fluorescence intensity was constant (Figure 5Ab).

Figure 5. Electrochemical analysis of the non-resistant *S. aureus* or RCd or RPb: (**A**) Gene expression with and without the application of antibiotics, (**a**) expression of *zntR* gene, (**b**) expression of 16S gene. (**B**) Determination of the amount of metallothionein (MT) in (**a**) *S. aureus*, RCd or RPb; and (**b**) *S. aureus*, RCd or RPb after application of 50 µM concentration of antibiotics; (**c**) comparison of the values of metallothionein in strains without application and after application of antibiotics. Presented values for ATBs is the sum of individual values for every antibiotic. All data represent mean ± s.d. NS, not significant, * $p < 0.05$, (**C**) Comparison of applied concentration of heavy metal ions (950 µM) with measured concentration using atomic absorption spectrometry.

A



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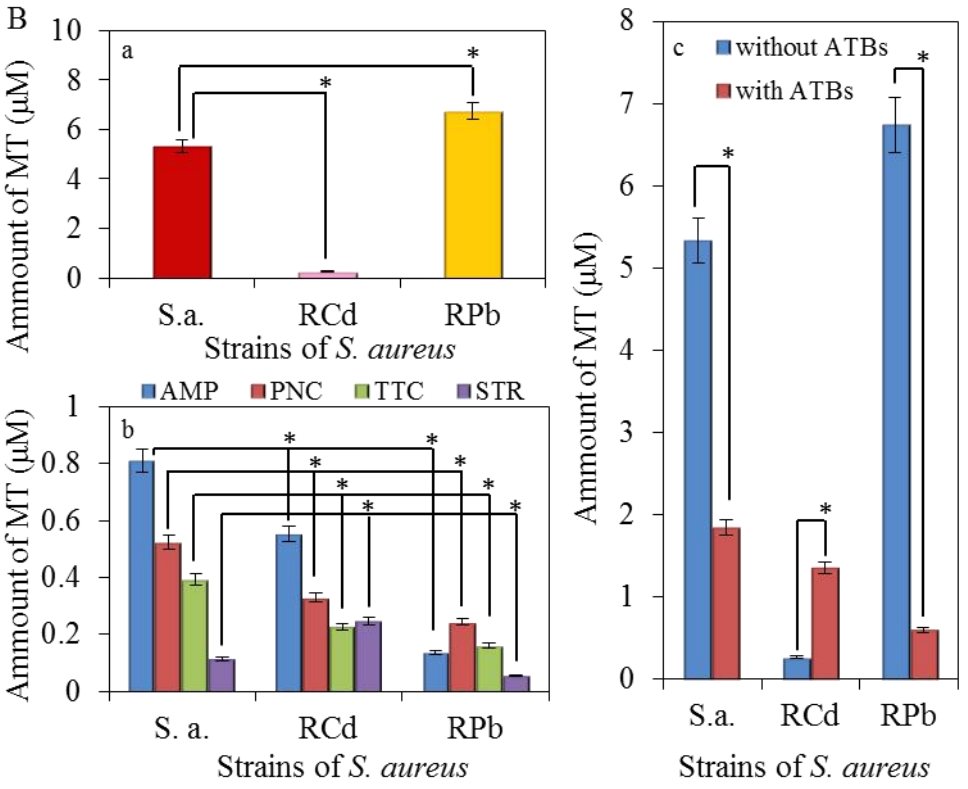

C

	Heavy metal ions (µM)		
	S. aureus	RCd	RPb
Without ATB	< 0.02	965.3	921.8
AMP	< 0.02	949.9	933.3
PNC	< 0.02	952.1	933.6
TTC	< 0.02	928.4	965.4
STR	< 0.02	936.6	917.2

Bacterial genome contains genes coding different proteins providing the tolerance to the heavy metal: metallo-regulatory genes [56]. These genes are usually from the family *ArsR/SmtB*. The sequences of these genes are usually homologous between the members of this family, but may differ in their active sites for binding metal [57]. Based on limited sequence homology, SmtB appears to be a member of a family of bacterial metalloregulatory proteins, including the ArsR proteins that are repressors of the arsenic-resistance operons and the CadC proteins which are the cadmium responsive repressors of expression of the cadmium efflux ATPase [57]. The SmtB, ArsR and CadC proteins contain conserved cysteine residues associated with the N-terminal extremity of putative DNA-binding helix-turn-helix motifs [58]. It has been predicted that these cysteine residues bind to metals via formation of metal-thiolate bonds and thus inhibit binding via the adjacent helix-turn-helix region [57,58]. Unlike ArsR/SmtB metalloregulatory proteins, above mentioned ZntR does not contain any cysteine residue and lacks the characteristic metal-binding elements [50]. However, it has two histidine-rich regions, one at the C-terminus and the other near the N-terminus. Similar histidine-rich regions have been reported for zinc and cobalt transporters which are thought to be domains for zinc-binding ions [36].

Metallothioneins (MTs) are known as proteins containing thiol groups in their structure, especially cysteine, which is the cause of affinity to metals—such as Cd, Pb, Hg, Cu or Zn [59–61]. The metallothionein gene, *smtA*, is controlled by the SmtB repressor [62], which also regulates a zinc-transporting P-type ATPase [56,57]. The primary function of MT is detoxification of heavy metals in living organisms, which became the subject of a number of studies [63–65]. Occurrence of metallothionein was observed in a variety of organisms and microorganisms such as bacteria, invertebrates or vertebrates [60,66–69].

In this study the presence of metallothionein in strains of *S. aureus* with resistance to the effects of heavy metals was determined. Much higher concentration of metallothionein (more than 6 μM) in RPb in comparison to the control has been reported and the other resistant strain RCd, where the concentration of metallothionein was much lower in comparison to the control *S. aureus*, values around 0.5 μM (Figure 5Ba). These results show possible variation in the metal resistance origin for cadmium and lead. While cadmium is mainly removed through the CadA transport system, lead is mainly removed after bounding to metallothioneins. CadA transport system was proofed as lead and cadmium transporter too [39]. Probably all the zinc/cadmium translocating P-type ATPases are also effective in lead ions export [39]. After the application of antibiotics the values of determined metallothionein in both resistant strains decreased, especially in RPb (Figure 5Bb). Only the application of streptomycin caused the growth of MT level in RCd compared to the control (*S. aureus*). Here, the presented results confirm the trends which are obvious in the evaluation of spectrophotometric analysis (Section 3.1.2.) and suggest that the mechanisms of cross resistance at ampicillin and streptomycin will be different because of various MT level in RCd. As it was mentioned above (Section 3.1.2.) the ampicillin and streptomycin caused cross resistance in RPb and here it is the confirmation of such deduction in the lowest MT levels for these combinations. It is necessary to remember that the presented results are related to the MT level inside of cells, therefore the higher change of MT level indicates the highest removal of complex MT-metal ions. The summarized view (Figure 5Bc) then shows a significant effect of antibiotic application, especially on RPb where the decrease of metallothionein is greatest. On the other hand, the application of the antibiotic caused an

increase in the amount of metallothionein in RCd (Figure 5Bc). These results confirm the results obtained from the expression of *zntR* gene (Figure 5A) and thus confirm further the direct relationship between *zntR* gene and metallothionein expression. The great question is about mechanism of this relationship because till now no explanation was published about the influence of *zntR* gene expression on metallothionein level. Because of the fact that metallothionein production is closely connected with zinc level (zinc-sensing transcriptional repressor SmtB) [62], and *zntR* gene expression too [36], and both genes are chromosomally located, we only assumed the creation of similar effect which was called in the cross resistance formation as co-resistance. The second possibility, and it is important to highlight that it is only in hypothesis, is the reaction of *zntR* receptor for the presence of other metal ions (lead and cadmium) but this assumption was not proofed elsewhere.

The content of heavy metals in the samples was determined by atomic absorption spectrometry measurements. The determined content of metal ions is presented in Figure 5C. The concentrations of cadmium and lead ions (950 μM) in both samples were detected in almost total applied concentration (940 μM). The same concentration was measured in samples after the application of antibiotics. In control strain, the concentration of metals was below the detection limit (Figure 5C).

The influence of metallothioneins in the metal resistance was tested in some bacteria strains [70]. It must be said that the lead and cadmium resistance (in combination with antibiotics) in point of view of various resistance mechanisms (efflux, metallothioneins) are very seldom tested and there is no integrated output model of *S. aureus* resistance to these agents.

4. Conclusions

The reported experiments were performed to study the effects of antibiotic drugs (ampicillin, streptomycin, penicillin and tetracycline) on non-resistant strains of *S. aureus* and *S. aureus* strains resistant to the effects of heavy metal ions (cadmium or lead). Our results pointed to a significant antimicrobial effect of penicillin and tetracycline in both the control strain and the strains resistant to heavy metal ions. Microscopic methods only confirmed the morphological changes of resistant strains in comparison with the control, independently of the application of the tested antibiotic drug. On the other hand, the lead resistant *S. aureus* strain showed resistance to the effect of ampicillin and streptomycin. Cross resistance was thus observed only in RPb after the application of these two antibiotics. The obtained results can be used for further experiments with bacterial strains in terms of a deeper understanding of bacterial resistance caused by environmental factors.

