VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

BRNO UNIVERSITY OF TECHNOLOGY

FAKULTA CHEMICKÁ
ÚSTAV CHEMIE MATERIÁLŮ

FACULTY OF CHEMISTRY
INSTITUTE OF MATERIALS SCIENCE

POLYMERIC MATERIALS FOR THE CONTROLLED DRUG DELIVERY AND CONTROLLED RELEASE OF ACTIVE SUBSTANCES

DIZERTAČNÍ PRÁCE
DOCTORAL THESIS

AUTOR PRÁCE — Ing. IVANA CHAMRADOVÁ
AUTHOR

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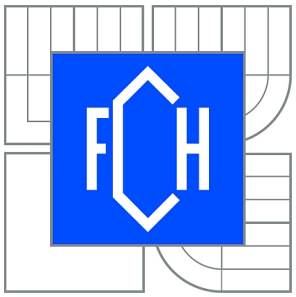

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POLYMERNÍ MATERIÁLY PRO ŘÍZENOU ADMINISTRACI LÉČIV A ŘÍZENÉ UVOLŇOVÁNÍ AKTIVNÍCH LÁTEK

DIZERTAČNÍ PRÁCE
DOCTORAL THESIS

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BRNO 2015

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Příprava kompozitních hydrogelů založených na termocitlivém kopolymeru a hydroxyapatitových částicích
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Sledování uvolňování hydroxyapatitu z polymerní matrice
Závěr

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Ing. Ivana Chamradová Student(ka)	Ing. Lucy Vojtová, Ph.D. Vedoucí práce	prof. RNDr. Josef Jančář, CSc. Ředitel ústavu

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Abstract

The literature review of proposed doctoral thesis summarizes knowledge of both present-day biomaterials and the new type of "smart" biomaterials such as thermosensitive copolymers. Among those copolymers whose aqueous solutions are liquid at laboratory temperature but turn into a solid gel as the temperature rises to 37 °C belong e.g. amphiphilic triblock copolymers based on the biodegradable hydrophobic polylactide, polyglycolide and hydrophilic poly(ethylene glycol) (PLGA−PEG−PLGA). Commercially available thermosensitive PLGA−PEG−PLGA copolymers, known as ReGel® or OncoGel® are used as injectable controlled drug delivery systems for diabetes or cancer treatment, respectively. However, PLGA−PEG−PLGA copolymers might be used as well as inorganic drug delivery carries and biodegradable implants for regenerative medicine in orthopedic and dental applications. For this reason the biocompatible hydroxyapatite (HAp), utilized in the bone remodeling process, was employed because of its majority representation in hard tissue.

The experimental part of this work is focused on the preparation of HAp/PLGA−PEG−PLGA composites, where used HAp was in the form of either nano- (n-HAp) or core-shell (CS) particles. Novel core-shell particles, prepared by double emulsion method, were consisted of "rigid" n-HAp core covered by PLGA−PEG−PLGA shell additionally end-functionalized by itaconic acid (ITA/PLGA−PEG−PLGA/ITA). ITA modification brought crosslinkable both double bonds and functional carboxylic groups to the ends of copolymer chains. Consequently, the ITA/PLGA−PEG−PLGA/ITA copolymer shell was chemically crosslinked to form life-time controlled 3D polymer network surrounded HAp core resulting in crosslinked core-shell particles (CS-x). The ATR-FTIR spectroscopy proved the presence of "new" ester bonds in polymer shell at 1021 cm^{-1} arising from the carbodiamide coupling reaction between -OH and -COOH groups. As mentioned above, both n-HAp and CS-x particles were mixed with the copolymer thermosensitive PLGA−PEG−PLGA matrix and flow/gelation behavior important for injectable materials were evaluated by rheological measurement. . It was found, that the addition of less than 5 wt.% of n-HAp particles or 10 wt.% of CS-x particles into the PLGA−PEG−PLGA polymer matrix retains its thermosensitive properties. However, adding higher amount of either n-HAp particles or CS-x particles in copolymer matrix changed the aqueous sol to permanent stiff gel while the temperature increases above 37 °C. Based on the ICP-OES analysis, the release of CS-x particles from 10 w/v % PLGA−PEG−PLGA gel matrix to the incubation medium at 37 °C was faster than in case of n-HAp particles (6 % vs. 3% in 9 days, respectively) which are more strongly bonded to matrix micellar structure. As a conclusion, composite based on n-HAP particles in copolymer matrix exhibiting stiff permanent gel at body temperature is suitable more as biodegradable bone adhesive while composite consisting of CS-x particles in thermosensitive copolymer matrix is useful as injectable drug delivery carrier.

Keywords

thermosensitive triblock copolymers, hydroxyapatite, drug release, rheology, core-shell particles

Abstrakt

Literární rešerše předložené dizertační práce shrnuje poznatky jak o současně používaných biomateriálech, tak i o tzv. „chytrých“ biomedicínských materiálech mezi které patří termocitlivé kopolymery. Mezi tyto kopolymery, jejichž vodné roztoky gelují při teplotě lidského těla (37 °C), řadíme amfifilní triblokové kopolymery skládající se z hydrofobního laktidu, glykolidu a hydrofilního polyethylen glykolu (PLGA−PEG−PLGA). Komerčně dostupné termocitlivé kopolymery známé pod názvem ReGel® or OncoGel® jsou v současné době využívány jako injekčně aplikovatelné nosiče s postupným uvolňováním léčiv, zejména při léčbě cukrovky nebo onkologického onemocnění. Nicméně PLGA−PEG−PLGA triblokový kopolymer může být použit I jako polymerní nosič anorganického léčiva případně jako biodegradabilní implantát v dentálních či ortopedických aplikacích. Z toho důvodu byl vybrán anorganický biokompatibilní hydroxyapatit (HAp) pro své majoritní zastoupení v tvrdých tkáních.

Experimentální část je zaměřena na přípravu HAp/PLGA−PEG−PLGA kompozitů, ve kterých je HAp buď ve formě nano- (n-HAp) nebo „core-shell“ částic (CS). Nové CS částice, připravené dvouemulzní metodou, jsou složeny z „tuhého“ HAp jádra obaleného termocitlivým kopolymerem, který je navíc funkcionalizován kyselinou itakonovou (ITA/PLGA−PEG−PLGA/ITA). Funkcionalizace pomocí ITA vnáší do původní struktury kopolymeru jak síťovatelné dvojné vazby, tak i koncové karboxylové skupiny. Volné karboxylové skupiny na koncích ITA/PLGA−PEG−PLGA/ITA kopolymerního obalu byly dále zesíťovány za vzniku 3D chemické sítě (CS-x), jejíž životnost je řízena a kontrolována. ATR-FTIR spektroskopie prokázala přítomnost „nových“ esterových vazeb vzniklých karbodiimidovou reakcí –OH a –COOH skupin, kterým náleží adsorpční pásy ve vlnové délce 1021 cm^{-1}.˙ n-HAp a CS-x částice byly přidány do kopolymerní termocitlivé matrice (PLGA−PEG−PLGA) za účelem charakterizace jejich reologického chování. Bylo zjištěno, že pokud bylo do polymerní matrice přidáno méně než 10 hm. % CS-x částic a jen 5 hm.% n-HAp kompozit si zachoval své termocitlivé vlastnosti. Na druhou stranu, přídavek vyššího množství HAp částic do polymerní matrice zajistil změnu vodného polymerního solu v permanentní gel při teplotě nad 37 °C. Analýza ICP-OES prokázala rychlejší uvolňování CS-x částic z 10 hm/obj. % PLGA−PEG−PLGA polymerní matrice do inkubačního média (6 % 9. den) než tomu bylo u n-HAp částic (jen 3 %), které jsou vázány více v micelární struktuře kopolymeru. Proto, kompozit na bázi n-HAP částic tvořící tuhý trvalý gel při tělesné teplotě, je vhodný více jako biologicky rozložitelné kostní lepidlo, zatímco kompozit z CS-x částic a termocitlivého kopolymeru je vhodný jako nosič léčiv pro injekční aplikace.

Klíčová slova

termocitlivé triblokové kopolymery, hydroxyapatit, uvolňování léčiv, reologie, core-shell částice

CHAMRADOVÁ, I. *Polymeric materials for controlled drug delivery and controlled release of active substances.* Brno University of Technology, Faculty of Chemistry, Institute of Materials Science, 2015. p. 102, supervisor doc. Ing. Lucy Vojtová, Ph.D.

Declaration

I declare that the doctoral thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete.

.....
student's signature

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1 INTRODUCTION

In 1986 the Consensus Conference of the European Society for Biomaterials defined a biomaterial as "a nonviable material used in a medical device intended to interact with biological systems" [1]. Biomaterials include a large and diverse array of materials that range from metallic orthopedic implants to polymeric materials which could be from natural or synthetic sources.

Advantage of biodegradable synthetic copolymers/polymers compared to natural polymers is the ability to tailor copolymer composition, mechanical properties, degradation kinetics of polymer and so control release of agents [2]. These synthetic polymers as resorbable sutures were firstly presented in 1960s [3]. The most studied groups are poly(α-hydroxy acids) such as poly(D,L or L-lactic acid) (PLA), poly(glycolic acid) (PGA), polycaprolactone (PCL) and their copolymers. Copolymerization of hydrophobic PLA, PGA or PCL with hydrophilic poly(ethylene glycol) forms the amphiphilic copolymer with thermo-sensitive properties which undergoes phase transitions (sol-gel and gel-suspension) depending on the surrounding temperature. The sol-gel transition is very attractive for medical applications because the bioactive agents (e.g. inorganic components, drug and other healing medicaments) can be mixed in the aqueous copolymer solutions at laboratory temperature (sol phase). After injection of copolymer into the body the “flowing” sol converts to stiff gel having entrapped drugs in its 3D hydrogel network [2, 4]. Synthetic copolymer may be tailored by functional groups or peptides and so provide rate-controlled release or target specificity in cancer treatment, especially.

Nowadays, on the market is unmet demand for new products in bone regenerative medicine. The lack of materials can be solved by thermosensitive copolymer. The goal of presented thesis was preparation of the hydroxyapatite/thermosensitive copolymer composites which are liquid at laboratory temperate and gels at body temperature *in situ*. The resulted hydroxyapatite composite with paste-like viscosity may be used in treatment of comminuted fracture or as filling material for bone cavity. The second aim of this inorganic-polymer composite was preparation of drug delivery carries in form of core-shell particles (CS-x). The CS-x/copolymer composite is able to form a temporary biodegradable implant *in situ*. By the degradation of copolymer matrix release the CS-x and treat directly at the site of the diseased tissue.

2 THEORETICAL PART

2.1 Biomaterials for medical applications

Biomaterials are those materials intended to interface with biological systems to replace, reconstruct, enhance or support either tissues or tissue function. Biomaterials have typically been *inorganic materials* such as metal, ceramic, glass, bioceramics and *organic materials* (e.g. polymers/copolymers) [5]. Bioceramics, especially hydroxyapatite, is widespread in bone regenerative medicine. The calcium cations are necessary for remodeling of damaged bone. At first, calcium crystals are removed from the bone skeleton by osteoclast cells and subsequently are used via osteoblast cells to produce new bone tissue. One have proved that the materials for bone tissue engineering having calcium-compounds in its structure shorten the recovery time [6].

2.2 Inorganic materials

Metals are more suitable for load-bearing applications compared with ceramics or polymeric materials due to their combination of high mechanical strength and fracture toughness. The most widely used metal material is stainless steel, titanium and cobalt–chromium-based alloys. A limitation of these current metallic biomaterials is the possible release of toxic metallic ions and/or particles through corrosion or wear processes (metal implants in medicine) [7, 8].
Ceramics and bioglass can be manufactured in porous scaffolds, powders and granules or in the form of coatings. Although the ceramics' implants are biocompatible, the body will react against them for their foreign nature and these ceramics are surrounded by collagen capsule which is isolated from the body [9]. The most commonly used inorganic material is hydroxyapatite (HAp).

