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Sialic acid as a recognition motif for host-originated glycoproteins in *Ixodes ricinus*

Bachelor's thesis

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Annotation

Glycans are found on the surface of every living cell and they are responsible for a countless number of functions. N-acetylneuraminic acid (often called sialic acid) is mostly found in vertebrate and only to a small degree in arthropods. The aim of this thesis was to find out if sialic acid is a recognition motif for the tick organism to take up and incorporate or recycle host originated proteins.

Affirmation

I hereby declare that I have worked on my [bachelor's dissertation] thesis independently and used only the sources listed in the bibliography. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor / master / dissertation thesis, in full / in shortened form resulting from deletion of indicated parts to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system

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Jakob Samek

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1. Introduction

The study of protein-carbohydrate interactions is part of glycobiology and has become more and more important over the last decade, however, it has not been given the proper attention and priority needed. This is prominent especially regarding vector-borne pathogens and their transmission, since glycans cover almost all living cells and are at the forefront of host-pathogen and host-vector interactions, cell attachment and cell invasion.

Even though sialic acids are typically found at the surface of vertebrate cells and only in minute concentrations in arthropods, previous studies showed the presence of sialylated N-glycans in tick salivary glands and the gut, and it was further shown that the majority of sialylated molecules in the adult tick originate in the host and is not synthesized by the tick. The near absence of tick originated sialylated molecules and the specific localization of host structures in the salivary glands and the saliva raises many questions on the role of these molecules in the tick physiology.

2. Glycobiology

Glycobiology is the study of biosynthesis, functions and structure of glycans.

2.1. Nomenclature

Glycans (also called oligosaccharides, or carbohydrates) are "compounds consisting of a large number of monosaccharides linked glycosidically" [1].

Glycosyltransferases are a family of enzymes that are responsible for assembling monosaccharides into branched or linear glycan chains [2].

Glycosylation is the most common and complex form of post- (or co-) translational modification which is the linkage of a glycan with a protein, or lipid.

Glycoconjugates are the products of glycosylation, they are generally classified as carbohydrates covalently linked with other chemical species like proteins, lipids, and peptides.

Glycoproteins, glycolipids, glycopeptides, etc. are products of glycosylation.

The **Glycome** is the totality of glycans produces by, e.g., a cell. It is analogous to genome or proteome, but it is dynamic, meaning it can change when the cell changes (e.g. from an embryonic cell to a differentiated cell)[2].

2.2. Glycans

Glycans are one of the most intricate organic molecules in nature and they can be found in every living cell [3]. The surfaces of all vertebrate cells are covered in a tightly packed diverse layer of glycan chains. They are mainly attached to the surface of proteins and lipids (glycoproteins and glycolipids) or proteoglycans [4]. Due to their ubiquity on cell surfaces and on secreted molecules, the functions of those glycoconjugates are vast and span from basic structural functions like the glycocalyx, which is a polysaccharide that forms a barrier around all eukaryotic cells [5], to pathogen-host interactions as part of the immune system of an organism [6]. The diversity of glycan function is due to the fact that their building blocks, monosaccharides, can be combined in multiple ways to create thousands of different complex glycans. Compared to protein or nucleic acid formation where, using a single type of bond, only linear sequences are formed, monosaccharides have two possible bond types (alpha or beta) and several bonding sites available which gives the possibility for the formation of an incredible number of different complex glycans [7]. Even though hundreds of monosaccharides are known, only a handful is commonly found in animal glycans (Figure 1).