Acknowledgments

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Authors Contributions

Dagmar Chudobova cultivated strains resistant to heavy metal ions with antibiotics and prepared samples for other analysis. Simona Dostalova participated on the data from molecular biology including isolation, amplification of genes. Iva Blazkova observed cells by microscopy in ambient

light. Petr Michalek participated on expression of genes. Branislav Ruttkay-Nedecky participated on the preparation of the manuscript and in the design of spectrophotometric experiment. Matej Sklenar participated on the data about determination of antimicrobial activity. Lukas Nejdl performed the analysis of the interaction of bacterial DNA fragment of zntR gene with heavy metal ions. Jiri Kudr performed the analysis of the interaction of bacterial DNA fragment of 16S gene with heavy metal ions. Jaromir Gumulec made the statistical analysis. Katerina Tmejova participated on determination of metallothionein. Marie Konecna performed the analysis of the data and participated in preparation of the manuscript. Marketa Vaculovicova treated molecular-biological data and participated in the preparation of the manuscript. David Hynek treated electrochemical data and participated in the preparation of the manuscript. Michal Masarik participated in preparation of the manuscript and in the design of microbiological experiment. Jindrich Kynicky participated in preparation of the manuscript and optimized the samples preparation. Rene Kizek participated in design and coordination of the study. Vojtech Adam conceived of the study, and participated in its design and drafted manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Levy, S.B.; Marshall, B. Antibacterial resistance worldwide: Causes, challenges and responses. *Nat. Med.* **2004**, *10*, S122–S129.
2. Zhou, F.; Wang, Y. Characteristics of antibiotic resistance of airborne *Staphylococcus* isolated from metro stations. *Int. J. Environ. Res. Public Health* **2013**, *10*, 2412–2426.
3. Gatica, J.; Cytryn, E. Impact of treated wastewater irrigation on antibiotic resistance in the soil microbiome. *Environ. Sci. Pollut. Res.* **2013**, *20*, 3529–3538.
4. Stone, N.D.; Lewis, D.R.; Lowery, H.K.; Darrow, L.A.; Kroll, C.M.; Gaynes, R.P.; Jernigan, J.A.; McGowan, J.E.; Tenover, F.C.; Richards, C.L. Importance of bacterial burden among methicillin-resistant *Staphylococcus aureus* carriers in a long-term care facility. *Infect. Control Hosp. Epidemiol.* **2008**, *29*, 143–148.
5. Bastug, A.; Yilmaz, G.R.; Kayaaslan, B.; Akinci, E.; Bodur, H. Risk factors for mortality in patients with nosocomial *Staphylococcus aureus* bacteremia. *Turk. J. Med. Sci.* **2012**, *42*, 1222–1229.
6. Tacconelli, E.; Pop-Vicas, A.E.; D'Agata, E.M.C. Increased mortality among elderly patients with meticillin-resistant *Staphylococcus aureus* bacteraemia. *J. Hosp. Infect.* **2006**, *64*, 251–256.
7. Cosgrove, S.E.; Qi, Y.L.; Kaye, K.S.; Harbarth, S.; Karchmer, A.W.; Carmeli, Y. The impact of methicillin-resistance in *Staphylococcus aureus* bacteremia on patient outcomes: Mortality, length of stay, and hospital charges. *Infect. Control Hosp. Epidemiol.* **2005**, *26*, 166–174.
8. Ammerlaan, H.S.M.; Harbarth, S.; Buiting, A.G.M.; Crook, D.W.; Fitzpatrick, F.; Hanberger, H.; Herwaldt, L.A.; van Keulen, P.H.J.; Kluytmans, J.; Kola, A.; *et al.* Secular trends in nosocomial bloodstream infections: Antibiotic-resistant bacteria increase the total burden of infection. *Clin. Infect. Dis.* **2013**, *56*, 798–805.

9. Ye, Y.; Li, S.L.; Li, Y.J.; Ren, T.S.; Liu, K.G. Mycoplasma pneumoniae 23S rRNA Gene Mutations and Mechanisms of Macrolide Resistance. *Labmedicine* **2013**, *44*, 63–68.
10. Aktan, Y.; Tan, S.; Icgen, B. Characterization of lead-resistant river isolate Enterococcus faecalis and assessment of its multiple metal and antibiotic resistance. *Environ. Monit. Assess.* **2013**, *185*, 5285–5293.
11. Hellweger, F.L. Simple model of tetracycline antibiotic resistance in aquatic environment: Accounting for metal coselection. *J. Environ. Eng.-ASCE* **2013**, *139*, 913–921.
12. My, N.H.; Hirao, H.; Van, D.U.; Morokuma, K. Computational studies of bacterial resistance to beta-lactam antibiotics: Mechanism of covalent inhibition of the penicillin-binding protein 2a (PBP2a). *J. Chem. Inf. Model.* **2011**, *51*, 3226–3234.
13. Yuan, W.C.; Hu, Q.W.; Cheng, H.; Shang, W.L.; Liu, N.; Hua, Z.Y.; Zhu, J.M.; Hu, Z.; Yuan, J.Z.; Zhang, X.; *et al.* Cell wall thickening is associated with adaptive resistance to amikacin in methicillin-resistant *Staphylococcus aureus* clinical isolates. *J. Antimicrob. Chemother.* **2013**, *68*, 1089–1096.
14. Anaya-Lopez, J.L.; Lopez-Meza, J.E.; Ochoa-Zarzosa, A. Bacterial resistance to cationic antimicrobial peptides. *Crit. Rev. Microbiol.* **2013**, *39*, 180–195.
15. Majzlik, P.; Strasky, A.; Adam, V.; Nemec, M.; Trnkova, L.; Zehnalek, J.; Hubalek, J.; Provaznik, I.; Kizek, R. Influence of zinc(II) and copper(II) ions on *Streptomyces bacteria* revealed by electrochemistry. *Int. J. Electrochem. Sci.* **2011**, *6*, 2171–2191.
16. Sobrova, P.; Zehnalek, J.; Adam, V.; Beklova, M.; Kizek, R. The effects on soil/water/plant/animal systems by platinum group elements. *Cent. Eur. J. Chem.* **2012**, *10*, 1369–1382.
17. Krizkova, S.; Huska, D.; Beklova, M.; Hubalek, J.; Adam, V.; Trnkova, L.; Kizek, R. Protein-based electrochemical biosensor for detection of silver(I) ions. *Environ. Toxicol. Chem.* **2010**, *29*, 492–496.
18. Pereira, P.M.; Filipe, S.R.; Tomasz, A.; Pinho, M.G. Fluorescence ratio imaging microscopy shows decreased access of vancomycin to cell wall synthetic sites in vancomycin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **2007**, *51*, 3627–3633.
19. Summers, A.O. Genetic linkage and horizontal gene transfer, the roots of the antibiotic multi-resistance problem. *Anim. Biotechnol.* **2006**, *17*, 125–135.
20. Resende, J.A.; Silva, V.L.; Fontes, C.O.; Souza, J.A.; de Oliveira, T.L.R.; Coelho, C.M.; Cesar, D.E.; Diniz, C.G. Multidrug-resistance and toxic metal tolerance of medically important bacteria isolated from an aquaculture system. *Microbes Environ.* **2012**, *27*, 449–455.
21. Ji, X.L.; Shen, Q.H.; Liu, F.; Ma, J.; Xu, G.; Wang, Y.L.; Wu, M.H. Antibiotic resistance gene abundances associated with antibiotics and heavy metals in animal manures and agricultural soils adjacent to feedlots in Shanghai, China. *J. Hazard. Mater.* **2012**, *235*, 178–185.
22. Malik, A.; Aleem, A. Incidence of metal and antibiotic resistance in *Pseudomonas* spp. from the river water, agricultural soil irrigated with wastewater and groundwater. *Environ. Monit. Assess.* **2011**, *178*, 293–308.
23. Adam, V.; Fabrik, I.; Kohoutkova, V.; Babula, P.; Hubalek, J.; Vrba, R.; Trnkova, L.; Kizek, R. Automated electrochemical analyzer as a new tool for detection of thiols. *Int. J. Electrochem. Sci.* **2010**, *5*, 429–447.

24. Adam, V.; Baloun, J.; Fabrik, I.; Trnkova, L.; Kizek, R. An electrochemical detection of metallothioneins at the zeptomole level in nanolitre volumes. *Sensors* **2008**, *8*, 2293–2305.
25. Adam, V.; Blastik, O.; Krizkova, S.; Lubal, P.; Kukacka, J.; Prusa, R.; Kizek, R. Application of the Brdicka reaction in determination of metallothionein in patients with tumours. *Chem. Listy* **2008**, *102*, 51–58.
26. Maret, W. Fluorescent probes for the structure and function of metallothionein. *J. Chromatogr. B* **2009**, *877*, 3378–3383.
27. Sobrova, P.; Vyslouzilova, L.; Stepankova, O.; Ryvolova, M.; Anyz, J.; Trnkova, L.; Adam, V.; Hubalek, J.; Kizek, R. Tissue specific electrochemical fingerprinting. *PLoS One* **2012**, *7*, e49654, doi:10.1371/journal.pone.0049654.
28. Brissonnoel, A.; Trieucuot, P.; Courvalin, P. Mechanisms of action of spiramycin and other macrolides. *J. Antimicrob. Chemother.* **1988**, *22*, 13–23.
29. Jenkins, R.; Burton, N.; Cooper, R. Manuka honey inhibits cell division in methicillin-resistant *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **2011**, *66*, 2536–2542.
30. Belley, A.; Harris, R.; Beveridge, T.; Parr, T.; Moeck, G. Ultrastructural effects of oritavancin on methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus*. *Antimicrob. Agents Chemother.* **2009**, *53*, 800–804.
31. Webster, D.; Rennie, R.P.; Brosnikoff, C.L.; Chui, L.; Brown, C. Methicillin-resistant *Staphylococcus aureus* with reduced susceptibility to vancomycin in Canada. *Diagn. Microbiol. Infect. Dis.* **2007**, *57*, 177–181.
32. Rouch, D.A.; Lee, B.T.O.; Morby, A.P. Understanding cellular-responses to toxic agents—A model for mechanism-choice in bacterial metal resistance. *J. Ind. Microbiol.* **1995**, *14*, 132–141.
33. Trajanovska, S.; Britz, M.L.; Bhave, M. Detection of heavy metal ion resistance genes in Gram-positive and Gram-negative bacteria isolated from a lead-contaminated site. *Biodegradation* **1997**, *8*, 113–124.
34. Nies, D.H.; Silver, S. Ion efflux systems involved in bacterial metal resistances. *J. Ind. Microbiol.* **1995**, *14*, 186–199.
35. Nies, D.H. Microbial heavy-metal resistance. *Appl. Microbiol. Biotechnol.* **1999**, *51*, 730–750.
36. Choudhury, R.; Srivastava, S. Zinc resistance mechanisms in bacteria. *Curr. Sci.* **2001**, *81*, 768–775.
37. Naik, M.M.; Dubey, S.K.; Khanolkar, D.; D'Costa, B. P-type ATPase and MdrL efflux pump-mediated lead and multi-drug resistence in estuarine bacterial isolates. *Curr. Sci.*, **2013**, *105*, 1366–1372.
38. Blaszak, M.; Bienkowska, D. Effect of soil pollution on bacterial resistance to lead ions: An experimental approach. *Pol. J. Ecol.* **2009**, *57*, 555–560.
39. Rensing, C.; Sun, Y.; Mitra, B.; Rosen, B.P. Pb(II)-translocating P-type ATPases. *J. Biol. Chem.* **1998**, *273*, 32614–32617.
40. Levinson, H.S.; Mahler, I.; Blackwelder, P.; Hood, T. Lead resistance and sensitivity in *Staphylococcus aureus*. *FEMS Microbiol. Lett.* **1996**, *145*, 421–425.
41. Deb, S.; Ahmed, S.F.; Basu, M. Metal accumulation in cell wall: A possible mechanism of cadmium resistance by *Pseudomonas stutzeri*. *Bull. Environ. Contam. Toxicol.* **2013**, *90*, 323–328.