2.2.1 Hydroxyapatite

Hydroxyapatite (HAp) - $Ca_{10}(PO_4)_6(OH)_2$ belongs to the family of calcium phosphate cements (CPCs) which were discovered in the 1980s by Brown and Chow [10] and LeGeros [11]. All CPCs are formed by combination of one or more calcium orthophosphate powder. CPCs developed up to now have only two different products, precipitated *hydroxyapatite* (HAp) or *brushite* (dicalcium phosphate dihydrade - DCPD) (Fig. 1) [12].

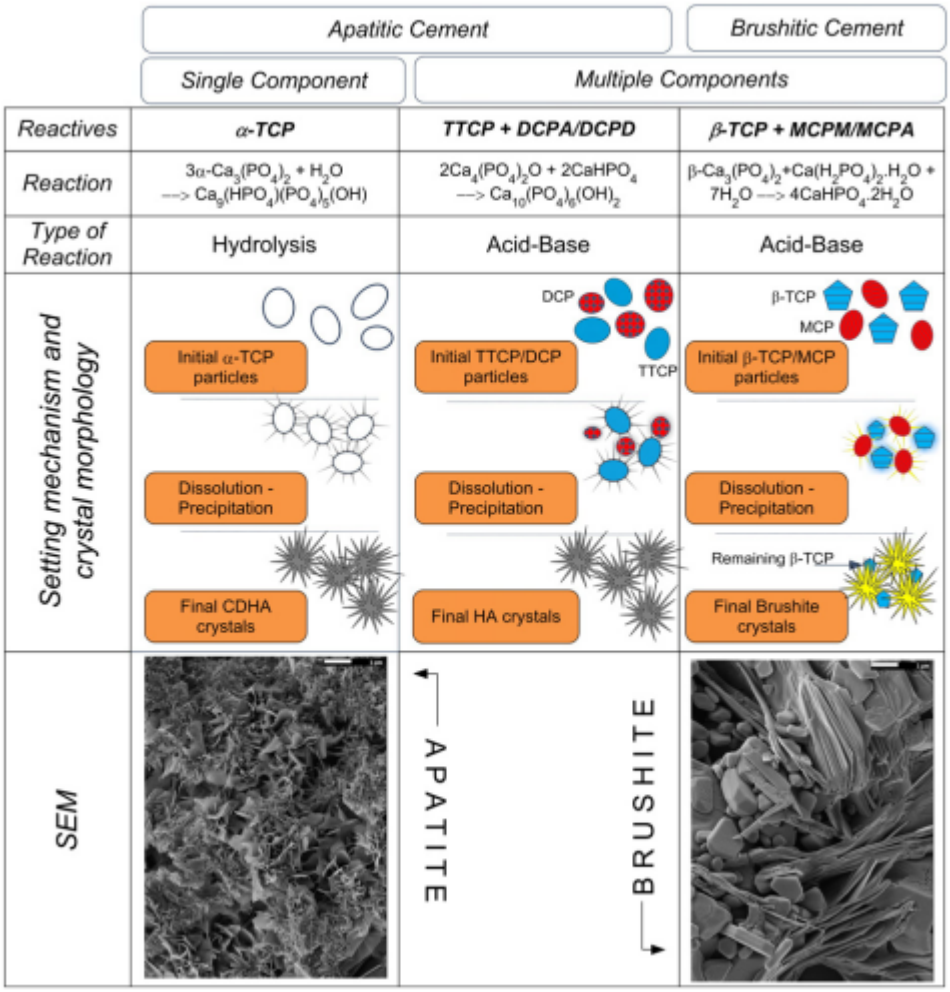

Fig. 1. Classification of calcium phosphate cements [12].

HAp can be prepared from biological sources (animal bones and corals) or can be synthetized from appropriate substrates [13, 14, 15]. Nowadays CPCs are widely used for reconstruction or regeneration in bone tissue engineering or coating of metal materials due their degradability, bioactivity, excellent biocompatibility, osteoconductivity and potential osteoinductivity. The first commercial CPCs products were introduced in the 1990s for treatment of maxillo-facial defects as well as for treatment of fracture [16, 17].

Synthetically produced HAp is preferable for their controlled microstructure and uniform composition. Extensive researches have been carried out to prepare HAP in powder form, thin films and by using gel-casting techniques to obtain pieces with complex shapes

2.2.1.1 Hydroxyapatite in medical applications

Hydroxyapatite is one of the most used bioceramic materials in the field of biomaterials and tissue engineering, because it is major mineral constituent of the bone matrix. Hydroxyapatite particles can be synthetized by variety of methods including solid state methods [18], wet chemical methods [19] or can be prepared by hydrothermal process [20] Preparation conditions have significant influence on crystallinity, Ca/P ratio and stoichiometric value of HAp [21]. Hydroxyapatite is used in combination with polymer matrices (biodegradable or non-degradable). The attention is paid to preparation on composites with individual (nano)particles without aggregation. To overcome the problem with agglomeration of HAp is

surface modification. The purpose of the surface treatment is not only to guarantee the even distribution of HAp particles but also to prevent or delay the debonding process of HAp particles from the polymer matrix [22]. It is very important to controlled interphase between polymer matrix and inorganic bioactive filler. However, surface modifiers must satisfy several requirements such biocompatibility or non-toxicity [23]. Table 1 summarized several modification of HAp.

Table 1. Surface modification of HAp

Surface modification	**References**
Hexanoic decanoic acid	Tanaka et al. [24]
Oleic acid	Kim et al. [25]
Stearic acid	Li et al. [26]
Hexamethyldisilazane (HMDS)	Tanaka [27]
Vinyl triethoxy silane	Wen at al. [28]
γ-aminopropyltriethoxysilane,	Furuzono et al. [9]
γ-methacryloxytrimethylsilane	Damia et al. [29, 30]
3-trimethoxysilylpropylmethacrylate	Wang et al. [31]
L-lactide	Hong et al. [32]
Polyacrylic acid (PAA)	Liu et al [33]

In most of these techniques, the modified agents were reacted with the hydroxyl groups (P-OH) on the surface of the HAp crystals [24−30]. The surface of HAp is often treated by polymer grafting (PAA, L-LA) to improve the interfacial condition [32, 33]. The surface of HAp containing OH^-, Ca^{2+}, PO_4^{3-} is attractive due their ability to form ionic interaction with drugs.

2.3 Polymers

The next group which is often used in biomaterials devices is polymer or copolymer. Polymers used in tissue engineering have to be biocompatible with low toxicity to the human (or animal) body. These polymers could be classified based on their biodegradability as fully or partially resorbable and nonresorable. *Resorbable polymers (or copolymers)* could be used for internal fracture fixation applications with degradation inside the body in a controllable rate or such as drug delivery carriers (polyesters). *Partially resorbable* polymers could be composed of a non-absorbable reinforcing materials and fully resorbable matrix materials (e.g. PLA with HAp, poly(hydroxybutyrate (PHB)/alumina or calcium carbonate). *Non-resorbable* biocomposites provide specific mechanical properties and stability (knee joint prostheses, dental posts, stems of hip) (e. g. carbon fiber/poly (ether ether ketone)) [9, 34].

The advantages of polymeric biomaterials, compared to metallic or ceramics materials, are simple manufacturing of orthopedics products in various shapes, adequate cost and wide range of physical and mechanical properties [12].

2.4 Biodegradable polymers

This class of polymeric materials can be synthetic or natural origin. They are capable of being cleaved into biocompatible byproducts through chemical or enzyme-catalyzed hydrolysis. This biodegradable property makes it possible to implant them into the body without the need of subsequent removal by the surgical operation. Biodegradable polymeric materials have been found to present innumberable applications, from surgical sutures and surgical glues to contact lenses, heart valves or drug delivery carriers [36, 37]. The release rates of the drugs from biodegradable polymers can be controlled by a number of factors, such as biodegradation kinetics of the polymers [16, 38] physicochemical properties of the polymers and drugs [39, 40], thermodynamic compatibility between the polymers and drugs [41] and the shape of the devices [42, 43].

2.4.1 Natural polymer

Natural polymer materials are derived from the proteins such as collagen, gelatin, silk fibroin and the polysaccharides such as cellulose, hyaluronate, chitosan or alginate. Although of natural polymer are known as biocompatible, but there are same disadvantages such degradation rate or deficiency in bulk quantity. They have been used as scaffolds for repair of nerves, cartilage or bones [19].

Collagen occurs as major component of connective tissue (such as bones, cartilage or blood vessels), giving strength and flexibility. It is one of the most applied scaffolds for tissue engineering and it is used in vitro for the culture of many different types of cells. The composite of collagen and hydroxyapatite is used as biodegradable synthetic bone graft replacement [44]. *Hyaluronic acid* is a glycosaminoglycan found in nearly every mammalian tissue and fluid. This material can be chemically cross-linked or combined with other materials in order to obtain the desired mechanical properties. It has been used in many different applications (cosmetics industry or for the treatment of osteoarthritis). *Alginate* is a polysaccharide obtained primarily from seaweed. It is widely used as cell transplantation vehicles to grow new tissues as well as wound dressing or drug stabilization for delivery applications. *Chitosan* is derived from chitin. This polysaccharide and its derivate is suitable candidate for scaffold materials for various tissue engineering including cartilage, skin, and bone [45].

2.4.2 Synthetic polymer

Synthetic polymers are suitable for tissue engineering. They can be reproducibly produced with specific molecular weights, composition of block structures, degradable linkages and cross-linking modes. These properties in turn, determine gel formation dynamics, cross-linking density and material mechanical and degradation properties.

Aliphatic polyesters have attracted significant interest as drug carriers. This class of polymers degrades via the hydrolytic cleavage of the ester bonds in their backbone. The degradation rate strongly depends on the crystallinity, hydrophobicity and molecular weight of the polymer. For aliphatic polyester is typical bulk erosion. This group includes polymers (or copolymers) such as poly(lactic acid) (L-LA or D,L-LA), poly(ethylene glycol) (PEG), poly(glycolic acid) (PGA), poly(ε-caprolactone) (PCL) [46, 47, 48].

Polyanhydrides are the hydrophobic polymers with hydrolytically labile anhydride linkages. In general, this class of polymers show minimal inflammatory reaction in vivo and degrade into monomeric acids as non-mutagenic and non-cytotoxic products [49].

Another group of synthethic polymers which are commonly used in medical applications are **polyphosphazenes.** They are typically synthesized as linear polymers, composed of an inorganic backbone with nitrogen and phosphorous atoms. They can degrade by both surface and bulk erosion, depending on the lability of the bond and hydrophobicity of the polymer [50] (Fig. 2).

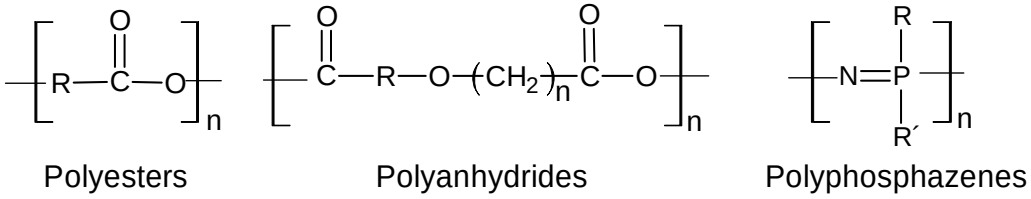

Fig. 2. Formulas of polyesters, polyanhydrides, polyphosphazenes.

Design of prepared polymers/copolymers strongly dependents on the applications use and environment into which the scaffold will be placed. Nowadays are known shapes as foams, hydrogels, films, porous scaffolds, wounds, fibrous scaffolds, etc. For example, hydrogel (prepared from polyesters) was designed as the drug carries, biological glue or cells-carries, therefore must be capable of being gelled without damage of entrapped cells, degradation products have to be nontoxic, has appropriate diffusion and require mechanical integrity and strength [51].