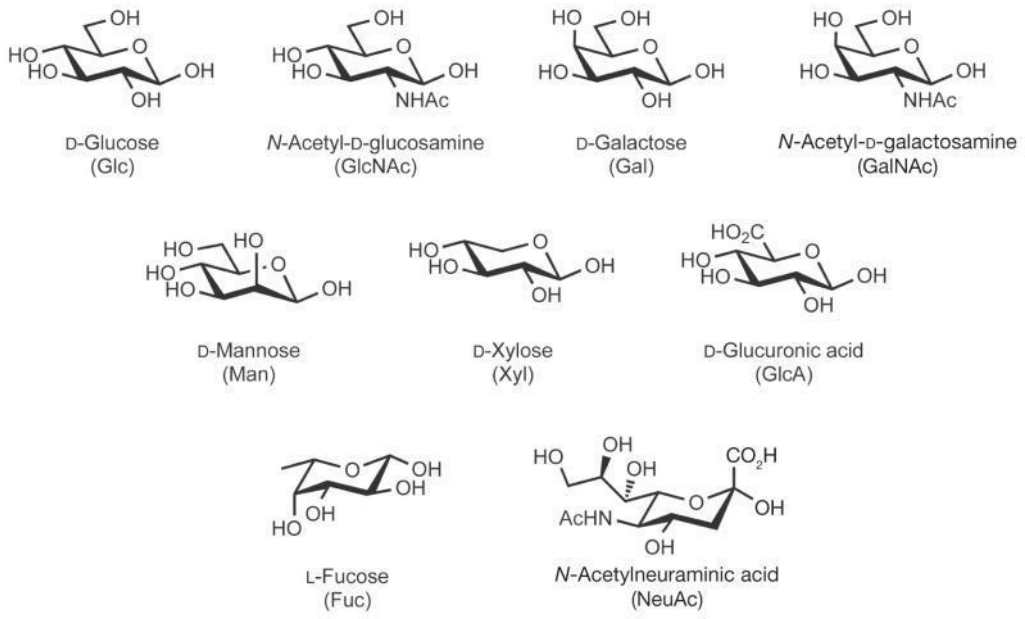

Figure 1: the most common monosaccharides found in vertebrates. (from literature [2]*)*

Pentose	five carbon sugar (Xyl)
Hexose	six carbon sugar (Glc, Gal, Man)
Hexosamine	Hexose with an amino group at the 2-position (GlcNAc, GalNAc)
Deoxyhexose	Hexose without the hydroxyl group at the 6-position (Fuc)
Uronic acid	Hexose with a negative charged carboxylate at the 6-position (GlcA)
Neuraminic acid (Sia)	Family of nine-carbon acidic sugars, of which the most common forms are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc).

Table 1: 6 types of sugars found in vertebrates

This set of monosaccharides represent the main building blocks for glycans of higher vertebrates but also of several "lower" animals and bacteria.

2.3. Glycosylation

The two main protein glycosylation types are of N- or O-type, meaning they are either attached to a nitrogen, on an asparagine (Asp), or to an oxygen, on a serine (Ser) or threonine (Thr) group.

N-glycosylation

N-glycosylation in humans and other Eukaryotes takes place in 3 major steps:

Synthesis of precursor glycan

Every N-glycan shares a common core glycan structure, two N-Acetyl-glucosamine (GlcNac) molecules followed by a branch of three mannose (Man) molecules (also called $Man_3GlcNAc_2$-Core), so every N-glycosylation starts out with a GlcNAc sugar bound to an ER-membrane-bound lipid molecule called dolichol. Next, the second GlcNAc residue is added followed by 5 Man. At this point the precursor glycan is flipped across the ER membrane where another four Man and three glucose molecules are added to the precursor [8] (Figure 2).