42. Baker-Austin, C.; Wright, M.S.; Stepanauskas, R.; McArthur, J.V. Co-selection of antibiotic and metal resistance. *Trends Microbiol.* **2006**, *14*, 176–182.
43. Chapman, J.S. Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *Int. Biodeterior. Biodegrad.* **2003**, *51*, 271–276.
44. Jensen, S.O.; Kwong, S.M.; Lyon, B.R.; Firth, N. Evolution of multiple drug resistance in *Staphylococci*. *Microbiol. Aust.* **2008**, *29*, 120–123.
45. Jensen, S.O.; Lyon, B.R. Genetics of antimicrobial resistance in *Staphylococcus aureus*. *Future Microbiol.* **2009**, *4*, 565–582.
46. Novick, R.P.; Roth, C. Plasmid-linked resistance to inorganic salts in *Staphylococcus aureus*. *J. Bacteriol.* **1968**, *95*, 1335–1342.
47. Chopra, I.; Roberts, M. Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* **2001**, *65*, 232–260.
48. Lacey, R.W.; Chopra, I. Evidence for mutation to streptomycin resistance in clinical strains of *Staphylococcus aureus*. *J. Gen. Microbiol.* **1972**, *73*, 175–180.
49. Derbise, A.; de Cespedes, G.; el Solh, N. Nucleotide sequence of the *Staphylococcus aureus* transposon, Tn*5405*, carrying aminoglycosides resistance genes. *J. Basic Microbiol.* **1997**, *37*, 379–384.
50. Singh, V.K.; Xiong, A.M.; Usgaard, T.R.; Chakrabarti, S.; Deora, R.; Misra, T.K.; Jayaswal, R.K. ZntR is an autoregulatory protein and negatively regulates the chromosomal zinc resistance operon znt of *Staphylococcus aureus*. *Mol. Microbiol.* **1999**, *33*, 200–207.
51. Yoon, K.P.; Misra, T.K.; Silver, S. Regulation of the cadA cadmium resistance determinant of *Staphylococcus aureus* plasmid PI258. *J. Bacteriol.* **1991**, *173*, 7643–7649.
52. Yoon, K.P.; Silver, S. A 2nd gene in the *Staphylococcus aureus* cadA cadmium resistance determinant of plasmid-PI258. *J. Bacteriol.* **1991**, *173*, 7636–7642.
53. Okkeri, J.; Haltia, T. Expression and mutagenesis of ZntA, a zinc-transporting P-type ATPase from *Escherichia coli*. *Biochemistry* **1999**, *38*, 14109–14116.
54. Stomeo, F.; Valverde, A.; Pointing, S.B.; McKay, C.P.; Warren-Rhodes, K.A.; Tuffin, M.I.; Seely, M.; Cowan, D.A. Hypolithic and soil microbial community assembly along an aridity gradient in the Namib Desert. *Extremophiles* **2013**, *17*, 329–337.
55. Xiong, A.M.; Jayaswal, R.K. Molecular characterization of a chromosomal determinant conferring resistance to zinc and cobalt ions in *Staphylococcus aureus*. *J. Bacteriol.* **1998**, *180*, 4024–4029.
56. Thelwell, C.; Robinson, N.J.; Turner-Cavet, J.S. An SmtB-like repressor from Synechocystis PCC 6803 regulates a zinc exporter. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 10728–10733.
57. Cook, W.J.; Kar, S.R.; Taylor, K.B.; Hall, L.M. Crystal structure of the cyanobacterial metallothionein repressor SmtB: A model for metalloregulatory proteins. *J. Mol. Biol.* **1998**, *275*, 337–346.
58. Shi, W.P.; Dong, J.; Scott, R.A.; Ksenzenko, M.Y.; Rosen, B.P. The role of arsenic thiol interactions in metalloregulation of the ars operon. *J. Biol. Chem.* **1996**, *271*, 9291–9297.
59. Prusa, R.; Kizek, R.; Trnkova, L.; Vacek, J.; Zehnalek, J. Study of relationship between metallothionein and heavy metals by CPSA method. *Clin. Chem.* **2004**, *50*, A28–A29.

60. Vasak, M. Advances in metallothionein structure and functions. *J. Trace Elem. Med. Biol.* **2005**, *19*, 13–17.
61. Skalickova, S.; Zitka, O.; Nejdl, L.; Krizkova, S.; Sochor, J.; Janu, L.; Ryvolova, M.; Hynek, D.; Zidkova, J.; Zidek, V.; *et al.* Study of interaction between metallothionein and CdTe quantum dots. *Chromatographia* **2013**, *76*, 345–353.
62. Huckle, J.W.; Morby, A.P.; Turner, J.S.; Robinson, N.J. Isolation of a prokaryotic metallothionein locus and analysis of transcriptional control by trace-metal ions. *Mol. Microbiol.* **1993**, *7*, 177–187.
63. Klaassen, C.D.; Liu, J.; Diwan, B.A. Metallothionein protection of cadmium toxicity. *Toxicol. Appl. Pharmacol.* **2009**, *238*, 215–220.
64. Templeton, D.M.; Cherian, M.G. Toxicological significance of metallothionein. *Method Enzymol.* **1991**, *205*, 11–24.
65. Sochor, J.; Pohanka, M.; Ruttkay-Nedecky, B.; Zitka, O.; Hynek, D.; Mares, P.; Zeman, L.; Adam, V.; Kizek, R. Effect of selenium in organic and inorganic form on liver, kidney, brain and muscle of Wistar rats. *Cent. Eur. J. Chem.* **2012**, *10*, 1442–1451.
66. Coyle, P.; Philcox, J.C.; Carey, L.C.; Rofe, A.M. Metallothionein: The multipurpose protein. *Cell. Mol. Life Sci.* **2002**, *59*, 627–647.
67. Henkel, G.; Krebs, B. Metallothioneins: Zinc, cadmium, mercury, and copper thiolates and selenolates mimicking protein active site features—Structural aspects and biological implications. *Chem. Rev.* **2004**, *104*, 801–824.
68. Adam, V.; Fabrik, I.; Eckschlager, T.; Stiborova, M.; Trnkova, L.; Kizek, R. Vertebrate metallothioneins as target molecules for analytical techniques. *TRAC-Trends Anal. Chem.* **2010**, *29*, 409–418.
69. Krizkova, S.; Ryvolova, M.; Hrabeta, J.; Adam, V.; Stiborova, M.; Eckschlager, T.; Kizek, R. Metallothioneins and zinc in cancer diagnosis and therapy. *Drug Metab. Rev.* **2012**, *44*, 287–301.
70. Naik, M.M.; Shamim, K.; Dubey, S.K. Biological characterization of lead-resistant bacteria to explore role of bacterial metallothionein in lead resistance. *Curr. Sci.* **2012**, *103*, 426–429.

6 ZÁVĚR

O rezistenci bakteriálních kmenů pojednává mnoho příspěvků, publikací a studií. Nadměrné užívání antibiotických léčiv výrazně urychlilo evoluci bakterií, které své vlastnosti přizpůsobily životu v prostředí antibiotických léčiv. Ve světě je v současné době monitorováno mnoho bakteriálních kmenů, na něž konvenční léčba nezabírá. Bakterie se bez větších potíží v prostředí léčiv množí a šíří a stávají se tak mnohdy nezvladatelným problémem medicínského prostředí.

Nutnost hledání alternativ v léčbě bakteriálních infekcí, zejména infekcí způsobených rezistentními kmeny bakterií, je tak na snadě. Po zjištění, že kovy, a to především ve formě iontů, mají významné antibakteriální účinky, se vědecké skupiny začaly intenzivně zabývat studiem různých typů kovů. Byly testovány kovy esenciální i neesenciální.

Postupem času se do popředí zájmu začaly dostávat nanotechnologie a s nimi i testování kovů ve formě nanočástic. Naše studie potvrdily výrazný vliv iontů stříbra i nanočástic fosforečnanu stříbrného na vznik oxidačního stresu u bakterie *Staphylococcus aureus* a zejména jejich antibakteriální efekt, který se dále podařilo posílit kombinací s taktéž antibakteriálními polymerními látkami tvorbou komplexů. Nedlouho poté byly nanočástice fosforečnanu stříbrného nahrazeny ještě účinějšími nanočásticemi selenu. Disertační práce se pak v neposlední řadě experimentálně zabývala rezistencí *Staphylococcus aureus* vůči samotným těžkým kovům a zároveň zkříženou rezistencí bakteriálních kmenů vůči těžkým kovům a antibiotickým léčivům.

Využití antibakteriálních komponent na bázi nanotechnologií je tak prokazatelně vhodnou cestou při léčbě bakteriálních infekcí, se kterými je stále častěji těžké bojovat. Našich výsledků může být přímo využito v medicínských oborech a mohou být zároveň využity jako podklad pro další případné modifikace komponent s antibakteriálními vlastnostmi.