2.4.3 Temperature-responsive hydrogel

Hydrogel is three-dimensional network composed of a polymer backbone and do not dissolve in water at physiological temperature and pH. Hydrophilic polar groups such as -OH (hydroxylic), -COOH (carboxylic), -CONH (amidic), $-CONH_2$ (primary amidic), $-SO_3H$ (sulphonic) present in hydrogel are capable of absorbing water (or body fluids) without undergoing dissolution. They swell considerably in an aqueous medium and quantity of water into polymeric network structure moving over 20 % [52, 53, 54]. Responsive hydrogels are a class of hydrogels with swelling properties dependent on environmental factors like pH, temperature, ionic strength, electric current and are known as “stimuli-responsive” or “smart” gels [55, 56, 57, 58]. The thermo-responsive hydrogels are mainly prepared from synthetic

polymers, but some natural polymers like xyloglucan may also form thermoreversible gels [59]. Hydrogels do offer several advantageous properties such as biocompatibility, biodegradability and biologically recognizable moieties that support cellular activities. Biodegradable products as well as their metabolites have to be non-toxic and non-carcinogenic.

Hydrogels have many different functions in the fields of tissue engineering e.g as space filling agents, as delivery vehicles for bioactive molecules (or drugs), and as three-dimensional structures that organize cells. Hydrogel is especially attractive due to their minimally invasive delivery procedure, providing reduced healing time, decreased risk of infection and ease of delivery compared with surgically implanted materials [60].

2.4.3.1 Composition of temperature-responsive hydrogel

Multiblocks temperature-responsive synthetic hydrogels are often consisting of a hydrophilic block A (PEG) and hydrophobic block B (PLLA, D,L-LA, PCL). Molecules having both hydrophilic and hydrophobic segments are known as amphiphilic molecules. Hydrophobic blocks are separated from the aqueous surrounding to form an inner core and hydrophilic segments consist of a wall around it [61, 62] (see Fig. 3).

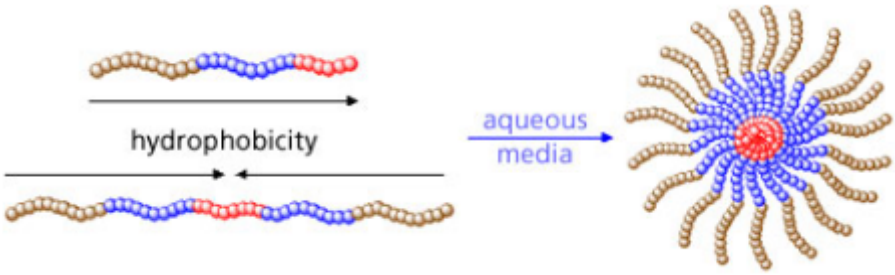

Fig. 3. Amphiphilic molecules in aqueous solution [63].

Temperature-sensitive hydrogels are probably the most commonly studied class of environment-sensitive polymer systems in drug delivery research. These hydrogels are able to swell or deswell as a result of change in the temperature of the surrounding fluid. The polymer solution is a free-flowing liquid at lower temperature and displayed low viscosity. As the temperature increases polymers undergo sol-gel transition, after that can be observed a gel (transparent, opaque or white) which exhibited viscoelastic behavior. At higher temperature occur gel-suspension phase transition and the gel loss elasticity (polymer are separated from the water) (Fig. 4)

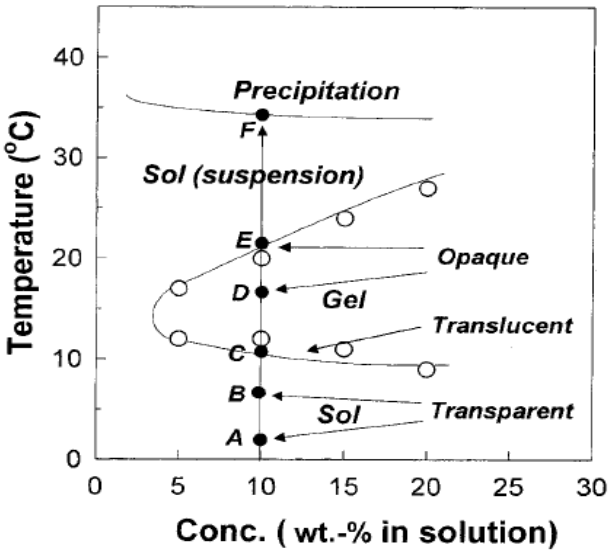

Fig. 4. A typical phase diagram of ABA-type PLGA-block-PEG-block-PLGA triblock copolymer in water [64].

The blocks of copolymers may have different architectures such as AB diblock or ABA/BAB triblock. Lee and co-workers prepared thermo-responsive copolymers consisting of PEG/PLLA (AB) and study their rheological properties using dynamical rheological analysis and by test tube inverting method. This copolymer exhibited a reverse thermal gelation in a temperature range from 30 to 45 °C [65].

The ABA-type triblock copolymers with central PEG demonstrated an interesting reversible sol-gel and gel-suspension transition in aqueous solution (see Fig. 5). Lee and Shim [64, 65] studied the solubility of these triblock copolymers with central PEG (M_n = 1000 g·mol^{-1}) block with different molecular weight of PLGA block (from 900 to 1600 g·mol^{-1}). The PLGA with higher mol. weight was insoluble in water, while triblock copolymer with PLGA block (M_n = 900 g·mol^{-1}) and lower was soluble in water but do not form hydrogel in water. The solubility of polymer depends strongly on the molecular weight of PLGA block.

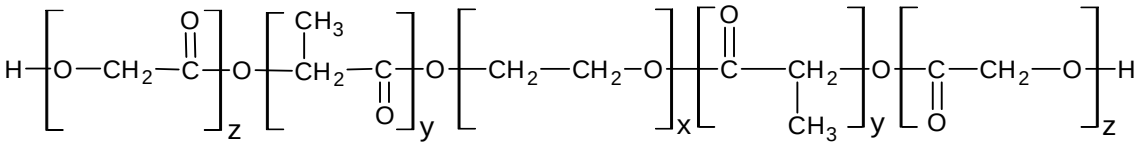

Fig. 5. Formula of ABA-type triblock copolymer PLGA—PEG—PLGA; x, y, z are numbers of poly(ethylene glycol), lactide and glycolide.

Effects of precipitated agents (methanol, hexane, diethyl ether) on copolymer (PLGA-PEG-PLGA) were investigated by Yu and co-worker. Option of precipitated agents exhibit changes in elastic modulus (G´), critical micelles concentration (CMC), micellar size and macroscopic states in water (some were sols, some were precipitates and some underwent sol-gel transition upon heating) [66].

Not only molecular weight, precipitated agents, but temperatures of polymerization change properties of PLGA-PEG-PLGA. The synthesis was performed at 130 °C or 160 °C and degradation rates were studied in phosphate buffer saline (PBS). Thermogels exhibited two optical states (transparent or opaque) and have similar mol. weight and the same ratio of PGA and PLA. However, the different gel strength and their change ways with degradation time were found [67].

Habbas et al. investigated rheological properties of triblock copolymers consisting of poly(ethylene glycol)-poly(propylene oxide)-poly(ethylene glycol) (PEG-PPO-PEG) (BAB). These copolymers are able to form micelles at higher temperature and concentration up to 30 wt % [68].

2.4.1 Crosslinking of Biodegradable Copolymers

Hydrogels are often used in controlled delivery systems and therefore is very important to control their degradation rate by crosslinking, composition (ratio of hydrophilic and hydrophobic blocks) and molecular weight. As mentioned, hydrogel is polymeric network, which absorb and retain large amounts of water due to the present of hydrophilic polymer chains in their backbone. Hydrogels are biodegradable and have labile bonds which can be broken under physiological conditions by hydrolysis.

Functional groups added in polymer chains have to be capable of crosslinking and the polymer network became denser. Polymer/copolymer precursors containing double bonds can be cured by thermal, redox- or photoinitiation to form crosslinked product. The final crosslinked polymer is stable in comparison with polymer without crosslinkable bonds. The Table *2* summarized the functional groups useable in polymer or copolymer modification.

Table 2. Crosslinkable agent used in functionalization of biodegradable polymers.

Polymer/Copolymer	**Funcionalization by**	**Reference**
D,L-LA	isocynates, methacrylates	Storey [69, 70]
PEG-PGA	acrylates, methacrylates 2-isocyanatoethyl methacrylates	Bencherif [71]
PEG-PCL	itaconic anhydride	Ramos and Huang [72]
PPF	acrylates, epoxides	Peter [73]
PCL	(3-isocyanatopropyl)triethoxysilane	Matsuda [74, 75]
PCL	itaconic anhydride	Turunen [76]
PLGA-PEG-PLGA	itaconic anhydride	Michlovská [77]

Aliphatic polyester have been widely investigated for biomedical applications, therefore they are tailored by introducing functional groups as carboxyl, hydroxyl, amino or double bonds [77]. Block of copolymers containing more functional crosslinkable groups on the hydrophobic block (PLA, PGA, PCL) allow for crosslinking the core of the micelles, while when the functional groups are situated on the hydrophilic block (PEG) they might form crosslinked shell (see Fig. 6) [78] .

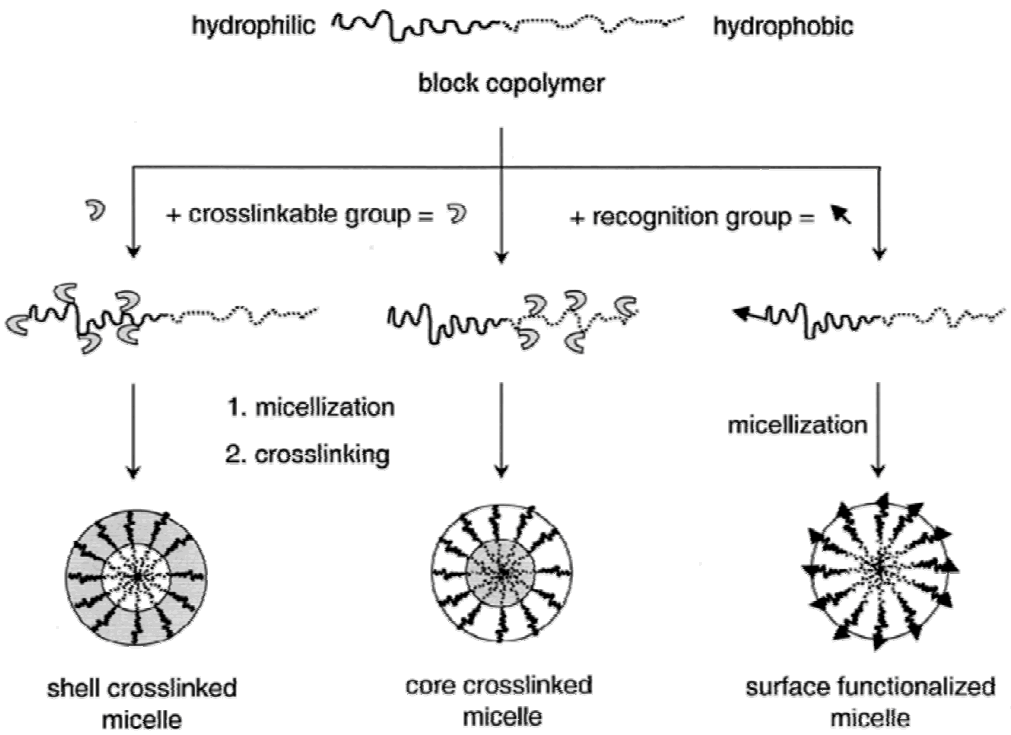

Fig. 6. Schematic representation of the three different classes of functional amphiphilic block copolymers - shell crosslinked micelles, core crosslinked micelles or surface functionalized micelles [79].

Chemical fixation of micelles by crosslinking of either core or shell is very interesting for a number of reason such as increasing the stability of the micelles, circularization times, allow drugs to be administered over longer periods of time [79].