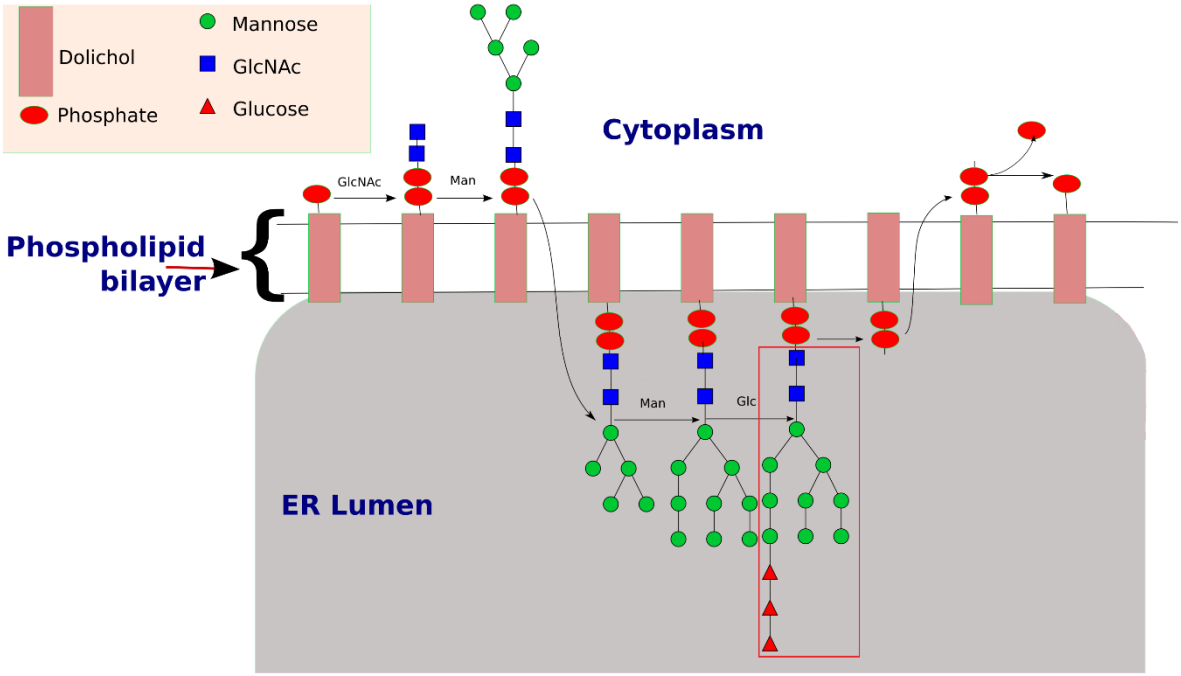

Figure 2: synthesis of N-linked precursor glycan.

Transfer to a protein.

N-glycosylation actually is a co-translational modification as it is attached to a protein while it is translated in the endoplasmic reticulum (ER). The asparagine must be located at a specific consensus sequence in the protein – the sequon (Asn-X-Ser or Asn-X-Thr) [9]. The enzyme oligosaccharyltransferase (OSTase) is responsible for recognizing the specific sequence and the transfer of the glycan. (Figure 3).

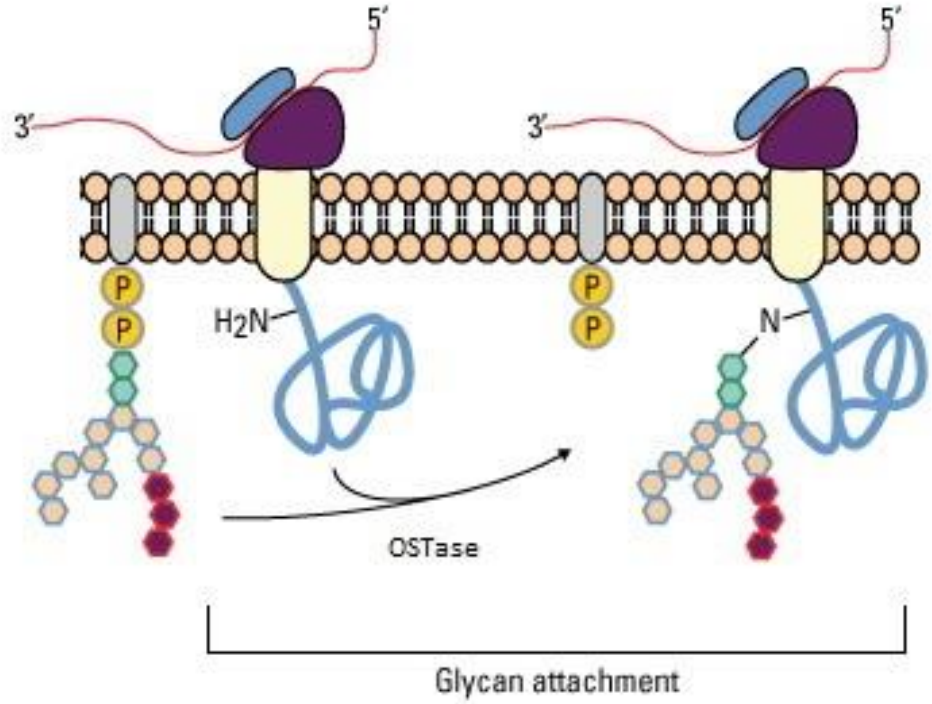

Figure 3: glycan attachment to a specific sequon via OSTase.