7 LITERATURA

1. Martinez-Finley, E.J., et al., *Cellular transport and homeostasis of essential and nonessential metals.* Metallomics, 2012. **4**(7): p. 593-605.
2. Foster, T.J., *The Staphylococcus aureus "superbug".* Journal of Clinical Investigation, 2004. **114**(12): p. 1693-1696.
3. Kayser, F.H. and K.A. Bienz, *Medical microbiology*2011: Thieme. 724.
4. Rao, A.S., *Introduction to microbiology*1997: PHI Learning Pvt. Ltd. 216.
5. Archunan, G., *Microbiology*2004: Sarup & Sons. 384.
6. Cheesbrough, M., *District laboratory practice in tropical countries.* Vol. 2. 2006: Cambridge university Press. 440.
7. Perl, T.M., et al., *Intranasal mupirocin to prevent postoperative staphylococcus aureus infections.* New England Journal of Medicine, 2002. **346**(24): p. 1871-1877.
8. Ginalska, G., et al., *Antibacterial activity of gentamicin-bonded gelatin-sealed polyethylene terephthalate vascular prostheses.* European Journal of Vascular and Endovascular Surgery, 2005. **29**(4): p. 419-424.
9. Bandyk, D.F., *Vascular surgical site infection: Risk factors and preventive measures.* Seminars in Vascular Surgery, 2008. **21**(3): p. 119-123.
10. Teebken, O.E., et al., *Recommendations for Reporting Treatment of Aortic Graft Infections.* European Journal of Vascular and Endovascular Surgery, 2012. **43**(2): p. 174-181.
11. O'Brien, R., N. Pocock, and F. Torella, *Wound infection after reconstructive arterial surgery of the lower limbs: Risk factors and consequences.* Surgeon-Journal of the Royal Colleges of Surgeons of Edinburgh and Ireland, 2011. **9**(5): p. 245-248.
12. Tatterton, M.R. and S. Homer-Vanniasinkam, *Infections in vascular surgery.* Injury-International Journal of the Care of the Injured, 2011. **42**: p. S35-S41.
13. Homer-Vanniasinkam, S., *Surgical site and vascular infections: treatment and prophylaxis.* International Journal of Infectious Diseases, 2007. **11**: p. S17-S22.
14. Bunt, T.J., *Vascular graft infections: an update.* Cardiovascular Surgery, 2001. **9**(3): p. 225-233.
15. Gagliardi, A.R., et al., *Identifying opportunities for quality improvement in surgical site infection prevention.* American Journal of Infection Control, 2009. **37**(5): p. 398-402.
16. Demaria, R.G., et al., *Topical negative pressure therapy. A very useful new method to treat severe infected vascular approaches in the groin.* Journal of Cardiovascular Surgery, 2003. **44**(6): p. 757-761.
17. Anderson, D.J., et al., *Severe surgical site infection in community hospitals: Epidemiology, key procedures, and the changing prevalence of methicillin-resistant staphylococcus aureus.* Infection Control and Hospital Epidemiology, 2007. **28**(9): p. 1047-1053.
18. Earnshaw, J.J., *Methicillin-resistant Staphylococcus aureus: Vascular surgeons should fight back.* European Journal of Vascular and Endovascular Surgery, 2002. **24**(4): p. 283-286.
19. Nasim, A., et al., *The impact of MRSA on vascular surgery.* European Journal of Vascular and Endovascular Surgery, 2001. **22**(3): p. 211-214.

20. Young, M.H., G.R. Upchurch, and P.N. Malani, *Vascular Graft Infections.* Infectious Disease Clinics of North America, 2012. **26**(1): p. 41-+.
21. Lew, W. and W. Moore, *Antibiotic-Impregnated Grafts for Aortic Reconstruction.* Seminars in Vascular Surgery, 2011. **24**(4): p. 211-219.
22. Ricco, J.B. and O. Assadian, *Antimicrobial Silver Grafts for Prevention and Treatment of Vascular Graft Infection.* Seminars in Vascular Surgery, 2011. **24**(4): p. 234-241.
23. Driscoll, A.J., et al., *Disk Diffusion Bioassays for the Detection of Antibiotic Activity in Body Fluids: Applications for the Pneumonia Etiology Research for Child Health Project.* Clinical Infectious Diseases, 2012. **54**: p. S159-S164.
24. Zuniga, R. and T. Nguyen, *Skin conditions: emerging drug-resistant skin infections and infestations.* FP essentials, 2013. **407**: p. 17-23.
25. Leucker, T.M., S.V. Reddy, and B.K. Moffett, *Methicillin-resistant Staphylococcus aureus Induced Reactive Arthritis Treated With Systemic Corticosteroid.* The American journal of the medical sciences, 2013. **346**(2): p. 172-3.
26. Duffy, J., et al., *Community-onset invasive methicillin-resistant Staphylococcus aureus infections following hospital discharge.* American Journal of Infection Control, 2013. **41**(9): p. 782-6.
27. Taylor, A.R., *Methicillin-resistant Staphylococcus aureus infections.* Primary care, 2013. **40**(3): p. 637-54.
28. Baek, K.T., et al., *beta-Lactam Resistance in Methicillin-Resistant Staphylococcus aureus USA300 Is Increased by Inactivation of the ClpXP Protease.* Antimicrobial Agents and Chemotherapy, 2014. **58**(8): p. 4593-4603.
29. Wan, M.T. and C.C. Chou, *Spreading of beta-lactam resistance gene (mecA) and methicillin-resistant Staphylococcus aureus through municipal and swine slaughterhouse wastewaters.* Water Research, 2014. **64**: p. 288-295.
30. Ogata, K., et al., *Molecular epidemiological study of community-acquired methicillin-resistant staphylococcus aureus (CA-MRSA) - an examination of commercially distributed meat as a possible vehicle for CA-MRSA.* Journal of UOEH, 2014. **36**(3): p. 179-90.
31. Fon Sing, S., et al., *Pilot-scale continuous recycling of growth medium for the mass culture of a halotolerant Tetraselmis sp. in raceway ponds under increasing salinity: a novel protocol for commercial microalgal biomass production.* Bioresource Technology, 2014. **161**: p. 47-54.
32. Sing, S.F., et al., *Pilot-scale continuous recycling of growth medium for the mass culture of a halotolerant Tetraselmis sp in raceway ponds under increasing salinity: A novel protocol for commercial microalgal biomass production.* Bioresource Technology, 2014. **161**: p. 47-54.
33. Banno, H., et al., *Characterization of multidrug-resistant group B streptococci with reduced penicillin susceptibility forming small non-Beta-hemolytic colonies on sheep blood agar plates.* Journal of clinical microbiology, 2014. **52**(6): p. 2169-71.
34. Nasiri, V., *Sheep blood-LB agar base medium (SLM) as a simple and suitable medium for the cultivation of Leishmania major promastigotes.* Parasitology Research, 2013. **112**(11): p. 3741-3742.
35. Goetz, K., et al., *Potential new method: A comparability study of M-coliblue24 and M-endo les agar for the verified recoveries of total coliforms and E. coli.*

Abstracts of the General Meeting of the American Society for Microbiology, 1997. **97**(0): p. 481.
36. Dvorkova Heroldova, M. and M. Dvorackova, *Medium with amikacin for laboratory diagnosis of beta-haemolytic streptococci.* Epidemiologie Mikrobiologie Imunologie, 2006. **55**(2): p. 73-75.
37. Kim, H.J., et al., *Optimal Formation of Genetically Modified and Functional Pancreatic Islet Spheroids by Using Hanging-Drop Strategy.* Transplantation Proceedings, 2013. **45**(2): p. 605-610.
38. Gupta, J.C., G. Pandey, and K.J. Mukherjee, *Two-stage cultivation of recombinant Saccharomyces cerevisiae to enhance plasmid stability under non-selective conditions: experimental study and modeling.* Enzyme and Microbial Technology, 2001. **28**(1): p. 89-99.
39. Voropaeva, L.A., *Mutants of Saccharomyces cerevisiae yeast with increased plasmid mitotic stability isolated following prolonged cultivation of transformants under nonselective conditions.* Molekulyarnaya Genetika Mikrobiologiya i Virusologiya, 1991(7): p. 8-12.
40. Gorelova, N.B. and S.V. Shcherbakov, *Modification of a medium for the cultivation and isolation of the lyme disease pathogen.* Meditsinskaya Parazitologiya i Parazitarnye Bolezni, 1991(3): p. 56-57.
41. Seitgel'dyev, N., et al., *Modification of A-5P medium and its test under experimental commercial Chlorella cultivation with solar energy use.* Izvestiya Akademii Nauk Turkmenistana Seriya Biologicheskikh Nauk, 1992. **0**(1): p. 46-51.
42. Schegoleva, R.A., *Modification of sauton liquid nutrient medium for cultivation of Mycobacterium tuberculosis.* Laboratornoe Delo, 1989(5): p. 78-78.
43. Tasca, T., et al., *Modification and adaptation in semi-defined media for cultivation of flagellate Tetratrichomonas didelphidis (Trichomonadidae Trichomonadinae) from the Didelphis marsupialis.* Parasitologia Al Dia, 1999. **23**(3-4): p. 121-122.
44. E, T.S., et al., *Criteria of bacteria count in "Emission Standard of Polluted Water from Hospital"--confirmation tests for the total number of E. coli group.* Zhonghua yu fang yi xue za zhi [Chinese journal of preventive medicine], 1983. **17**(6): p. 339-41.
45. Tennant, A.D., et al., *Coliform bacteria in sea water and shellfish .2. ec confirmation test for Escherichia coli.* Canadian Journal of Microbiology, 1961. **7**(5): p. 733-&.
46. Hirvonen, J.J., et al., *Rapid confirmation of suspected methicillin-resistant Staphylococcus aureus colonies on chromogenic agars by a new commercial PCR assay, the GenomEra MRSA/SA Diagnose.* European Journal of Clinical Microbiology & Infectious Diseases, 2012. **31**(8): p. 1961-1968.
47. Johnson, J.L. and C.P. Lattuada, *Comparison of nucleic-acid hybridization assays and biochemical-characterization tests for the confirmation of Listeria monocytogenes.* Journal of Food Protection, 1993. **56**(10): p. 834-840.
48. Zourob, M., S. Elwary, and A.P.F. Turner, *Principles of bacterial detection: Biosensors, recognition receptors and microsystems*2008: Springer Science & Business Media. 1002.
49. Ryll, M., et al., *Isolation and differentiation of a cytochromoxidase-negative strain of Ornithobacterium rhinotracheale from turkeys.* Berliner Und Munchener Tierarztliche Wochenschrift, 2002. **115**(7-8): p. 274-277.