2.4.1.1 Functionalization by Itaconic Anhydride (ITA)

Functionalization of triblock copolymer (PLGA-PEG-PLGA) by ITA was recently published by our group [77]. ITA can be obtained from renewable resources, either by distillation of citric acid or by pyrolysis of 2-methylidenebutanedioic acid (itaconic acid) which can be prepared by the large-scale fermentation of polysaccharides (e.g. molasses) by *Aspergilus terreus.* The degradation of ITA was studied by Adler and degradation products were detected as acetate, lactate and carbon dioxide [80]. ITA introduces carboxyl groups and double bond in their structure. Carboxyl groups at the end of ITA/PLGA-PEG-PLGA/ITA can cause physical crosslinking and double bonds can be further crosslinked chemically by covalent bonding (e.g. using photopolymerization) (Fig. 7). Both chemical and physical crosslinking produce a new strength high density 3D-hydrogel network [81, 82].

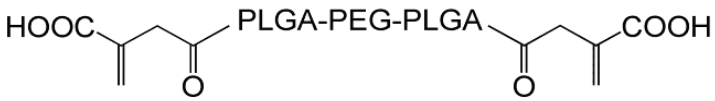

Fig. 7. Formula of ITA/ PLGA-PEG-PLG/ITA triblock copolymer.

2.5 Controlled drug delivery

The first idea of controlled release from polymers dates to the 1960s through the employment of silicone rubber and polyethylene [83], but the lack of biodegradability in presented systems implies the requirement of eventual surgical removal and limits their applicability. In the 1970s biodegradable polymers were suggested as appropriate drug delivery materials circumventing the requirement of removal [84]. Over the past 25 years a lot of researches have also been focused on degradable polymer microspheres for drug delivery. In present day hydrogel seems to be a suitable candidate for drug delivery system.

Present conventional oral drug administration does not usually provide rate-controlled release or target specificity. Moreover, these drugs provide sharp increase of drug concentration at potentially toxic levels, following a relatively short period at the therapeutic level and finally rapid decrease in concentration (Fig. 8). The drug activity has to remain between a maximum represent a toxic level and a minimum value below which the drug is no longer effective.

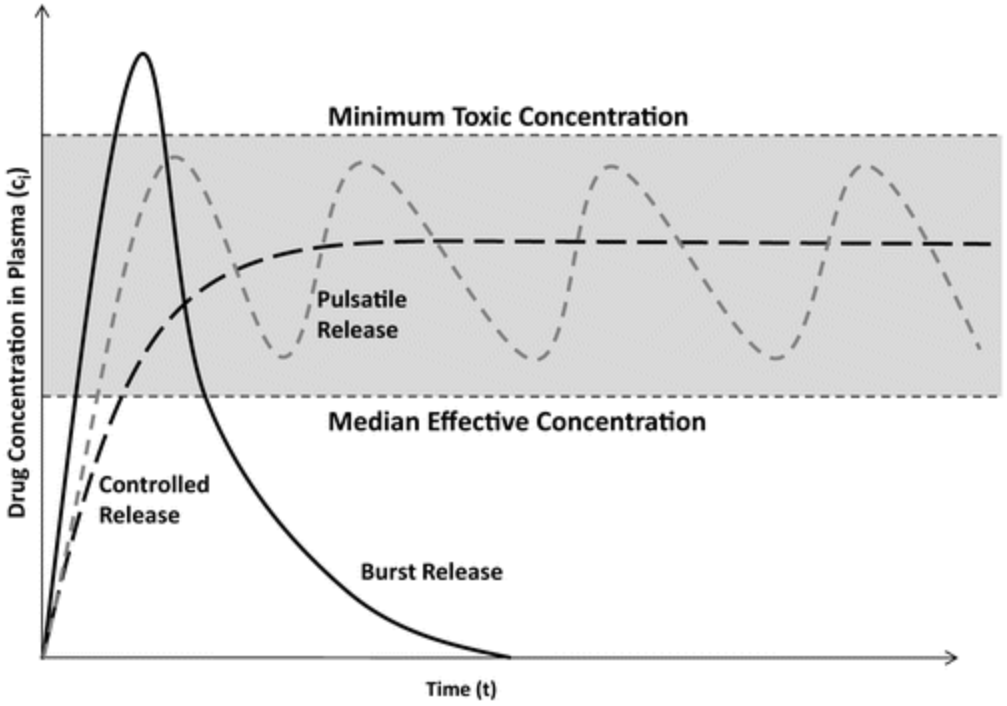

Fig. 8. Therapeutic band showing impact of burst release, pulsatile release, and controlled release relative to effective and toxic concentration [85].

Controlled administration is constant between the desired maximum and minimum for an extended period of time. Controlled drug carriers often combine polymer/copolymer with a drug or active agent. Degradation of polymer/copolymer part of drug carries to ensure gradually release of active agents [85].

2.5.1 Mechanism of Drug Release from Polymer Matrix

The term “release mechanism” has been defined as a description of the way in which drug molecules are transported or released. Knowledge of the release mechanisms and the physico-chemical processes that influence the release is vital in order to develop controlled-release drug delivery systems (DDSs). The drug has many of possible ways to be released from

delivery system. The release mechanisms of PLGA-based DDSs were studied by Fredenberg at el. Water is absorbed by polymer immediately upon immersion in water or administrative *in vivo.* Fig. 9 shows physico-chemical process in PLGA matrices [86]. **Hydrolysis** starts immediately upon contact with water, cleave the ester bonds and subsequent decrease in M_w. Hydrolysis creates acids, which catalyzed hydrolysis (auto-catalytic phenomena) [87] and caused faster degradation especially inside of the polymer than at the surface [88]. **Erosion** starts when the dissolved polymer degradation products are able to diffuse into the release medium. In this way polymer lose their mass.

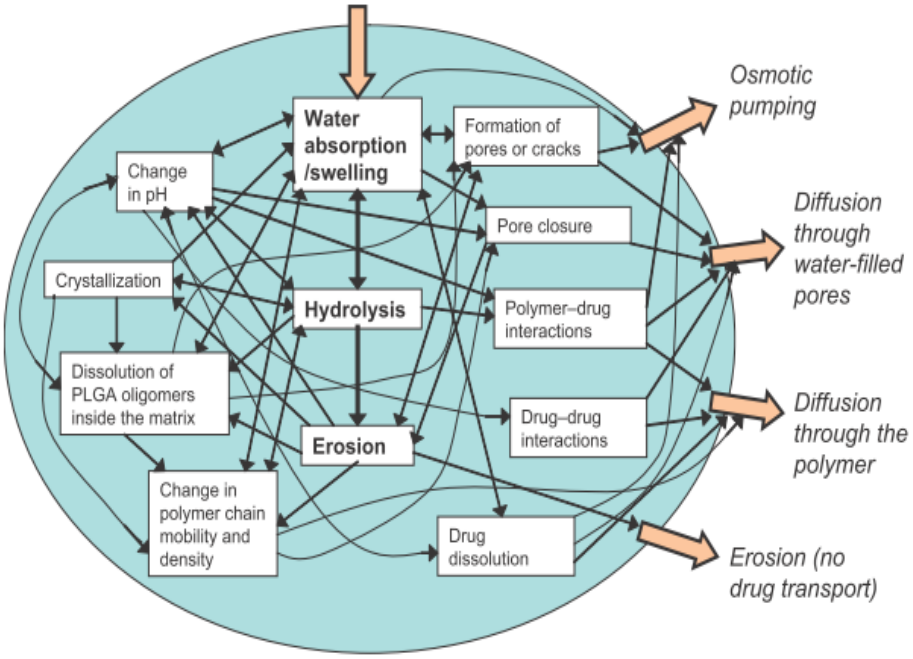

Fig. 9. The complex pictures of physico-chemical processes taking place within PLGA matrices [86].

The encapsulated drug may affect many of the process in polymer matrix. Properties of drug (hydrophilicity/hydrophobicity) can change the original properties of polymer. Incorporated drug enhanced or inhibited water absorption and hydrolysis, increased/decreased rate of hydrolysis due to acid or base neutralization, drug-drug interaction and degree of encapsulated substance. The release of drug depends on properties of polymer, drug and their interactions. The Fig. 10 shows the complex factors that influence drug release from PLGA matrices.

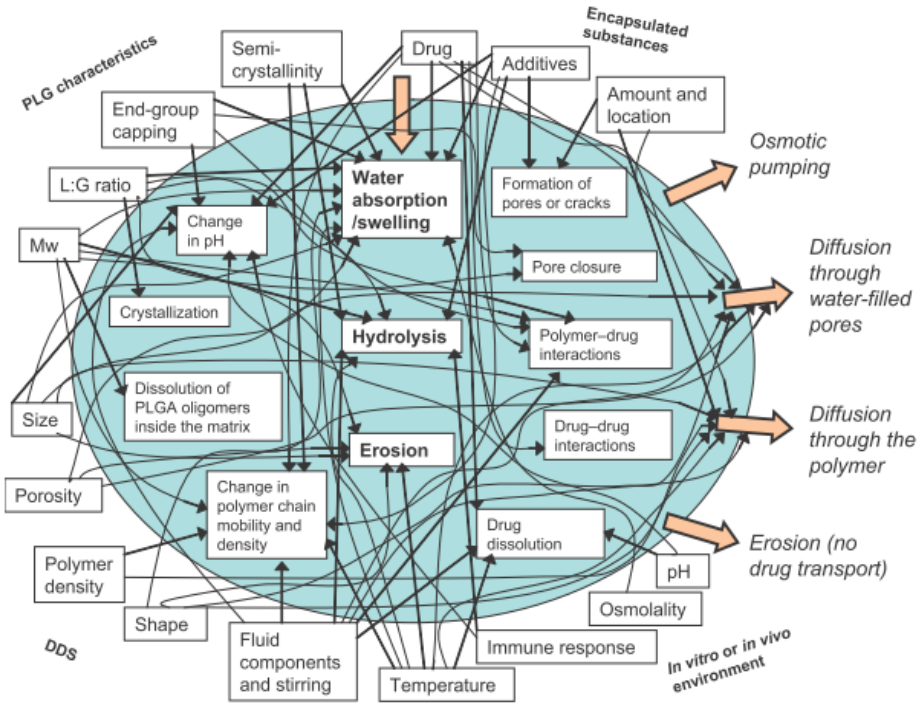

Fig. 10. The complex factors which influence drug release from PLGA matrix in the presence of drugs [86].

2.5.2 Release Profiles of Drugs

Fredenburg at al. described release profiles consisting of different phases. Mono-phasic release is very rare, however bi-phasic or triphasic profile is most common (see Fig. 11). Large particles often exhibit tri-phase system due to heterogeneous degradation. Small particles or coated particles exhibit often bi-phasic release profile.

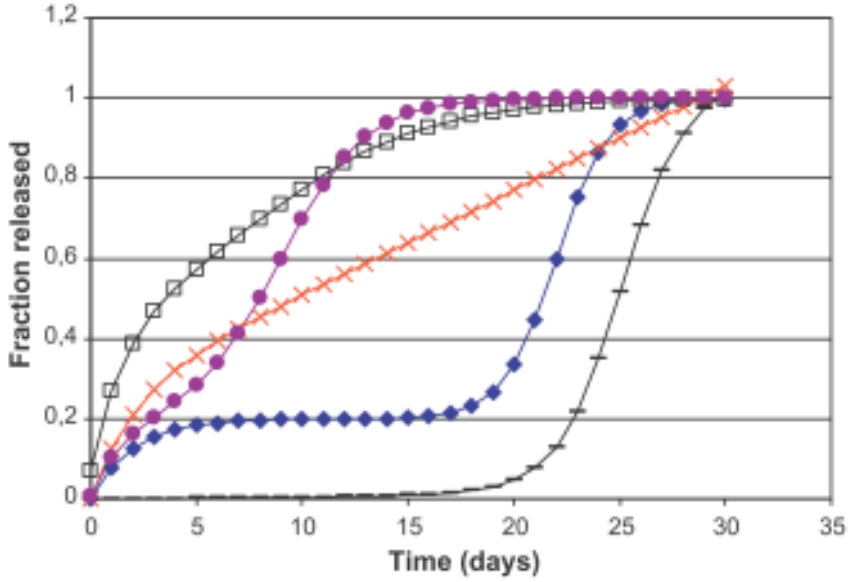

Fig. 11. Release profiles. Open square: burst and rapid phase II. Filled circles: tri-phasic release with a short phase II. Crosses: burst and zero-order release. Filled diamonds: tri-phasic release. dashes: bi-phasic release - similar to tri-phasic but without burst release [86].