Processing of glycans

Trimming of the glycan takes place in the ER and the Golgi but it serves different purposes.

In the ER, the first two glucose residues of the glycan chain are hydrolyzed by glycosidase I and II, enzymes which cut glucose monosaccharides at the non-reducing end of the glycan [8]. Then two, so called chaperon lectin molecules, calnexin and calreticulin bind and assist in folding of newly synthesized proteins that contain a monoglucosylated N-linked glycan. If the protein is not properly folded it can re-do the previous steps of Glc addition and removal until the protein is properly folded [10], [11]. Once the protein is folded properly it will be transported to the Golgi.

Up until this point all N-linked glycoproteins have the same precursor glycan, which means the maturation in the Golgi is where the vast diversity of glycans stems from. In the Golgi a set of specific enzymes play the key role in determining if the resulting glycan is either a high-mannose type (oligomannose) by only adding additional mannose residues, or a complex type, where any number of any other monosaccharide

can be added, or of hybrid type where one branch only carries mannose and the other is a complex-type branch [12], [13] (Figure 4).

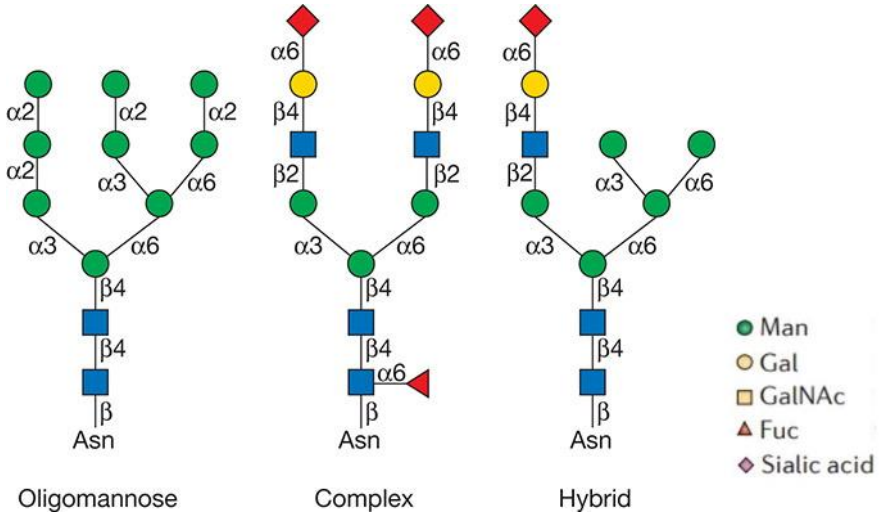

Figure 4: Types of N-glycans

2.4. Functions of glycans

The biological roles of glycans fall into two broad categories: (1) structural and regulatory functions that only involve the glycan itself or the protein it is bound to and (2) specific recognition of glycans by glycan-binding proteins (GBPs), which can be intrinsic or extrinsic to the organism [4]. Lectins are an important group of GBPs, they are cell adhesion proteins that bind highly selective to specific glycans.

As mentioned before, structural and regulatory roles include the formation of a physical barrier on the outside of cells, nutritional storage, protection from protease and immune recognition, water solubility for macromolecules, and many more [2], [4].

Intrinsic functions include the already mentioned intracellular glycoprotein folding, degradation, and trafficking, and they play an important part in fertilization and reproduction [4], [14], [15].

Extrinsic functions often involve GBPs as numerous pathogens and symbionts evolved to interact highly selectively with the dense layer of glycans in a host organism. Their interactions and functions range from bacterial, parasite and fungal adhesins, which help via specific recognition of glycans to adhere to host cells, to viral agglutinins, which are viral GBPs, that play key roles in infection processes of viruses like the H1N1

group. Differences between the host and pathogen surface glycans, detected by specific receptors, is commonly the basis for innate immune response [2].