50. Yoshida, M., *Clinical significance of beta-glucuronidase analysis in biochemical laboratory tests.* Nihon rinsho. Japanese journal of clinical medicine, 1989. **48 Suppl**: p. 270-3.
51. Fearon, W.R., *A Study of some Biochemical Tests. No. 2: The Adamkiewicz Protein Reaction. The Mechanism of the Hopkins-Cole Test for Tryptophan. A New Colour Test for Glyoxylic Acid.* The Biochemical journal, 1920. **14**(5): p. 548-64.
52. Horowitz, R., et al., *Confirmation of an excess of the high enzyme activity COMT val allele in heroin addicts in a family-based haplotype relative risk study.* Drug and Alcohol Dependence, 2000. **60**(Suppl. 1): p. S93-S94.
53. Kendrick, P.S., et al., *Confirmation of the presence of a domoic acid (DA)-specific antibody in naturally exposed California sea lion (Zalophus californianus) serum samples as detected via an indirect enzyme-linked immunosorbent assay (ELISA).* Figshare, 2013.
54. Abadom, V., et al., *IFA confirmation of HLV-I-II antibodies.* Sixth International Conference on Aids. Sixth International Conference on Aids, Vols. 1-3. Pagination Varies Sixth International Conference on Aids University of California San Francisco: San Francisco, California, USA. Illus. Maps. Paper, 1990: p. 309.
55. Israil, A.-M., et al., *Discordancies between classical and API 20E microtest biochemical identification of Vibrio and Aeromonas strains.* Bacteriologia, virusologia, parazitologia, epidemiologia (Bucharest, Romania : 1990), 2003. **48**(2-3): p. 141-3.
56. Hascelik, G., *A comparison between classic manual methods and Enterotube II Roche for the identification of the enteric bacteria isolated from feces.* Mikrobiyoloji Bulteni, 1987. **21**(4): p. 274-278.
57. Negut, N., et al., *Trial of a new microtest system, Enterotest IC, for identification of Enterobacteriaceae.* Revista de igiena, bacteriologie, virusologie, parazitologie, epidemiologie, pneumoftiziologie. Bacteriologia, virusologia, parazitologia, epidemiologia, 1984. **29**(2): p. 135-44.
58. Rojickova, R. and E. Pakrova, *Evaluation of the NEFERMtest for identification of gram-negative non-fermentative rods in clinical specimens.* Epidemiologie Mikrobiologie Imunologie, 1994. **43**(3): p. 107-110.
59. Lazareva, E.B., et al., *Identification of staphylococci isolated from patients with pyoinflammatory processes by means of STAPHYtest system.* Klinicheskaya Laboratornaya Diagnostika, 1995. **0**(1): p. 45-47.
60. Svec, P., I. Sedlacek, and E. Pakrova, *Evaluation of the STREPTOtest kit for the identification of enterococci isolated from human clinical material.* Epidemiologie Mikrobiologie Imunologie, 1996. **45**(4): p. 153-157.
61. Kpikpi, E.N., et al., *Identification of Bacillus species occurring in Kantong, an acid fermented seed condiment produced in Ghana.* International journal of food microbiology, 2014. **180**: p. 1-6.
62. Lee, K., M. Lee, and Y. Lee, *Safety assessment of commercial Enterococcus probiotics in Korea.* Journal of Microbiology and Biotechnology, 2008. **18**(5): p. 942-945.
63. Baldwin, M.A., *Mass spectrometers for the analysis of biomolecules*, in *Biological Mass Spectrometry*, A.L. Burlingame, Editor 2005. p. 3-48.

64. Seng, P., et al., *Ongoing Revolution in Bacteriology: Routine Identification of Bacteria by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry.* Clinical Infectious Diseases, 2009. **49**(4): p. 543-551.
65. Balazova, T., et al., *The influence of culture conditions on the identification of Mycobacterium species by MALDI-TOF MS profiling.* Fems Microbiology Letters, 2014. **353**(1): p. 77-84.
66. Paterson, G.K., E.M. Harrison, and M.A. Holmes, *The emergence of mecC methicillin-resistant Staphylococcus aureus.* Trends in Microbiology, 2014. **22**(1): p. 42-47.
67. O'Grady, K.A.F., et al., *Mailed versus frozen transport of nasal swabs for surveillance of respiratory bacteria in remote Indigenous communities in Australia.* Bmc Infectious Diseases, 2013. **13**.
68. Passow, H. and A. Rothstein, *The binding of mercury by the yeast cell in relation to changes in permeability.* Journal of General Physiology, 1960. **43**(3): p. 621-633.
69. Altimira, F., et al., *Characterization of copper-resistant bacteria and bacterial communities from copper-polluted agricultural soils of central Chile.* Bmc Microbiology, 2012. **12**.
70. Gutteridge, J.M.C. and B. Halliwell, *The measurement and mechanism of lipid-peroxidation in biological-systems.* Trends in Biochemical Sciences, 1990. **15**(4): p. 129-135.
71. Rouch, D.A., B.T.O. Lee, and A.P. Morby, *Understanding cellular-responses to toxic agents - a model for mechanism-choice in bacterial metal resistance.* Journal of Industrial Microbiology, 1995. **14**(2): p. 132-141.
72. Nakahara, H., et al., *Linkage of mercury, cadmium, and arsenate and drug-resistance in clinical isolates of Pseudomonas-aeruginosa.* Applied and Environmental Microbiology, 1977. **33**(4): p. 975-976.
73. Marques, A.M., F. Congregado, and D.M. Simonpujol, *Antibiotic and heavy metal resistance of Pseudomonas aeruginosa isolated from soils.* Journal of Applied Bacteriology, 1979. **47**(2): p. 347-350.
74. Harnett, N.M. and C.L. Gyles, *Resistance to drugs and heavy-metals, colicin production, and biochemical characteristic of selected bovine and porcine Escherichia-coli strains.* Applied and Environmental Microbiology, 1984. **48**(5): p. 930-935.
75. Belliveau, B.H., M.E. Starodub, and J.T. Trevors, *Occurrence of antibiotic and metal resistance and plasmid in bacillus strains isolated from marine sediment.* Canadian Journal of Microbiology, 1991. **37**(7): p. 513-520.
76. Wang, Y.T. and H. Shen, *Bacterial reduction of hexavalent chromium.* Journal of Industrial Microbiology, 1995. **14**(2): p. 159-163.
77. Silver, S. and G.G. Ji, *Newer systems for bacterial resistances to toxic heavy-metals.* Environmental Health Perspectives, 1994. **102**: p. 107-113.
78. Harnett, N.M. and C.L. Gyles, *Resistance to drugs and heavy-metals, colicin production, and biochemical characteristic of selected bovine and porcine Escherichia-coli strains* Applied and Environmental Microbiology, 1984. **48**(5): p. 930-935.
79. McEntee, J.D., J.R. Woodrow, and A.V. Quirk, *Investigation of cadmium resistance in an alcaligenes spp.* Applied and Environmental Microbiology, 1986. **51**(3): p. 515-520.

80. Schwarz, S. and H. Blobel, *Plasmids and resistance to antimicrobial agents and heavy-metals in Staphylococcus-hyicus from pigs and cattle.* Journal of Veterinary Medicine Series B-Zentralblatt Fur Veterinarmedizin Reihe B-Infectious Diseases and Veterinary Public Health, 1989. **36**(9): p. 669-673.
81. Sklenar, M., et al., *General Mechanisms of Microbial resistance to metals.* Journal of Metallomics and Nanotechnologies, 2014. **1**(1): p. 15-17.
82. Nies, D.H. and S. Silver, *Ion efflux systems involved in bacterial metal resistances.* Journal of Industrial Microbiology, 1995. **14**(2): p. 186-199.
83. Schreurs, W.J.A. and H. Rosenberg, *Effect of silver ions on transport and retention of phosphate by escherichia-coli.* Journal of Bacteriology, 1982. **152**(1): p. 7-13.
84. Nieboer, E. and D.H.S. Richardson, *The replacement of the non-descript term heavy-metals by a biologically and chemically significant classification of metal-ions.* Environmental Pollution Series B-Chemical and Physical, 1980. **1**(1): p. 3-26.
85. Nies, D.H., *Microbial heavy-metal resistance.* Applied Microbiology and Biotechnology, 1999. **51**(6): p. 730-750.
86. Silver, S. and M. Walderhaug, *Gene-regulation of plasmid-determined and chromosome-determined inorganic-ion transport in bacteria.* Microbiological Reviews, 1992. **56**(1): p. 195-228.
87. Misra, T.K., et al., *Mercuric ion-resistance operons of plasmid r100 and transposon tn501 - the beginning of the operon including the regulatory region and the 1st 2 structural genes.* Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences, 1984. **81**(19): p. 5975-5979.
88. Ji, G.Y. and S. Silver, *Bacterial resistance mechanism for heavymetals of enviromental concern.* Journal of Industrial Microbiology, 1995. **14**(2): p. 61-75.
89. Bagg, A. and J.B. Neilands, *Ferric uptake regulation protein acts as a repressor, employing iron(ii) as a cofactor to bind the operator of an iron transport operon in escherichia-coli.* Biochemistry, 1987. **26**(17): p. 5471-5477.
90. Braun, V., K. Gunter, and K. Hantke, *Transport of iron across the outer-membrane.* Biology of Metals, 1991. **4**(1): p. 14-22.
91. Cervantes, C. and S. Silver, *Inorganic cation and anion transport-systems of Pseudomonas*. Pseudomonas : Biotransformations, Pathogenesis, and Evolving Biotechnology, ed. S. Silver, et al.1990, Washington: Amer Soc Microbiology.
92. Boquet, P. and E. Lemichez, *Bacterial virulence factors and Rho GTPases*2006: Springer Science & Business Media. 208.
93. Lederberg, J., *Microbial evolution and co-adaptation: A tribute to the life and scientific legacies of Joshua Lederberg*2009. 332.
94. Poiani, A., *Pragmatic evolution: Applications of evolutionary theory*2012: Cambridge University Press. 326.
95. Flower, D.R. and Y. Perrie, *Immunomic discovery of adjuvants and candidate subunit vaccines*, 2012, Springer Science & Business Media.
96. Buboltz, A.M., *Molecular evolution of virulence and antigenic diversity in Bordetella bronchiseptica*2008: ProQuest.
97. Rasool, R., S. Hasnain, and N. Nishat, *Metal-based Schiff base polymers: preparation, spectral, thermal and their in vitro biological investigation.* Designed Monomers and Polymers, 2014. **17**(3): p. 217-226.