Phase I in the tri-phasic release profile is usually connected with burst release and has been attributed to non-encapsulated drug particles on the surface and so they are accessible to hydration [85]. *Phase II* is often a slow release phase, during which the drug diffuse slowly. In this phase polymer starts to degrade or hydrate. *Phase III* is usually a period of faster release, often attributed to the onset of erosion. This phase is sometimes called the *second burst* [89]. For the analysis of released drugs and characterization of release profile high-performance liquid chromatography (HPLC) or ultraviolet–visible spectroscopy (UV-VIS) were used. In the Table 3 there are shown the analytical methods for different drugs incorporated in PLGA-PEG-PLGA.

Table 3. Methods used for analysis of released drug.

copolymer	drugs	analysis	references
PLGA–PEG–PLGA	doxorubicin	UV-VIS spectroscopy	Yu et al. [90]
PLGA–PEG–PLGA	isoniazid	UV-VIS spectroscopy	Gajendiran [91]
PLGA–PEG–PLGA	ganclicovir	HPLC	Duvvuri [92]

2.6 Type of Drug Delivery Carriers

In present days, drug carriers such as core-shell particles, spheres, micelles, dendritic polymers (see Fig. 12) are known. Core-shell nanoparticles and nanospheres have spherical structures ranging around 100 nm in size. Core-shell nanoparticles have drug entrapped inside the core. When the drug is entrapped in or adsorbed on the surface of a matrix it forms “nanospheres”. A wide variety of medicines can be delivered using nanoparticles such as drugs, vaccines and biological macromolecules [87, 88].

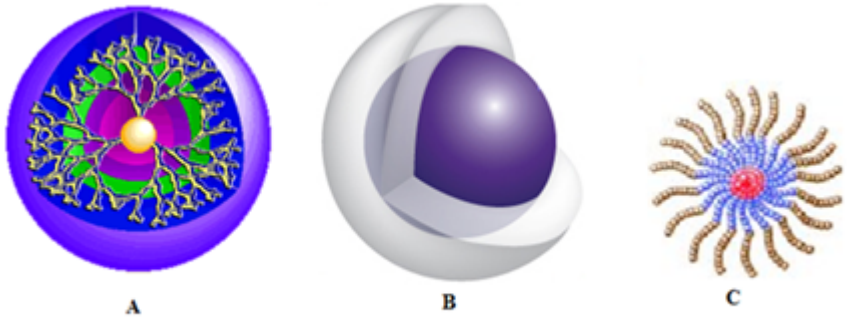

Fig. 12. Schema of (A) denrimers [93], (B) core-shell spheres [94], (C) polymer micelles [63]..

The polymer *micelles* consist of copolymer blocks, which are self-assemble into spherical micelles in water. A polymer micelle is amphiphilic and has usually a diameter of about 20 - 50 nm [95]. The drug molecules can be trapped in the inner hydrophobic core while the outer shell is hydrophilic and soluble in aqueous media. However, below a critical micelle concentration (CMC), the polymeric aggregates dissociate into free chains, leading to the sudden release of the drug [96]. *Dendrimers* are synthetic, highly branched, spherical, mono-disperse macromolecules of nanometer dimension. The most studied class of dendrimers

investigated for drug delivery is the polyamidoamine (PAMAM). Dendritic polymers were first synthetized by Tomalia and Newkome in 1980s. These dendritic micelles contain hydrophobic interior and hydrophilic surface functionality to overcome problem with CMC [97].

The major problems of some drugs are their low solubility in aqueous solution. Therefore their encapsulation in the PLGA-PEG-PLGA copolymer was crucial [98]. Gajendiran at el. have used hydrogel of poly(ε-caprolactone-co-glycolide)-poly-(ethylene glycol)-poly(ε-caprolactone-co-glycolide) [P(CL-GL)-PEG-P(CL)] triblock for in vitro release of isoniazid (INH) [91]. INH was encapsulated in shell based on PLGA-PEG-PLGA using W/O/W emulsion method by Lin et al.

2.6.1.1 Target specificity useable in cancer chemotheraphy

The surface of drug delivery carriers can be functionalized by „receptors“ e.g. peptides, antibodies, proteins, polysaccharides, glycolipids and glycoproteins (Fig. 13). Particles functionalized by receptors are known as *targeting* and have been used in chemotherapy. Chemotherapeutic agents damage healthy tissues, leading to systemic toxicity and adverse effects that greatly limit the maximum tolerated dose of anti-cancer drugs and thus restricts their therapeutic efficiency [98, 99].

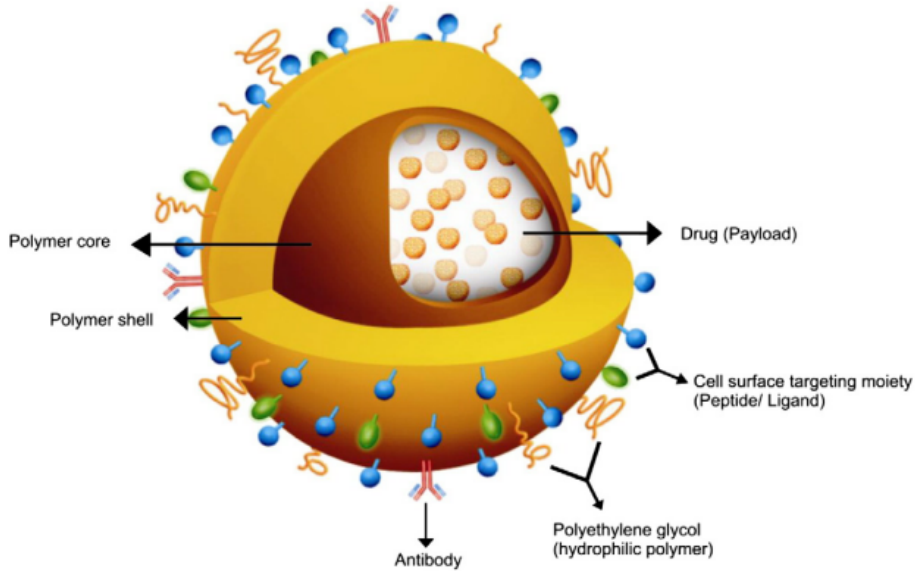

Fig. 13. Schema of multifunctional polymeric nanohybrid devices for targeted drug delivery [99].

Gold nanorods or rod-shaped gold nanoparticles are often used in cancer treatment - as contrast agents for in vivo bioimagination and as thermal converters for photothermal due therapy photothermal effect [100]. The present of gold nanoparticles in human body cause immunogenic reaction and are captured by specific cells of the body. Therefore, the drug is coated with polymeric materials that do not cause an immunogenic response [101].

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2.6.2 Biological barriers for drug carries

The common types of drugs are often detected in body like foreign substances and are cleared by the reticuloendothelial system (RES). RES are known as Kupffer cells in liver and they are primary cellular component of the scavenging system of the body and very effectively clear bacteria, colloidal particles and foreign from the body [102]. Therefore, drug entrapped into hydrophilic/amphiphilic polymer have “invisible” manner for RES and thus prolonged circulatory in the body.

For example, in mice intravenously injected with unprotected colloidal gold nanoparticles (gold particles containing drugs on its surface), was observed that 90-95 % of these gold nanoparticles are cleared from the circulation in 5-10 min after injection [103].

2.6.1 Storage of (nano)particles

The particles made from degradable polymers/copolymers are often stored in dry form for greater stability. Thus, the nanosuspensions are lyophilized and kept at low temperature (in frigde). Sometimes it is necessary to control the relative humidity to avoid hydrolysis [104].
Particles which are retained in the solution should be stabilized by a suitable stabilizing agent, because colloidal dispersions are unstable system due their high interfacial energy [105].

2.7 Determination of DDC parameters

It is necessary to known parameters (pacticles size, distribution, shape or thickness of the shell) of prepared DDC for biodistribution. Microscopy is suitable for determination of shape, but cannot be a method for the accurate determination of average shell thicknesses, it only gives an estimate. Microscopy is appropriate to supplement the method as dynamic light scattering (DLS) or small angle neutron scattering (SANS), which provide statistically averaged values. Combination of both methods gives comprehensive information about investigated materials. The Table 4 is the overview of used equipments for characterization of DDC.

Table 4. Equipments used for determination of DDC parameters.

device	type of DDc	polymer	references
SEM	particles	PLGA	Arnold [106]
	core-shell	PLGA-PLA	Zhao [107]
TEM	particles	PLGA-PEG-PLGA	Gajendiran [91]
	micelles	PLGA-PEG-PLGA	Song [98]
FESEM	particles	PLGA-PEG-PLGA	Gajendiran [91]
	core-shell	PLGA	Vukomanovic [108]
	particles	PLGA	Falco [109]
SANS	capsules	PLA	Rübe [110]
DLS	capsules	PLA	Rübe [110]

2.7.1 ^{1}H NMR relaxometry

Relaxometry is based on the relaxation time of water hydrogen, which is strongly dependent on the environmental conditions. This method it is possible to study water in the pores, swelling or solid-water interaction. It seems to be suitable method for studium of nanocomposites or CS particles. The relaxation parameter allows evaluation of the (nano)particles and molecular interaction between both nanocomposite components and its surroundings.

Almeida studied composites of poly(L-lactid acid) (PLLA)/silica or clay by ^{1}H NMR relaxometry. Silica promoted an increase in rigitidy, due to the strong intramolecular forces in comparison of clay which did not cause any significant change in the molecular mobility in the nanocomposites, probably due to non-affinity of the chemical structure [111]. However, this method has been used to study non-invasively the gel microstructure and gelation dynamics of alginates. Relaxation time (T_2) of the water protons in gels is a sensitive indicator of the state of gelation. The changes of relaxation time indicate the changes in structure like gelation, swelling or degradation [112].

2.8 Copolymer/HAp drug delivery carriers

The strategy to prolong drug release has been the preparation of calcium phosphate cements (CPC)/copolymer composites. The amount of HAp is in the range between 0.5 - 6 wt.% Release of the drug depends on the bond between hydroxyapatite and also on the properties of hydrogel (such as crosslinking of hydrogel network, composition of copolymer, molecular weight and degradation kinetic). The possibility to use HAp is not only as carriers for local and controlled supply of drugs carriers but also bone substitutes. Combination of bioactive inorganic material with the sol phase (polymer solution) form viscous moldable paste which in some instances can be injected with minimally invasive procedure (Fig. 14). In this way, they can be filled large or small bone defects, which can be caused by disease, tumor, infections or fractures [113, 114].

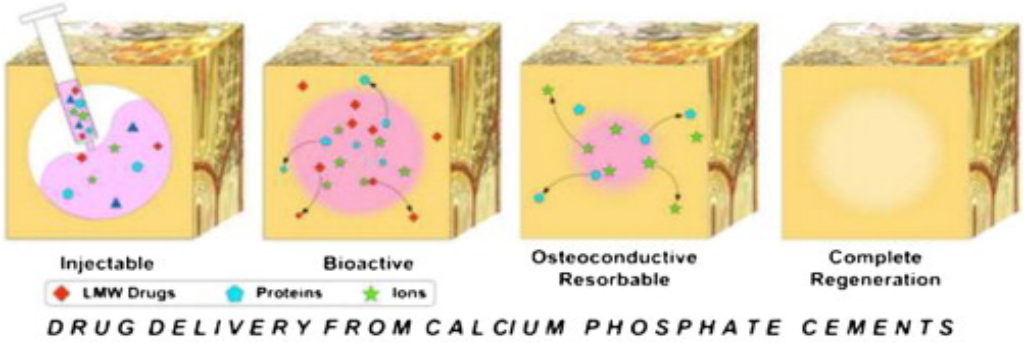

Fig. 14. Drug delivery from calcium phosphate cements [11].

The polymer with the weak mechanical properties changes their original properties with the adding of CPC. Laurencin studied mechanical properties and degradation rate of pure PLGA and PLGA/HAp composites. PLGA exhibited elastic modulus of about 293 MPa, while copolymer modified by 50 wt.% of HAp exhibited 1459 MPa. After six weeks of degradation under physiological conditions, the reinforcing effect had diminished. Elastic modulus of pure copolymer was 10 MPa after six weeks [115]. The advantage of HAp/copolymer is fabrication in various shapes.