2.5. Sugars in signaling

Signaling is an important part of the functions that sugars have in an organism. Not only glycans but also simple sugars (such as glucose, fructose, and sucrose) employ various sensing systems often linked with the metabolism of the sugar. In plants, various sugar imitated signals have been studied extensively. Some famous examples are the Nod factor as a signal for the nitrogen-fixing symbiosis of *Rhizobium* bacteria in legume [16]. The glycan-initiated signal in plant defense relies on the recognition of pathogen signatures by the host, the so-called transmembrane pattern recognition receptors (PRRs) [17]. Those PRRs are also active on animal cell surfaces and include receptors such as toll-like receptors (TLRs) and C-type lectins (CTLs), which play an important part for the innate immune system where they participate in the recognition of pathogens-associated molecular patterns (PAMPs). A well-studied example is the endotoxin Lipid A of Gram-negative bacteria, which is detected by TLR-4 to trigger an innate immune response [18]. CTLs are the largest family of known GBPs, they are Ca^{2+} dependent binding proteins that include other groups such as selectins, collectins or the Ashwell-Morell receptor (AMR) [19]. The AMR (also called asialogylcoprotein receptor), are located on hepatocytes on the liver and they are responsible for the rapid binding and clearing of asialoglycoproteins (glycoproteins from which sialic acid residues have been removed). This is achieved by receptor-mediated endocytosis by the AMRs [19], [20].

Collectins are type of (PRRs) with critical functions in innate immunity. By binding to surfaces of microbes or fungi expressing (PAMPs), they stimulate phagocytosis and production of cytokines and promote leukocyte chemotaxis [21]. Selectins are a family of cell adhesion molecules (CAMs) and they are one of the best described CTLs as they play a critical role in leukocyte extravasation during infection. Leukocytes normally circulate unattached in the blood, the presence of an infection or an inflammation triggers a signal which activates p-type lectin production on the surface of endothelial cells that line the blood vessels. The p-type lectins weakly binds glycan groups on the leukocyte surface forcing it to roll along the vessel wall, where with the help of platelet

activating factor (PAF) and $\alpha_L\beta_2$ integrin receptors the leukocytes bind to intracellular adhesion molecule 1 (ICAM-1) to start the extravasation migration [22].

3. Sialic acid

N-acetylneuraminic acid (Neu5Ac) and the closely related N-glycolylneuraminic acid (Neu5Gc) (see Figure 5), also called sialic acid, are the most common form of the Neuraminic acid sugars(Table 1), they are of special interest for this body of work and also the key to many of the aforementioned functions of glycans. Neu5Ac is a negatively charged sugar and is often found in alpha 2,3-linked or alpha 2,6-linked configuration with galactose (Figure 6). As mentioned above, sialylated glycoproteins and glycolipids make up the majority of glycoconjugates on cell surfaces and of secreted molecules in vertebrates, some invertebrate and in some bacteria. The high expression of sialic acid on the outer cell surface and on secreted proteins in vertebrates implies that they have general functions as stabilizers for membranes and molecules and also modulate and interact with their environment. For example, the negative charge of the sialic acid helps in the binding and transport of positively charged molecules but also repulses unwanted interactions, for example, with proteases. The repulsive forces also act as a separator between molecules which is important to ensure the proper folding conformations of proteins, but also is responsible for the high viscosity in mucins lining in the intestine or the surface of the eye. [23], [24]. Signaling and recognition processes are some of the main functions of this carbohydrate, they can be viewed in a dualistic role, either masking recognition sites or acting as a target for receptors. Masking of the penultimate sugars, like galactose, is of great importance as they are also designed to be recognized by receptors, so, for example, desialylation of human blood cells causes phagocytes to bind to the now exposed galactose via a galactose-specific receptor, which ultimately leads to degradation of the blood cells [25]. Many pathogens, including viruses like influenza, bacteria like *Escherichia coli* or *Heliobacter pylori*, and toxins produced by pathogens, e.g. cholera toxin, abuse the sialic acid on host cells to bind to those receptors. The previously discussed lectin family includes a big group that bind specific to sialic acid residues, some famous examples are the wheat germ agglutinin (WGA), *Sambucus nigra* agglutinin (SNA), and *Maackia amurensis* agglutinin (MAA). These bind specifically to alpha-2,6 or

alpha-2,3 linked sialic acid residues, which can be used for detection and quantification of sialic acids [26].