98. Magdolenova, Z., et al., *Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles.* Nanotoxicology, 2014. **8**(3): p. 233-278.
99. Alexander, J.W., *History of the Medical Use of Silver.* Surgical Infections, 2009. **10**(3): p. 289-292.
100. Unger, C. and C. Luck, *Inhibitory effects of silver ions on Legionella pneumophila grown on agar, intracellular in Acanthamoeba castellanii and in artificial biofilms.* Journal of Applied Microbiology, 2012. **112**(6): p. 1212-1219.
101. Rai, M.K., et al., *Silver nanoparticles: the powerful nanoweapon against multidrug-resistant bacteria.* Journal of Applied Microbiology, 2012. **112**(5): p. 841-852.
102. Sadhasivam, S., P. Shanmugam, and K. Yun, *Biosynthesis of silver nanoparticles by Streptomyces hygroscopicus and antimicrobial activity against medically important pathogenic microorganisms.* Colloids and Surfaces B-Biointerfaces, 2010. **81**(1): p. 358-362.
103. Cho, K.H., et al., *The study of antimicrobial activity and preservative effects of nanosilver ingredient.* Electrochimica Acta, 2005. **51**(5): p. 956-960.
104. Kittler, S., et al., *The influence of proteins on the dispersability and cell-biological activity of silver nanoparticles.* Journal of Materials Chemistry, 2010. **20**(3): p. 512-518.
105. Choi, O., et al., *The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth.* Water Research, 2008. **42**(12): p. 3066-3074.
106. Moser, A., et al., *Comparison of genomic and antimicrobial resistance features of latex agglutination test-positive and latex agglutination test-negative Staphylococcus aureus isolates causing bovine mastitis.* Journal of Dairy Science, 2013. **96**(1): p. 329-334.
107. Furr, J.R., et al., *Antibacterial activity of actisorb-plus, actisorb and silver-nitrate* J. Hosp. Infect., 1994. **27**(3): p. 201-208.
108. Jung, W.K., et al., *Antifungal activity of the silver ion against contaminated fabric.* Mycoses, 2007. **50**(4): p. 265-269.
109. Klasen, H.J., *Historical review of the use of silver in the treatment of burns. I. Early uses.* Burns, 2000. **26**(2): p. 117-130.
110. Li, W.R., et al., *Antibacterial effect of silver nanoparticles on Staphylococcus aureus.* Biometals, 2011. **24**(1): p. 135-141.
111. Low, W.L., et al., *Antimicrobial efficacy of silver ions in combination with tea tree oil against Pseudomonas aeruginosa, Staphylococcus aureus and Candida albicans.* Int. J. Antimicrob. Agents, 2011. **37**(2): p. 162-165.
112. Blecher, K. and A. Friedman, *Nanotechnology and the Diagnosis of Dermatological Infectious Disease.* Journal of Drugs in Dermatology, 2012. **11**(7): p. 846-851.
113. Jung, W.K., et al., *Antibacterial activity and mechanism of action of the silver ion in Staphylococcus aureus and Escherichia coli.* Applied and Environmental Microbiology, 2008. **74**(7): p. 2171-2178.
114. Park, H.J., et al., *Silver-ion-mediated reactive oxygen species generation affecting bactericidal activity.* Water Research, 2009. **43**(4): p. 1027-1032.
115. Lushchak, V.I., *Oxidative stress and mechanisms of protection against it in bacteria.* Biochem.-Moscow, 2001. **66**(5): p. 476-489.

116. Barriere, C., S. Leroy-Setrin, and R. Talon, *Characterization of catalase and superoxide dismutase in Staphylococcus carnosus 833 strain.* Journal of Applied Microbiology, 2001. **91**(3): p. 514-519.
117. Bir, F., et al., *Electrochemical depositions of fluorohydroxyapatite doped by Cu2+, Zn2+, Ag+ on stainless steel substrates.* Applied Surface Science, 2012. **258**(18): p. 7021-7030.
118. Estevao, M.S., et al., *Analysis of the antioxidant activity of an indole library: cyclic voltammetry versus ROS scavenging activity.* Tetrahedron Letters, 2011. **52**(1): p. 101-106.
119. Gomes, A., et al., *Cyclic voltammetric analysis of 2-styrylchromones: Relationship with the antioxidant activity.* Bioorganic & Medicinal Chemistry, 2008. **16**(17): p. 7939-7943.
120. Nyska, A. and R. Kohen, *Oxidation of biological systems: Oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification.* Toxicologic Pathology, 2002. **30**(6): p. 620-650.
121. Chevion, S. and M. Chevion, *Antioxidant status and human health - Use of cyclic voltammetry for the evaluation of the antioxidant capacity of plasma and of edible plants*, in *Reactive Oxygen Species: From Radiation to Molecular Biology: A Festschrift in Honor of Daniel L Gilbert*, C.C. Chiueh, Editor 2000, New York Acad Sciences: New York. p. 308-325.
122. Khan, M.A.M., et al., *Structural and spectroscopic studies of thin film of silver nanoparticles.* Applied Surface Science, 2011. **257**(24): p. 10607-10612.
123. Banfi, G., V. Degiorgio, and D. Ricard, *Nonlinear optical properties of semiconductor nanocrystals.* Advances in Physics, 1998. **47**(3): p. 447-510.
124. Banyai, L., et al., *2-photon optical nonlinearities in semiconductor quantum dots.* J. De Phys., 1988. **49**(C-2): p. 225-228.
125. Khlebtsov, N.G. and L.A. Dykman, *Optical properties and biomedical applications of plasmonic nanoparticles.* Journal of Quantitative Spectroscopy & Radiative Transfer, 2010. **111**(1): p. 1-35.
126. Limbach, L.K., et al., *Exposure of engineered nanoparticles to human lung epithelial cells: Influence of chemical composition and catalytic activity on oxidative stress.* Environmental Science & Technology, 2007. **41**(11): p. 4158-4163.
127. Choi, O., et al., *The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth.* Water Res., 2008. **42**(12): p. 3066-3074.
128. Kanmani, P. and J.W. Rhim, *Physicochemical properties of gelatin/silver nanoparticle antimicrobial composite films.* Food Chemistry, 2014. **148**: p. 162-169.
129. Green, J.B.D., T. Fulghum, and M.A. Nordhaus, *Review of immobilized antimicrobial agents and methods for testing.* Biointerphases, 2011. **6**(4): p. MR13-MR28.
130. Osinska-Jaroszuk, M., et al., *Vascular Prostheses with Covalently Bound Gentamicin and Amikacin Reveal Superior Antibacterial Properties than Silver-impregnated Ones - An In Vitro Study.* European Journal of Vascular and Endovascular Surgery, 2009. **38**(6): p. 697-706.
131. Sharma, B.K., et al., *Synthesis and characterization of polyaniline-ZnO composite and its dielectric behavior.* Synthetic Metals, 2009. **159**(5-6): p. 391-395.

132. Liu, S., et al., *Antibacterial and anti-adhesion effects of the silver nanoparticles-loaded poly(L-lactide) fibrous membrane.* Materials Science & Engineering C-Materials for Biological Applications, 2013. **33**(3): p. 1176-1182.
133. Shahzad, M.N. and N. Ahmed, *Effectiveness of Aloe Vera Gel compared with 1% silver sulphadiazine cream as burn wound dressing in second degree burns.* Journal of the Pakistan Medical Association, 2013. **63**(2): p. 225-230.
134. Grigor'eva, A., et al., *Fine mechanisms of the interaction of silver nanoparticles with the cells of Salmonella typhimurium and Staphylococcus aureus.* Biometals, 2013. **26**(3): p. 479-488.
135. Wang, Q. and T.J. Webster, *Nanostructured selenium for preventing biofilm formation on polycarbonate medical devices.* Journal of Biomedical Materials Research Part A, 2012. **100A**(12): p. 3205-3210.
136. Tran, P.A. and T.J. Webster, *Selenium nanoparticles inhibit Staphylococcus aureus growth.* International Journal of Nanomedicine, 2011. **6**.
137. Ramos, J.F., et al., *Selenium Nanoparticles for the Prevention of PVC-related Medical Infections*. 2012 38th Annual Northeast Bioengineering Conference2012.
138. Dowling, D.P., et al., *Anti-bacterial silver coatings exhibiting enhanced activity through the addition of platinum.* Surface & Coatings Technology, 2003. **163**: p. 637-640.
139. Ruden, S., et al., *Synergistic Interaction between Silver Nanoparticles and Membrane-Permeabilizing Antimicrobial Peptides.* Antimicrobial Agents and Chemotherapy, 2009. **53**(8): p. 3538-3540.
140. de Mel, A., et al., *A silver nanocomposite biomaterial for blood-contacting implants.* Journal of Biomedical Materials Research Part A, 2012. **100A**(9): p. 2348-2357.
141. Fujita, M., et al., *Inhibition of vascular prosthetic graft infection using a photocrosslinkable chitosan hydrogel.* Journal of Surgical Research, 2004. **121**(1): p. 135-140.
142. Rhim, J.W., L.F. Wang, and S.I. Hong, *Preparation and characterization of agar/silver nanoparticles composite films with antimicrobial activity.* Food Hydrocolloids, 2013. **33**(2): p. 327-335.
143. Pishbin, F., et al., *Single-step electrochemical deposition of antimicrobial orthopaedic coatings based on a bioactive glass/chitosan/nano-silver composite system.* Acta Biomaterialia, 2013. **9**(7): p. 7469-7479.
144. Sandri, G., et al., *Wound dressings based on silver sulfadiazine solid lipid nanoparticles for tissue repairing.* European Journal of Pharmaceutics and Biopharmaceutics, 2013. **84**(1): p. 84-90.
145. Ghosh, A. and M.A. Ali, *Studies on physicochemical characteristics of chitosan derivatives with dicarboxylic acids.* Journal of Materials Science, 2012. **47**(3): p. 1196-1204.
146. Mohamed, C., et al., *Antimicrobial and physical properties of edible chitosan films enhanced by lactoperoxidase system.* Food Hydrocolloids, 2013. **30**(2): p. 576-580.
147. Chang, H.W., et al., *Effects of chitosan characteristics on the physicochemical properties, antibacterial activity, and cytotoxicity of chitosan/2-glycerophosphate/nanosilver hydrogels.* Journal of Applied Polymer Science, 2013. **127**(1): p. 169-176.