Kim et al prepared porous composite (PLGA/HAp) scaffolds by the gas foaming/particulate leaching (GF/PL) and solvent casting/particulate leaching (SC/PL) methods. The fabricated scaffolds were seeded by cells onto the tops of scaffolds. Histological analysis showed bone formation was more extensive on the GF/PL scaffolds than on the SC/PL scaffolds due higher exposure of HA nanoparticles on the scaffold surface [116]. Composites for bone tissue engineering are commonly fabricated in form of the fiber. PLGA/HAp fibers content DNA/chitosan nanoparticles were prepared by electrospinning method. HAp as additive could aid the release of DNA from fibers, enhances cell attachment and protects cells [117].

PLGA/HAp nanoparticles (or suspension) composites have been extensively investigated due their biocompatibility and high drug encapsulating. Wand and co-workers prepared poly(lactide-*co*-glycolide) (PLGA) encapsulated hydroxyapatite microspheres as injectable depot for sustained delivery of antibiotic Doxycycline (Doxy). Encapsulation of HAp into the PLGA shell decreased burst release of antibiotics [118]. Table 5 summarized different CPCs formulation and drugs which have been used in medical applications. All of them exhibited burst release.

Table 5. Low molecular weight drugs incorporated in CPC.

Drugs	kind of drugs	Type of CPC	Liquid phase	References
antibiotics	gentamicin	B	0,9 % NaCl, water	119, 120, 121
		A	water, PAA	122, 123
	vancomycin	B	citric cid	124, 125
		A	solution A	126
	cephalexin	B	Na_2HPO_4, water	127, 128
anti-inflammatory	ibuprofen	B	$Ca(OH)_2$, H_3PO_4	129
	indomethacin	B	(11-20mN) H_3PO_4	130, 131
anticancer	cisplatin	B	solution A	132
	doxorubicin	B	solution A	133
	methotrexate	B	0,05 % H_3PO_4	134
anti-osteoporotic	alendronate	B	Na_2HPO_4	135
other	caffeine	B	water	136
	clorhexidine	A	800 mM citric acid	137

A and B apatite and brushite CPC, solution A: 5 % sodium chondroitin succinate, 0.3 % $NaHSO_4$, 82,7 % H_2O

2.8.1 Mechanisms of HAp-based Drug Delivery Carriers

Calcium phosphate cements provide some benefits for bone tissue engineering such as biocompatibility, proliferation and ability to harden *in vivo*. When liquid phase (water or polymer solution containing drugs) and powder are mixed, progressive dissolution of the ceramic particles takes place and a new mineral phase precipitates. The drug dissolving into the liquid phase is not expected inside in the crystalline lattice of the precipitated crystals, but is entrapped between the entangled crystals (Fig. 15).

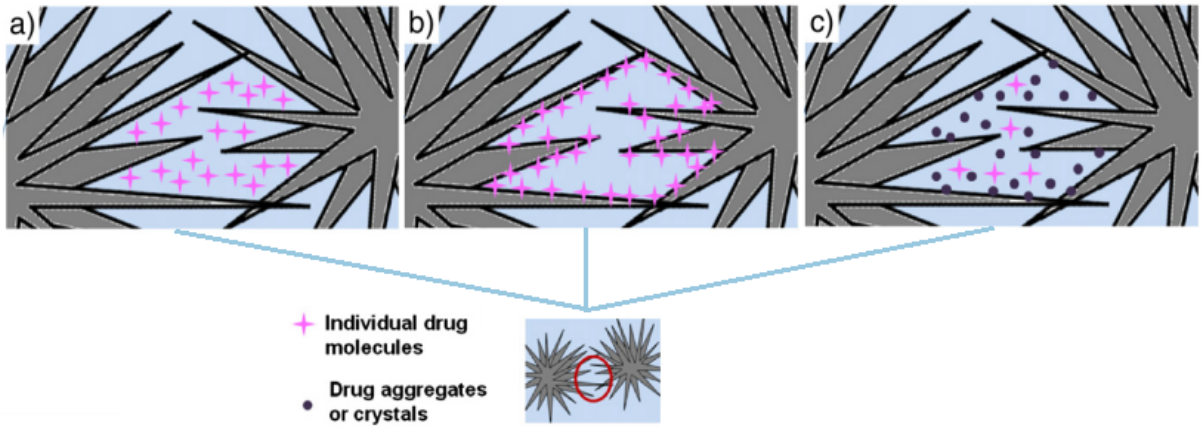

Fig. 15. Schematic presentation of the different ways a drug can be found in CPC matrix (a) as individual molecules dissolved in the liquid within pores (b) absorbed or chemically bonded to the crystal structures (c) drug crystal or aggregates [12].

CPCs (such **as hydroxyapatite**) can be used as drug carriers. Incorporation of drugs to the CPC strongly dependents on size of incorporated drug, microstructures of CPC porosity, surface area, permeability and interaction between drugs and CPC [11].

There are some methods of incorporation of the drug into the CPCs. Usually drugs are incorporated to CPCs by blending drug powder with the solid phase or dissolving it within the liquid phase. In both cases the drug is incorporated throughout the whole volume of the material, although a more homogenous distribution will be achieved when incorporated in the liquid phase. A different approach is to incorporate the drug by impregnation of pre-set CPC solid blocks or granulates with a drug solution.

2.8.2 Thermoresponsive Copolymers/CPC Composites

Some thermosensitive copolymers behave like sol at laboratory temperature and gel at body temperature. Thus polymer aqueous solution could be is easily mixed with drugs, proteins, cells or bioactive substances at lower temperature. While the temperature increases the sol changed into gel and the substances are entrapped in the gel. The physical properties of original thermosensitive copolymers changed significantly with adding of CPC, therefore it is very important to evaluate the changes in viscosity or elasticity.

In 2004 HAp/Fisiograft composite was prepared in three forms: powder, gel and sponge block. Fisiograft is commercial name for copolymer based on the PEG and PLGA (ratio 75:25). It was proved that the most suitable form of material for application in bone defects is a combination of gel and powder, in a weight pro-portion of 50:50 [138].

Lin characterized diblock copolymer (PLGA-PEG) by rheology and investigated the changes in storage modulus (G´) and viscosity of pure hydrogel and hydrogel/HAp composites. HAp significantly affected the magnitude on increasing of G´. The storage modulus of hydrogels increased with increasing HAp content [16].

Triblock copolymer of poly(ethylene glycol) - poly(ε- caprolactone) - poly(ethylene glycol) (PEG-PCL-PEG, PECE) was modified by n-HAp (up to 30 wt %. The present of n-HAp caused the shift of gelation temperature to the higher temperature in comparison with original copolymer solution without n-HAp (from 32°C to 40°C). The morphologies observed using SEM of n-HAp/PECE hydrogel nanocomposites exhibited interconnected pores with irregular shapes. With the addition of n-HAp the pores changed slightly and became less interconnected pores [139].

An important drawback of synthetic polymers is their acidic degradation products can lower the local solution pH. The resultant acidic medium accelerates further degradation in an autocatalytic manner, which triggers inflammatory and foreign body reactions in vivo. The addition of HAp (or generally CPC) to the polymer increased pH of polymer solution [83].

2.9 The Common Techniques Used in Preparation of Drug Carriers

Controlled release drug delivery employs devices such as polymer-based disks, rods, pellets, or microparticles which encapsulate drug that release at controlled rates for relatively long periods of time. While a variety of devices have been used for controlled release drug delivery, biodegradable polymer microspheres are one of the most common types and hold several advantages. Microspheres can encapsulate many types of drugs including small molecules proteins, nucleic acids or bioactive inorganic materials and are easily administered through a syringe needle. Microspheres can be prepared in a large number of ways e.g. by emulsion method, spraying techniques, etc. Emulsion processes may be controlled by stirring rate, solvent and/or by particles size adding to the primary solution [140].

2.9.1 Single Emulsion Process (W/O method)

Polymer is dissolved in volatile organic solvent (e.g. dichloromethane) in order to prepare a single phase solution. The particles of drug (or other healing medicament) are added to the solution to produce dispersion in the solution (Fig. 16).

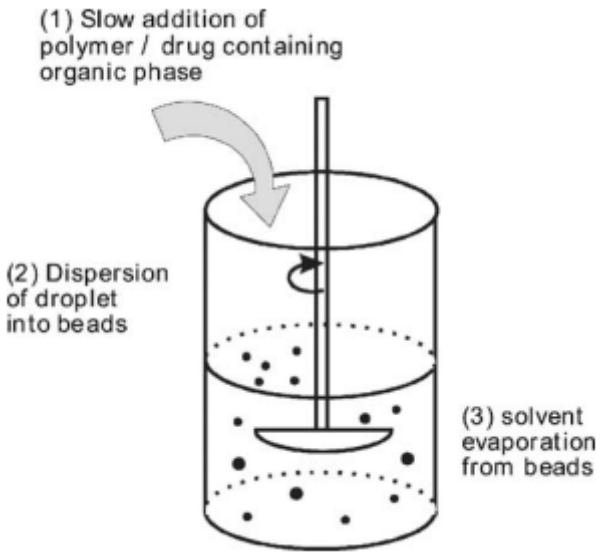

Fig. 16. Spheres formation by W/O process [2].

The polymer/drug suspension is then emulsified in large volume of water in the presence of emulsifier (polyvinyl alcohol (PVAl)) in appropriate temperature with stirring. The organic solvent is then allowed to evaporate or extracted to harden the oil droplets under applicable conditions. W/O emulsion method is ideal for water-insoluble drugs like steroids [141, 142].

2.9.2 Double Emulsion Process

2.9.2.1 Water-in-oil-in-water process (W/O/W)

Water-in-oil-in-water emulsion methods are best suited to encapsulate water-soluble drugs like peptides, proteins, and vaccines. At first the drug is dissolved in aqueous phase (deionized water) and then this drug solution is added to organic phase consisting of organic solvent and some copolymer (or polymer). These two phases are stirred to form water-in-oil emulsion (W/O). Emulsion prepared like these way is added to PVAl (concentration is about

0.5 wt.%) aqueous solution and further emulsified (W/O/W) (Fig. 17). The organic solvent is then allowed to evaporate or is extracted in the same manner as oil-in-water emulsion techniques [143, 144, 145].

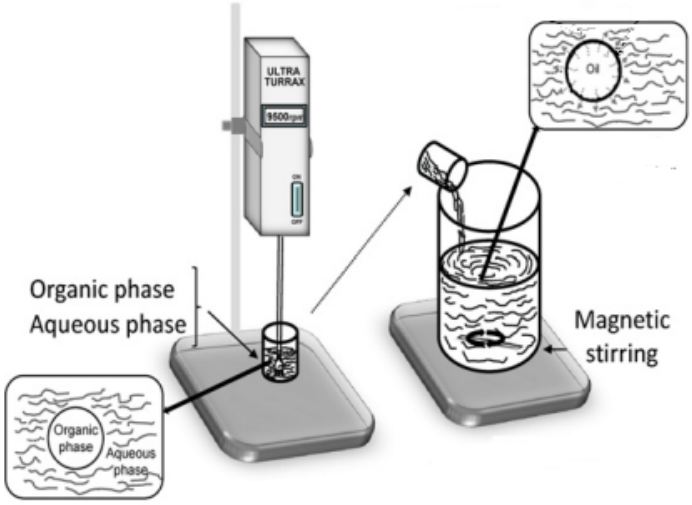

Fig. 17. Preparation of nanospheres by W/O/W method [146].]
Chyba! Nenalezen zdroj odkazů.

2.9.2.2 Solid-in-oil-in-water (S/O/W)

The emulsion system consists of two processes. Solid-in-oil (S/O) phase is prepared by dispersing solid phase in polymer solution to form oil phase. Then the S/O phase is dropped in the water phase containing emulsifier and stirred vigorously to obtain S/O/W emulsion [146, 147, 148]. S/O/W method is an alternative encapsulation procedure for W/O/W.