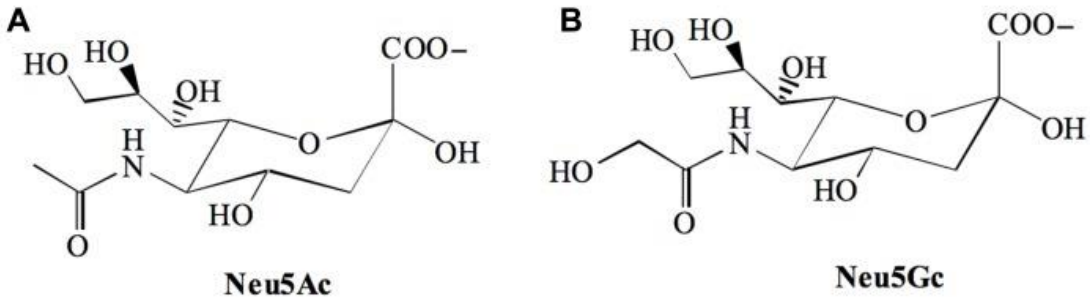

Figure 5: The two major sialic acid found in mammals. (A) N-acetylneuraminic acid (Neu5Ac) and, (B) N-glycolylneuraminic acid (Neu5Gc) (from literature [28])

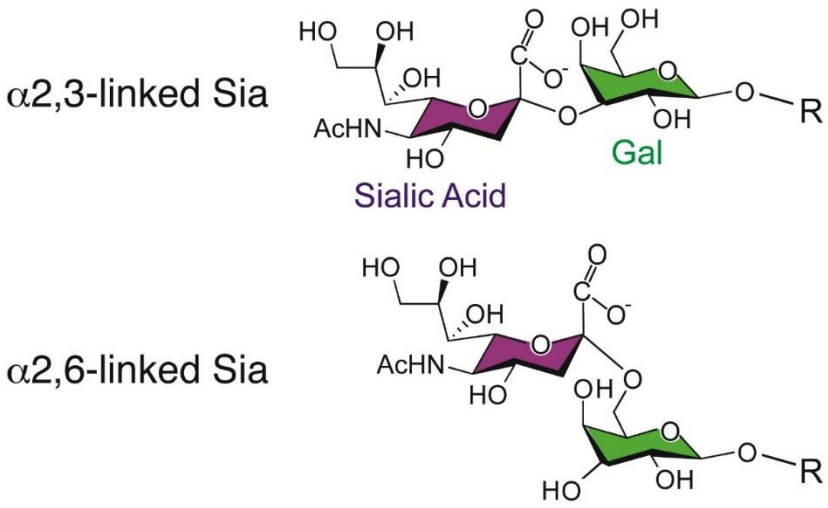

Figure 6: Neu5Ac in two very common and important linked configurations. (from literature [27]*)*

3.1. Sialic acid in ticks

In invertebrates, sialic acid has already been described in the embryo during early neural development of *Drosophila melanogaster* and in cicada *Philaenus spumaris* [28]–[30]. Also Drosophila are able to recycle sialic acid from host blood meal and incorporate it into their own glycoproteins [31]. In tick it was previously shown

lectins are involved in the innate immunity of arthropods (Vasta et al. 1994)

Soluble or membrane-bound invertebrate lectins take part in the processes of cell adhesion, opsonization, phago- cytosis and cytolysis (Vasta & Marchalonis, 1983). Sialic acid (Neu5Gc) - proteins were detected via Anti-Neu5Gc antibody staining of which the 95 kDa and 72 kDa structures were the most prominent ones in the tick organs

[32]. The tick lectin Dorin M has been identified in the plasma of the soft tick *Ornithodoros moubata* and has since been fully characterized as a glycoprotein with three N-linked glycosylation sites that plays a role in the innate immune system of the tick [32]–[34]. The three glycosylation sites are shown to be modified by high mannose and core-fucosylated glycans. Furthermore Dorin M also shows binding activity for sialic acid [34], [35].