148. Jayakumar, R., et al., *Biomedical applications of chitin and chitosan based nanomaterials-A short review.* Carbohydrate Polymers, 2010. **82**(2): p. 227-232.
149. Pires, N.R., et al., *Sulfated chitosan as tear substitute with no antimicrobial activity.* Carbohydrate Polymers, 2013. **91**(1): p. 92-99.
150. Madhumathi, K., et al., *Development of novel chitin/nanosilver composite scaffolds for wound dressing applications.* Journal of Materials Science-Materials in Medicine, 2010. **21**(2): p. 807-813.
151. Huang, G.Q., et al., *Complex coacervation of soybean protein isolate and chitosan.* Food Chemistry, 2012. **135**(2): p. 534-539.
152. Lee, S.B., et al., *Preparation and characteristics of hybrid scaffolds composed of beta-chitin and collagen.* Biomaterials, 2004. **25**(12): p. 2309-2317.
153. Hrabarova, E., et al., *Structural characterisation of thiol-modified hyaluronans.* Cellulose, 2012. **19**(6): p. 2093-2104.
154. Kenne, L., et al., *Modification and cross-linking parameters in hyaluronic acid hydrogels-Definitions and analytical methods.* Carbohydrate Polymers, 2013. **91**(1): p. 410-418.
155. Teh, B.M., et al., *A review on the use of hyaluronic acid in tympanic membrane wound healing.* Expert Opinion on Biological Therapy, 2012. **12**(1): p. 23-36.
156. Hrabarova, E., et al., *High-molar-mass hyaluronan degradation by Weissberger's system: Pro- and anti-oxidative effects of some thiol compounds.* Polymer Degradation and Stability, 2009. **94**(10): p. 1867-1875.
157. Sintzel, M.B., et al., *Biomaterials in ophthalmic drug delivery.* European Journal of Pharmaceutics and Biopharmaceutics, 1996. **42**(6): p. 358-374.
158. Stern, R., *Devising a pathway for hyaluronan catabolism: are we there yet?* Glycobiology, 2003. **13**(12): p. 105R-115R.
159. Bastow, E.R., et al., *Hyaluronan synthesis and degradation in cartilage and bone.* Cellular and Molecular Life Sciences, 2008. **65**(3): p. 395-413.
160. Evanko, S.P. and T.N. Wight, *Intracellular localization of hyaluronan in proliferating cells.* Journal of Histochemistry & Cytochemistry, 1999. **47**(10): p. 1331-1341.
161. Kogan, G., et al., *Hyaluronic acid: a natural biopolymer with a broad range of biomedical and industrial applications.* Biotechnology Letters, 2007. **29**(1): p. 17-25.
162. Goa, K.L. and P. Benfield, *Hyaluronic acid a review of its pharmacology and use as a surgical aid in opthalmology, and its terapeutic potential in joint disease and wound healing.* Drugs, 1994. **47**(3): p. 536-566.
163. Becker, L.C., et al., *Final Report of the Safety Assessment of Hyaluronic Acid, Potassium Hyaluronate, and Sodium Hyaluronate.* International Journal of Toxicology, 2009. **28**: p. 5-67.
164. Kim, J., et al., *Characterization of low-molecular-weight hyaluronic acid-based hydrogel and differential stem cell responses in the hydrogel microenvironments.* Journal of Biomedical Materials Research Part A, 2009. **88A**(4): p. 967-975.
165. Noble, P.W., *Hyaluronan and its catabolic products in tissue injury and repair.* Matrix Biology, 2002. **21**(1): p. 25-29.
166. Ortonne, J.P., *A controlled study of the activity of hyaluronic acid in the treatment of venous leg ulcers.* Journal of Dermatological Treatment, 1996. **7**(2): p. 75-81.

167. Guibal, E., *Interactions of metal ions with chitosan-based sorbents: a review.* Separation and Purification Technology, 2004. **38**(1): p. 43-74.
168. Abdel-Mohsen, A.M., et al., *Green synthesis of hyaluronan fibers with silver nanoparticles.* Carbohydrate Polymers, 2012. **89**(2): p. 411-422.
169. Broekaert, W.F., et al., *Plant defensins - novel antimicrobial peptides as components of the host-defense system.* Plant Physiology, 1995. **108**(4): p. 1353-1358.
170. Lehrer, R.I. and T. Ganz, *Antimicrobial peptides in mammalian and insect host defence.* Current Opinion in Immunology, 1999. **11**(1): p. 23-27.
171. Dathe, M., et al., *General aspects of peptide selectivity towards lipid bilayers and cell membranes studied by variation of the structural parameters of amphipathic helical model peptides.* Biochimica Et Biophysica Acta-Biomembranes, 2002. **1558**(2): p. 171-186.
172. Mani, R., et al., *Solid-state NMR investigation of the selective disruption of lipid membranes by protegrin-1.* Biochemistry, 2004. **43**(43): p. 13839-13848.
173. Hoskin, D.W. and A. Ramamoorthy, *Studies on anticancer activities of antimicrobial peptides.* Biochimica Et Biophysica Acta-Biomembranes, 2008. **1778**(2): p. 357-375.
174. Rivas, L., J.R. Luque-Ortega, and D. Andreu, *Amphibian antimicrobial peptides and Protozoa: Lessons from parasites.* Biochimica Et Biophysica Acta-Biomembranes, 2009. **1788**(8): p. 1570-1581.
175. Marr, A.K., W.J. Gooderham, and R.E.W. Hancock, *Antibacterial peptides for therapeutic use: obstacles and realistic outlook.* Current Opinion in Pharmacology, 2006. **6**(5): p. 468-472.
176. Zasloff, M., *Antimicrobial peptides of multicellular organisms.* Nature, 2002. **415**(6870): p. 389-395.
177. Papo, N. and Y. Shai, *Can we predict biological activity of antimicrobial peptides from their interactions with model phospholipid membranes?* Peptides, 2003. **24**(11): p. 1693-1703.
178. Yeaman, M.R. and N.Y. Yount, *Mechanisms of antimicrobial peptide action and resistance.* Pharmacological Reviews, 2003. **55**(1): p. 27-55.
179. Epand, R.F., A. Mor, and R.M. Epand, *Lipid complexes with cationic peptides and OAKs; their role in antimicrobial action and in the delivery of antimicrobial agents.* Cellular and Molecular Life Sciences, 2011. **68**(13): p. 2177-2188.
180. Wimley, W.C. and K. Hristova, *Antimicrobial Peptides: Successes, Challenges and Unanswered Questions.* Journal of Membrane Biology, 2011. **239**(1-2): p. 27-34.
181. Almeida, P.F. and A. Pokorny, *Mechanisms of Antimicrobial, Cytolytic, and Cell-Penetrating Peptides: From Kinetics to Thermodynamics.* Biochemistry, 2009. **48**(34): p. 8083-8093.
182. Mc Grath S., v.S.D., *Bacteriophage: Genetics and Molecular Biology.* 2007: p. 344.
183. Wommack K. Eric, C.R.R., *Virioplankton: viruses in aquatic ecosystems.* Microbiol. Mol. Biol. Rev., 2000. **64**(1): p. 69-114.
184. DiMauro, A.J., et al., *Crystallization of phi29 spindle-shaped nano-bar anti-receptor with glycosidase domain.* Journal of Nanoscience and Nanotechnology, 2007. **7**(8): p. 2616-2622.