Protein solution exposed in an organic phase containing polymer could resulted in protein denaturation. In S/O/W method dehydrated protein is in the form of powder and being used to create a suspension in an organic phase, followed by emulsification in aqueous solution to allow microspheres formation. This procedure eliminates W/O emulsion step and because the absence water/organic solvent interface, it might in turn increase protein stability within encapsulation procedure [149].

2.9.3 Spray-drying Techniques

Spray-drying (SD) can be defined as the transformation of a material from a fluid state into a dried particulate form by spraying the feed into a hot drying gas medium. This process is very rapid, convenient and with few processing parameters makes it suitable for industrial scalable processing. The main disadvantage of this process is the adhesion of the microparticles to the inner walls of the spray-dryer. Using SD drug/protein/peptide microspheres might be prepared [150].

2.9.4 The preparation methods used for amphiphilic copolymer

The presented methods are focus on the structure types of thermosensitive copolymers (PLGA-PEG, respectively) and their structure-influenced applications in drug delivery.
These **polymeric micelles** have a number of significant advantages, for example it is a thermo-dynamically stable system, the solubilization of insoluble drugs is possible by their

encapsulation into hydrophobic cores the particle size of micelles is between 10 nm and 100 nm and therefore it is relatively easy for micelles (on nanoparticles) to pass through tumor leaky vasculature. The DDS based on the micellar structure can be prepared by dialysis (penetration of solvent through membrane), film-casting (evaporation of organic solvent to form dry polymer film), freeze drying or co-solvent evaporation.
Preparation of thermosensitive (nano, micro) **particles** is mostly the same as for another "non-thermosensitive" biocompatible polymers/copolymers (PLGA). The particles size strongly depends on the chosen method, amount of drug-loading, the length of polymer hydrophobic/hydrophilic blocks, molecular weight, etc.
Thermosensitive hydrogels have enormous advantages to gel in situ upon injection into the body and the encapsulation of proteins or drugs. A new method called ring-opening polymerization, using microwave radiation to synthesize PLGA–PEG–PLGA, has many significant advantages compared with traditional methods; for example, no solvent is required during the synthesis and purification process [151] (Fig. 18).

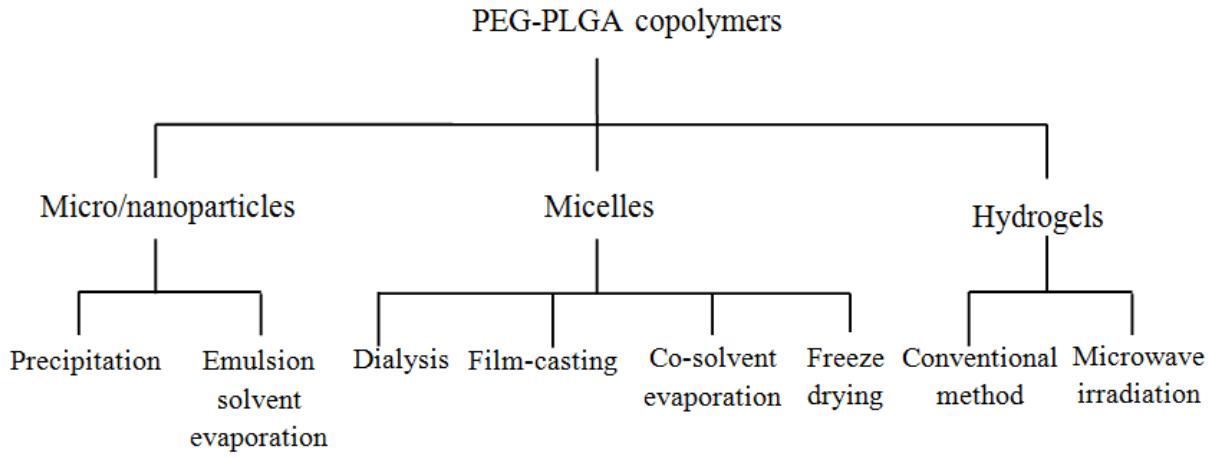

Fig. 18. The preparation methods for various types of thermosensitive DDS.

2.10 Rheology

The term rheology was first used in physics and chemistry by E. C. Bingham and M. Reiner at 1929 when the American Society of Rheology was founded in Columbus. Since then the rheology is desired tools for materials characterization [152]. Rheological testing includes materials from water-like liquid over paste and composite to solid materials. Especially in (micro or nano)composites materials rheology can predict filler and matrix interactions a therefore this method was chosen.

Nanocomposites represent system, where the specific filler surface area is around 100 g/m^2 (microfiller have surface area usually less than 10 g/m^2). Moreover, the nanofiller particles dimension is comparable with dimension of polymer chains. Incorporating randomly nanoparticles into the copolymer matrix leads to the interaction of polymer chain and causes conformational restrictions of them [153] (Fig. 19).

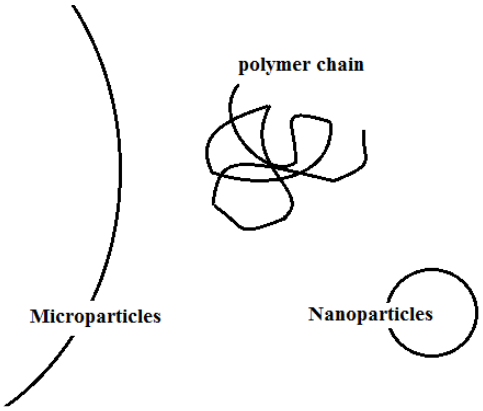

Fig. 19. Schematic approach the size of nanoparticles, microparticles and polymer chain [153].

Rheological properties of particulate suspension change significantly with favorable particles-matrix interactions compared to non-interaction system or strong particle-particle interactions [154].

Many research groups have been investigated the aggregation/flocculation process in shear flow condition. The process of aggregation or dislocation of nanoparticles in solution under shear flow by perikinetic/orthokinetic conditions is controlled. *Perikinetic* aggregation is due the Brownian motions (Fig. 20a). On the other hand, orthokinetics aggregation is due higher shear rate that tends to bring particles close each other (hydrodynamic parameter). High applied shear rate leads to the thickening of nanofluid (Fig. 20c) [155].

But almost of rheological flow measurements are in the range from 10^{-2} to 10^{3} s^{-1}. The motion of the particles with the fluid flow in a nonuniform field of velocity will lead to a shear thinning caused by the fact that the nanoparticles will adopt a more flow oriented arrangement (Fig. 20b).

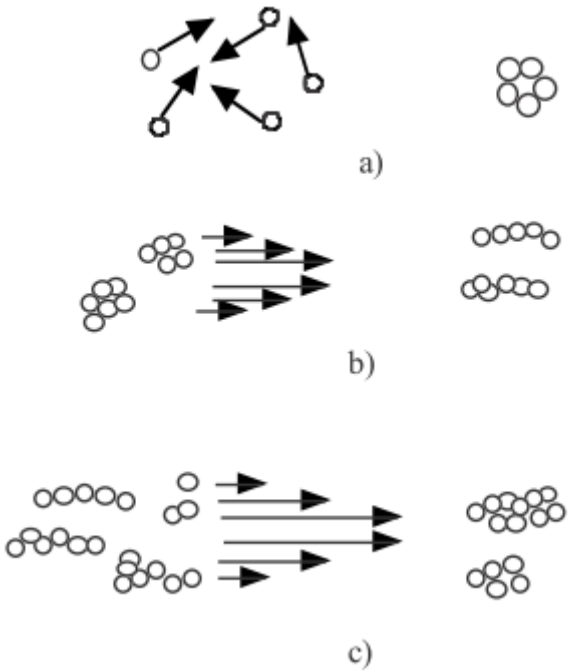

Fig. 20 Perikinetics aggregation (a), shear thinning of nanofluids (b) and orthokinetics aggregation - high shear rate [154].

The nanofluid, containing spherical SiO_2 nanoparticles, was submitted to strong shear rates ($250 \cdot 10^3\ s^{-1}$). Increase in volume fraction of nanoparticles in the system tends to the present of agglomerates. The smaller particles (9 and 11 nm) have the stronger tendency to agglomerate due Van der Waals forces [156]. Existence of particles aggregations in the liquid suspension is indicated generally by pseudoplastic behavior. Nanosuspension of TiO_2 suspension exhibited pseudoplastic behaviorand for the determination of yield stress various empiric model were used (Bigham, Casson, Herschel-Bulkley model) [157].

The aggregation process can be study also by dynamic light scattering or microscopy (TEM, SEM). However, rheological properties can provide the knowledge on the structure within the nanofluids, under both static and dynamic conditions. After that one can anticipate nanoparticles behavior in fluid and its tendency to agglomeration/aggregation.

The reinforcement mechanisms and the influence of n-HAp and CS particles in thermosensitive copolymer matrix by rheological measurements were investigated. It is necessary to characterize nanocomposite behavior in flow condition (at higher shear rate) for its future application such an injectable bone adhesive, drug delivery carriers.

3 MAIN AIMS OF DISSERTATION WORK

The main goal of presented doctoral thesis was preparation of the nano-hydroxyapatite/thermosensitive copolymer composites which are liquid at laboratory temperate and gels at body temperature.

The first type of composite consists of rigid hydroxyapatite particles and thermosensitive copolymer matrix (PLGA−PEG−PLGA). In the second case, the core-shell particles are incorporated in thermosensitive copolymer matrix. The in the core are situated the n-HAp particles which are covered by copolymer shell of thermosensitive triblock copolymer functionalized by itaconic anhydride to form α, ω-itakonyl[(polylactid-co-polyglycolid)-b-polyethylenglycol-b-(polylactid-co-polyglycolid)] (ITA/PLGA−PEG−PLGA/ITA). The carboxyl-end groups are able to further chemical crosslinking.

The individual steps of the proposed doctoral thesis can be summarized in following steps:

1. Synthesis thermosensitive PLGA–PEG–PLGA and novel functionalized ITA/PLGA–PEG– PLGA/ITA copolymers.
2. Clarification of temperature-dependent micellization behavior of both thermosensitive triblock copolymers.
2. Preparation of “core-shell” structure (CS), including chemical crosslinking (CS-x).
3. Rheological characterization of CS-x and n-HAp particles in thermosensitive copolymer matrix.
4. The release study of CS-x and n-HAp particles from thermosensitive matrix into the water incubation medium.

4 EXPERIMENTAL PART

4.1 Chemicals

PEG (M_n = 1 500 g·mol^{-1}), Sn (II)2-ethylhexanoate (95 %), N-Ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), poly(vinyl alcohol) (PVAl) (Mw = 130 000, 99 % hydrolyzed) were purchased from Sigma-Aldrich (Germany), d,l-lactide (LA, 99.9%) and glycolide (GA, 99.9%) were supplied from Polysciences (Pennsylvania, USA). Itaconic anhydride (ITA 97 %) was obtained from FLUKA (Switzerland). Ultrapure water (ultrapure water of type II according to ISO 3696) was prepared on our Elix 5 UV Water Purification System (Millipore, Merck spol. s r. o.). Acetone was purchased from Lach-ner (Czech Republic). Nano-hydroxyapatite (n-Hap) was prepared by Ing. Klára Částková, Ph.D. (CEITEC VUT, Brno).

4.2 Materials and Methods

4.2.1 Polymer synthesis

The PLGA—PEG—PLGA triblock copolymer with weight ratio of PLGA/PEG equal to 2.4 and molar ratio of LA/GA equal to 2.9 (PDI = 1.15, M_n = 5 050 g·mol^{-1} for PLGA—PEG—PLGA, M_n = 5 200 g·mol^{-1} for ITA/PLGA—PEG—PLGA/ITA) was synthesized via ring opening polymerization method in a bulk under nitrogen atmosphere according to Michlovska et. al [77] and was used pro polymer matrix. Briefly, PEG was degassed and dewatered at 130 °C for 3 h under the vacuum and D,L-lactide and glycolide monomers were added against the nitrogen outflow. After the homogenization by stirring, Sn(II)2-ethylhexanoate as organic catalyst was injected in order to start up the copolymerization at 130 °C for 3 hours (PLGA−PEG−PLGA). Functionalization with ITA proceeded at 110 °C for 1 hour (ITA/PLGA−PEG−PLGA/ITA). The content of end-capped ITA was 65 %.
Both copolymers were purified from unreacted monomers by dissolving in cold ultrapure water and heating the solution up to 80 °C. Precipitated polymer was separated by decantation and dried in freezer dryer until the constant weight. The purifying process was repeated three times. ITA/PLGA−PEG−PLGA/ITA copolymer was further used for double emulsion method to from polymer shell of a core from hydroxyapatite particles.