185. De Gusseme, B., et al., *Biogenic Silver for Disinfection of Water Contaminated with Viruses.* Applied and Environmental Microbiology, 2010. **76**(4): p. 1082-1087.
186. Krebs, J., *Naturally dangerous: Surprising facts about food, health, and the environment.* Nature, 2002. **415**(6868): p. 117-117.
187. Lee, T.J., C. Schwartz, and P.X. Guo, *Construction of Bacteriophage Phi29 DNA Packaging Motor and its Applications in Nanotechnology and Therapy.* Annals of Biomedical Engineering, 2009. **37**(10): p. 2064-2081.
188. Jing, P., et al., *Robust properties of membrane-embedded connector channel of bacterial virus phi29 DNA packaging motor.* Molecular Biosystems, 2010. **6**(10): p. 1844-1852.
189. Rakonjac, J., et al., *Filamentous Bacteriophage: Biology, Phage Display and Nanotechnology Applications.* Current Issues in Molecular Biology, 2011. **13**(2): p. 51-75.
190. Zhang, H., et al., *RNA Ring-Geared Bacteriophage phi29 DNA Packaging Nanomotor for Nanotechnology and Gene Delivery*, in *Nanomedicine Design of Particles, Sensors, Motors, Implants, Robots, and Devices*, M.J. Schulz, V.N. Shanov, and Y. Yun, Editors. 2009, Artech House: Norwood. p. 211-+.
191. Guo, P.X. and T.J. Lee, *Viral nanomotors for packaging of dsDNA and dsRNA.* Molecular Microbiology, 2007. **64**(4): p. 886-903.
192. Petrenko, V.A., *Evolution of phage display: from bioactive peptides to bioselective nanomaterials.* Expert Opinion on Drug Delivery, 2008. **5**(8): p. 825-836.
193. Singh, P., M.J. Gonzalez, and M. Manchester, *Viruses and their uses in nanotechnology.* Drug Development Research, 2006. **67**(1): p. 23-41.
194. Ivanovska, I.L., et al., *Bacteriophage capsids: Tough nanoshells with complex elastic properties.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(20): p. 7600-7605.
195. Petrenko, V.A., *Landscape phage as a molecular recognition interface for detection devices.* Microelectronics Journal, 2008. **39**(2): p. 202-207.
196. Krumpe, L.R.H. and T. Mori, *The use of phage-displayed peptide libraries to develop tumor-targeting drugs.* International Journal of Peptide Research and Therapeutics, 2006. **12**(1): p. 79-91.
197. Li, L., et al., *Evaluation of specific delivery of chimeric phi29 pRNA/siRNA nanoparticles to multiple tumor cells.* Molecular Biosystems, 2009. **5**(11): p. 1361-1368.
198. Ni, J.P., et al., *Molecular orientation of a ZnS-nanocrystal-modified M13 virus on a silicon substrate.* Journal of Polymer Science Part B-Polymer Physics, 2004. **42**(4): p. 629-635.
199. Abramov, G., O. Morag, and A. Goldbourt, *Magic-Angle Spinning NMR of a Class I Filamentous Bacteriophage Virus.* Journal of Physical Chemistry B, 2011. **115**(31): p. 9671-9680.
200. Tsen, K.T., et al., *Observation of the low frequency vibrational modes of bacteriophage M13 in water by Raman spectroscopy.* Virology Journal, 2006. **3**.
201. Tsen, K.T., et al., *Raman scattering studies of the low-frequency vibrational modes of bacteriophage M13 in water - observation of an axial torsion mode.* Nanotechnology, 2006. **17**(21): p. 5474-5479.
202. Guo, P.X., et al., *Engineering RNA for Targeted siRNA Delivery and Medical Application.* Advanced Drug Delivery Reviews, 2010. **62**(6): p. 650-666.

203. Shu, D., et al., *Dual-Channel Single-Molecule Fluorescence Resonance Energy Transfer to Establish Distance Parameters for RNA Nanoparticles.* Acs Nano, 2010. **4**(11): p. 6843-6853.
204. Khaled, A., et al., *Controllable self-assembly of nanoparticles for specific delivery of multiple therapeutic molecules to cancer cells using RNA nanotechnology.* Nano Letters, 2005. **5**(9): p. 1797-1808.
205. Shu, D., et al., *Construction of phi29 DNA-packaging RNA monomers, dimers, and trimers with variable sizes and shapes as potential parts for nanodevices.* Journal of Nanoscience and Nanotechnology, 2003. **3**(4): p. 295-302.
206. Suthiwangcharoen, N., et al., *M13 Bacteriophage-Polymer Nanoassemblies as Drug Delivery Vehicles.* Nano Research, 2011. **4**(5): p. 483-493.
207. Alm, E.W., et al., *Patterns and persistence of antibiotic resistance in faecal indicator bacteria from freshwater recreational beaches.* Journal of Applied Microbiology, 2014. **117**(1): p. 273-85.
208. Machado, A. and A.A. Bordalo, *Prevalence of antibiotic resistance in bacteria isolated from drinking well water available in Guinea-Bissau (West Africa).* Ecotoxicology and environmental safety, 2014. **106**: p. 188-94.
209. Nedbalcova, K., et al., *Resistance to selected beta-lactam antibiotics.* Veterinary microbiology, 2014. **171**(3-4): p. 328-36.
210. Nguyen, H.N.K., et al., *Molecular characterization of antibiotic resistance in Pseudomonas and Aeromonas isolates from catfish of the Mekong Delta, Vietnam.* Veterinary microbiology, 2014. **171**(3-4): p. 397-405.
211. Pesavento, G., et al., *Prevalence and antibiotic resistance of Enterococcus spp. isolated from retail cheese, ready-to-eat salads, ham, and raw meat.* Food microbiology, 2014. **41**: p. 1-7.
212. Rodriguez, C., et al., *Multilocus sequence typing analysis and antibiotic resistance of Clostridium difficile strains isolated from retail meat and humans inBelgium.* Food microbiology, 2014. **42**: p. 166-71.
213. Krizkova, S., et al., *Metallothioneins and zinc in cancer diagnosis and therapy.* Drug Metabolism Reviews, 2012. **44**(4): p. 287-301.
214. Babula, P., et al., *Mammalians' metallothioneins and their properties and functions.* Metallomics, 2012. **4**(8): p. 739-750.
215. Eckschlager, T., et al., *Metallothioneins and cancer.* Curr. Protein Pept. Sci., 2009. **10**(4): p. 360-375.
216. Marambio-Jones, C. and E.M.V. Hoek, *A review of the antibacterial effects of silver nanomaterials and potential implications for human health and the environment.* J. Nanopart. Res., 2010. **12**(5): p. 1531-1551.
217. He, D., J.J. Dorantes-Aranda, and T.D. Waite, *Silver Nanoparticle-Algae Interactions: Oxidative Dissolution, Reactive Oxygen Species Generation and Synergistic Toxic Effects.* Environmental Science & Technology, 2012. **46**(16): p. 8731-8738.
218. Calas-Blanchard, C., et al., *Electrochemical Biosensors for the Determination of the Antioxidant Capacity in Foods and Beverages Based on Reactive Oxygen Species.* Curr. Anal. Chem., 2012. **8**(4): p. 428-435.
219. Klayraung, S. and S. Okonogi, *Antibacterial and antioxidant activities of acid and bile resistant strains of Lactobacillus fermentum isolated from miang. .* Braz. J. Microbiol., 2009. **40**(4): p. 757-766.
220. Zitka, O., et al., *Nanotechnology for more efficient blood vessel replacements.* Chem. Listy, 2013. **107**(1): p. 24-29.

221. Ye, Y., et al., *Mycoplasma pneumoniae 23S rRNA Gene Mutations and Mechanisms of Macrolide Resistance.* Labmedicine, 2013. **44**(1): p. 63-68.
222. Rai, M., A. Yadav, and A. Gade, *Silver nanoparticles as a new generation of antimicrobials.* Biotechnology Advances, 2009. **27**(1): p. 76-83.
223. Sudha, S.S., K. Rajamanickam, and J. Rengaramanujam, *Microalgae mediated synthesis of silver nanoparticles and their antibacterial activity against pathogenic bacteria.* Indian Journal of Experimental Biology, 2013. **51**(5): p. 393-399.
224. Wang, Q. and T.J. Webster, *Short communication: inhibiting biofilm formation on paper towels through the use of selenium nanoparticles coatings.* International Journal of Nanomedicine, 2013. **8**: p. 407-411.
225. Dlugosz, M., et al., *Hybrid calcium carbonate/polymer microparticles containing silver nanoparticles as antibacterial agents.* Journal of Nanoparticle Research, 2012. **14**(12).
226. Levy, S.B. and B. Marshall, *Antibacterial resistance worldwide: causes, challenges and responses.* Nature Medicine, 2004. **10**(12): p. S122-S129.
227. Gatica, J. and E. Cytryn, *Impact of treated wastewater irrigation on antibiotic resistance in the soil microbiome.* Environmental Science and Pollution Research, 2013. **20**(6): p. 3529-3538.
228. Ammerlaan, H.S.M., et al., *Secular trends in nosocomial bloodstream infections: antibiotic-resistant bacteria increase the total burden of infection.* Clinical Infectious Diseases, 2013. **56**(6): p. 798-805.

8 SEZNAM OBRÁZKŮ A TABULEK

9 SEZNAM ZKRATEK

ABTS	2,2´-azino-bis(3-ethylbenzotiazolin-6-sulfonová) kyselina
AFM	Mikroskopie atomárních sil
AMP	Antimikrobiální peptidy
AMR	Antimikrobiální rezistence
ATP	Adenosintrifosfát
CA-MRSA	Komunitní methicilin-rezistentní *Staphylococcus aureus*
DNA	Deoxyribonukleová kyselina
dNTP	Deoxyribonukleotid trifosfát
DPPH	2,2-difenyl-1-pikrylhydrazyl
ELISA	Enzymatická imunoanalýza
FRAP	Antioxidační potenciál redukce železa
G^-	Gramnegativní
G^+	Grampozitivní
HA-MRSA	Nozokomiální (nemocniční) methicilin-rezistentní *Staphylococcus aureus*
CHIP	Inhibiční bílkoviny chemotaxe
IFA	Nepřímá imunofluorescence
MALDI-TOF	Matricí asistovaná laserová desorpce/ionizace s analyzátorem doby letu
MRSA	Methicilin-rezistentní *Staphylococcus aureus*
NMR	Spektroskopie nukleární magnetické rezonance
PBP2A	Penicilin vázající protein
PCR	Polymerázová řetězová reakce
pRNA	Nekódující ribozomální RNA
PVL	Panton-Valentine leukocidin
RNA	Ribonukleová kyselina
ROS	Volné kyslíkové radikály
SCCmec	Stafylokoková chromozomová kazeta mec
SEM	Skenovací (rastrovací) elektronová mikroskopie
SeNPs	Selenové nanočástice
SPNPs	Nanočástice fosforečnanu stříbrného

TAE	Tris-acetát-EDTA
UV	Ultrafialový