4.2.2 Nano-hydroxyapatite particles

The needle-shaped nano-hydroxyapatite particles (n-HAp) were obtained from Ing. Klára Částková. Ph.D. (Institute of Materials Science and Engineering, Brno and CEITEC BUT, CZ). Briefly, the nanoparticles were prepared by precipitation method of calcium nitrate and ammonium phosphate in the presence of ammonium hydroxide. The particles were treated hydrothermally at 150 °C for 1 hour and ground on a planet mill for 4 hours.

4.2.3 Core-shell particles

This part had to be removed due to papent priority.

4.3 Characterization of Materials

4.3.1 Proton Nuclear Magnetic Resonance (^{1}H NMR)

Molecular weight, PLGA/PEG and LA/GA ratios were confirmed using ^{1}H NMR spectroscopy on 500 MHz Bruker AVANCE III instrument using 128 scans in $CDCl_3$ solvent.

4.3.2 Gel Permeation Chromatography (GPC)

Number average molecular weights (M_n) and polydispersity index (M_w/M_n) of the copolymers were determined by GPC method using Agilent Technologies 1100 Series instrument equipped with isocratic pump, autosampler, RI and UV-VIS detector, fraction collector, column thermostat up to 80 °C and 300 × 7.5 mm PLgel 5 μm MIXED E column with THF as the eluent at a flow rate of 1 mL·min^{-1} against linear polystyrene standards.

4.3.3 Dynamic Rheological Analysis

The thermosensitive and mechanical properties of the pure copolymer solution, n-HAp and CS modified were investigated by a dynamic stress-controlled rheometer (AR-2 TA Instruments) with Cone Plate geometry (angle 2°, diameter 40 mm and gap 60 μm).
Cold polymer solution (600 μL) was transferred to the Peltier (temperature control system) by micropipette. Before each measurement the solvent trap was filled with distilled water and the liquids were used to prevent evaporation of the sample.

Thermosensitive properties were study by dynamic oscillatory mode. Dynamic oscillatory mode was set at constant angular frequency of 1 rad·s^{-1} with temperature ramp from 15 to 60 °C (with temperature increments of 0.5 °C·min^{-1}) and shear stress at 0.4 Pa. All measurements lie in within linear region and in which dynamic moduli are independent on measurement conditions. Before measurements were the samples equilibrated for 2 minutes.

The dependence of neat copolymer solution and n-HAp and/or CS viscosity in copolymer matrix on the shear rate and influence of the system composition was studied. The shear rate range was carried out from 10^{-2} to 10^{2} rad·s^{-1}. The samples were equilibrated for 2 minutes at first step conditioning and in the second step conditioning following 10 minutes to ensure sufficiently isothermal conditions into the entire sample volume.

4.3.3.1 Kinetics of crosslinking

The kinetic of crosslinking by oscillatory mode was study. The experiments were carried out at 23 °C (sol phase). The rheological study of crosslinking is necessary for core-shell(crosslinked) preparation. ITA-modified copolymer solution and crosslinked agent were transferred on Peltier and measured immediately. The measurement was switched on without pre-shear test.

4.3.1 Fourier Transformed Infra-Red spectroscopy (ATR-FTIR)

Infra-Red spectra of triblock copolymers and chemical functionalization with ITA were confirmed by FT-IR spectrometer (Bruker Tensor 27) in range of 4000−650 cm^{-1} using attenuated total reflectance (ATR) sampling technique.

4.3.2 Environmental scanning electron microscopy/Energy Dispersive X-ray (ESEM/EDX)

The CS structure was investigated by environmental scanning electron microscopy (FEI Quanta 250). Samples were prepared by placing a droplet of CS aqueous suspension onto aluminium stub. The samples were dried at laboratory temperature and then measured at the operating voltage of 30 kV (spot size 2.5) and working distance of 10.00 mm.
The presence of the calcium and phosphorus ions (n-HAp) in the copolymer core can be detected by Energy Dispersive X-ray (EDX) analysis.

4.3.3 Transmission electron microscopy (TEM)

CS particles and n-HAp particles were observed using transmission electron microscopy (Tecnai Sphera 20 and Tecnai G2, both FEI). The samples was dissolved in 50 µl of 50/50 vol. % (ethanol/miliQ water), treated by Vortex, 4 ul of suspension was applied on grid (Cu 400 mesh + continous carbon - 50; glow discharge 10 s at 15 mA), the excess of samples was washed away.

4.3.4 Dynamic light scattering (DLS) and zeta potential

Dynamic light scattering (DLS, DynaPro Nanostar, Wyatt) (laser wavelength 658 nm) using digital autocorrelator was carried out to determine the size distribution of micelles in the aqueous copolymer system. Measurements were then performed at increasing temperatures from 25 °C to 55 °C. The DLS device (Zetasizer Nano ZS, Malvern) was employed for measuring of zeta potential at 4 mV by He-He laser.

4.3.5 Brunauer-Emmet-Teller method (BET method)

BET method is based on the adsorption/desorption of nitrogen gas molecules on the material surface and is suitable for determination of specific surface area ($g \cdot m^{-2}$) of gained n-HAp particles. The measurement was performed on Quantachrome NovaWin 2200e Instrument. The sample was degassed under vacuum at 300 °C for 20 hours.

4.3.6 Particles size of HAp powder

The mean size and particles size distribution by HELOS Particles Size Analysis (Sympatec) was determinated. HELOS is powerful instrument for measuring of the particles in wet/dry form and thus can predict new interactions in aqueous environment tend to agglomeration and/or aggregation.

4.3.7 Inductively coupled plasma optical emission spectrometry (ICP-OES)

ICP-OES analytical technique was selected for the detection of released calcium (phosphorus) ions from hydroxyapatite. Homogenuous n-HAp/copolymer composite (a) was kept at 37 °C in incubator for 1 hour to form a gel. The gel was poured 2 ml of water (b). After 0, 3, 6, 9 and 12 days the water was decanted and added to 2 ml of 1M HCl for ICP-OES analysis. Analysis was performed on ICP-OES (Ultima 2, Horiba Jobin Yvon, France) equipped with Mainhard type nebuliser and cyclonic spray chamber. The plasma gas flow (Ar) was 12 l/min, auxiliary gas flow (Ar) 0.5 l/min, generator power 1200 W and nebuliser pressure 0,29 MPa. The measured spectral lines were 213,667 nm (P) and 422,673 nm (Ca), integration time was

0.5 s for both spectral lines. The calibration solutions of P and Ca for the ICP-OES were prepared by dissolution of knowing amount of hydroxyapatite in 0.1 M hydrochloric acid.

Before analysis, the HAp particles had to be dissolved in 1 M HCl (equat. 1) to give calcium cations. For each analysis, the separate vial with the composite was prepared. The amount of released ions was calculated according to calibration curve.

$$Ca_{10}(PO_4)_6(OH)_2 \rightarrow 10Ca^{2+} + 6PO_4^{3-} + 2OH^- \quad (1)$$

4.3.8 ^{1}H nuclear magnetic resonance relaxometry (^{1}H NMR relaxometry)

Nuclear magnetic resonance measurement for the analysis of the relaxation time of hydrogen proton on Minispec (Bruker, Germany) was used. Device was operating at the Lamour frequency of 7.5 MHz for protons. T_2 (spin-spin relaxation time) decay were obtained by applying the CPMG (Carr-Purcell-Meiboom-Gill) pulse sequence and the results were fitted by RIWinFIT software (Version 2.4, Resonance Instrument Ltd. Oxfordshire, UK). The relaxation delay was 12 s, number of echoes (6000), scan (32) and interecho constant spacing 2 ms. The measurement of newly-formed CS particles and (another components used for CS particles preparation) as n-HAp particles and water were carried out in glass NMR tube. All experiments were carried out at 23 °C.

5 CONCLUSION

In the first experimental part, PLGA−PEG−PLGA (ABA, M_n = 5 050 g·mol^{-1}) and ITA/ PLGA−PEG−PLGA/ITA (ITA/ABA/ITA, M_n = 5 200 g·mol^{-1}) copolymers with PLGA/PEG ratio 2.4 and LA/GA ratio 2.9 were synthetized by ring-opening polymerization and purified. The copolymer structure, molecular weights and the amount of ITA bonded to original ABA copolymer were confirmed by ^{1}H NMR. Both copolymers exhibited narrow polydispersity index (1.15).

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5.1 List of Abbreviations

A1, A2, A3	water content in individual domains, in ^{1}H NMR relaxometry
ABA	viz. PLGA−PEG−PLGA
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infrared spectroscopy
BET method	Brunauer-Emmet-Teller method
$Ca(OH)_2$	calcium hydroxide
CMC	critical micellar concentration
CPCs	calcium phosphate cements
CS	core-shell particles (with physically shell)
CS-x	core-shell particles (with chemically crosslinked shell)
DCPD	dicalcium phosphate dihydrade
DDC	drug delivery carries
DLS	dynamic light scattering

DNA	deoxyribonucleic acid
EDC	N-Ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride
G´, G´´	elastic and viscous shear modulus
GCV	ganclicovir
GF/PL	gas foaming/particulate leaching method
GPC	gel permeation chromatography
D,L-LA	D,L-lactide
DDSs	drug delivery systems
HAp	hydroxyapatite
^{1}H NMR	proton nuclear magnetic resonance
H_3PO_4	phosphoric acid
HPLC	high-performance liquid chromatography
IHN	isoniazid
ICP-OES	inductively coupled plasma optical emission spectrometry
ITA	itaconic anhydride
ITA/ABA/ITA	viz. ITA/PLGA−PEG−PLGA/ITA
ITA/PLGA−PEG−PLGA/ITA	α, ω-itakonyl[(polylactid-co-polyglycolid)-b-polyethylenglycol-b-(polylactid-co-polyglycolid)]
L-LA	L-lactide
MEEP	poly[di(methoxyethoxy-ethoxy)phosphazene
M_w/M_n	polydispersity index
n-HAp	nano-hydroxyapatite
Na_2HPO_4	sodium dihydrogen phosphate
NaCl	sodium chloride
NMR	nuclear magnetic resonance
^{1}H NMR relaxometry	nuclear magnetic resonance relaxometry
PAA	polyacrylic acid
PAMAM	polyamidoamine
PBS	phosphate buffer saline
PCL	poly(ε-caprolactone)
PCPP	poly[di(carboxylatophen-oxy)phosphazene]
PDI	polydispersity index
PEG	poly(ethylene glycol)
PGA	poly(glycolic acid)
PHB	poly(hydroxybutyrate)
PLGA	poly(lactic-*co*-glycolic acid)
PLGA−PEG−PLGA	poly(D,L-lactic acid-co-glycolic acid)-b-poly(ethylene glycol)-b- poly(D,L-lactic acid-co-glycolic acid)
PPO	poly(propylene oxide)
PVAl	polyvinyl alcohol
SA	sebatic anhydride
SANS	small angle neutron scattering
SC/PL	solvent casting/particulate leaching method
SD	spray-drying technique

SEM	scanning electron microscope
SEM/EDX	scanning electron microscopy with energy-dispersive X-ray spectroscopy
S/O/W	solid in oil in water method
tan δ	tan delta
T_AH, T_BH, T_CH method	the relaxation times of water protons used in relaxometry
TEM	transmission electron microscopy
TTIM	test tube inverting method
UV-VIS	ultraviolet–visible spectroscopy
W/O	water in oil method
W/O/W	water in oil in water method

5.2 List of Figures

5.3 List of Tables