Sialic acid residues were previously detected in various tick organs using sialic acid binding lectins (SNA, MAA II) [36]. Later, the presence of Neu5Ac and Neu5Gc was confirmed using mass spectrometry in salivary glands, gut, Malpighian tubes, and ovaries [37]. Via quantitation of total sialic acid and comparison to biorthogonal labeled sialylated molecules, a host origin of sialylated glycoproteins was proposed [38]. It is speculated that sialic acid on tick glycans is engaged in molecular mimicry and the tick itself produces sialic acid in ovaries, eggs, and larva [39]. Even though there is evidence that some insect cells encode for the synthesis of sialic acid but very low levels of insect originated sialic acid suggests that this biosynthesis is not a major pathway and it is suggested that insects have a sialic acid salvaging pathway [31]. Sialyltransferase genes, to encode for enzymes to attach sialic acid to glycans, were also detected in *I. scapularis* [37].

3.2. Tick feeding

Hard ticks, like *I. ricinus*, need a blood meal at every stage of their life cycle and depending on the stage they are in, the blood meal can take several days up to weeks. Thus, it is not surprising that ticks developed a sophisticated cocktail of pharmacologically active molecules in their saliva to evade several host immune responses. During feeding, the female ticks can get up to 100x their original size and while blood is being taken up via the mouthparts, most of the water and ion content of the blood meal is injected back into the host to maintain the homeostasis [40].

Once the mouthparts penetrate the skin a cement secreted from the salivary glands anchors the tick in place and protects from the host immune system [41].

Tick saliva has several functions but the first hurdle for the tick is to stop a hemostatic response, which normally controls blood loss and ensures proper blood flow. Several bioactive components in the tick saliva counteract the host injury responses, like

hemostasis inhibitors, pain and itch blockers and, innate and adaptive immune modulators [40].

4. Labeling

4.1. Glycan labeling

Since the interest in glycoconjugates increased in the last decade, better and more specific labeling methods for glycans were developed. Most methods involve some kind of glycan derivatization step to introduce a chromophore or fluorophore for later detection or isolation [42]. Sialic acid specific labeling has improved immensely in the last years and many different strategies can be employed to label and detect specific glycans. The aforementioned sialic acid specific lectins (SNA, MAA, WGA, and others) are used in affinity chromatography for isolating and separating sialic acids [43]. It is also possible to label sialylated glycoproteins metabolically by treating cells or living animals with analogs of N-acetylmannosamine (ManNAc) [44]. This kind of bioorthogonal chemistry works by providing cells with an analog of a particular metabolite that is introduced into the cell's metabolic pathway where it competes with native metabolites. This analog is then naturally used as a building block and incorporates it into the cell structure. A probe or a small tag can be attached which is detected later. In this work sialic acid specific labeling was done using a mild periodate oxidation to generate an aldehyde on the sugar followed by an oxime ligation according to literature [45]. Different dyes or labels can be used to react with the produced aldehyde such as aminooxy-biotin, Fluorescein-5-thiosemicarbazide (FTSC), Alexa Fluor™ 488 Hydroxylamine, and many more.

4.2. Protein labeling

Fluorescent spectroscopy is an invaluable tool for analyzing protein structures and FITC has been utilized broadly since its introduction as a fluorescent marker for antibodies [2] and it keeps on being one of the most generally utilized fluorescent labels. Amine-reactive dyes, like FITC, are often used for uses in immunochemistry, receptor binding, and cell tracing. Often those bioconjugates will be subjected to post-processing steps like washing, fixing, and mounting so the bond between the molecule and the dye is important to preserve the integrity of the bioconjugate otherwise a generated signal

would no longer have any useful relationship to the abundance or localization of the labeled target. Optimal labeling using FITC depends on the reactive group isothiocyanate but generally good labeling is achieved using high pH, temperature, and concentration of proteins. FITC is soluble in water but more often it is dissolved in DMSO, which gives better control and predictability of the reaction (Goding, 1976).

Sometimes a more specific probe is needed to better understand protein-protein or protein-nucleic acid interactions, then a labeling method based on maleimide modification can be used. It works by binding a maleimide group, attached to a dye, to a sulfhydryl group on a protein. At neutral pH this method is highly specific but at higher pH it again reacts significantly with primary amines [46]. Other specialized tags are also available, e.g. fluorescein-NHS which employs a Carboxyfluorescein succinimidyl ester (CFSE) group to couple to lysine groups. other flurophores like Rhodamine

5. Goals

Previous studies show that sialylated glycoproteins present in ticks are of host origin and it is speculated that host proteins are incorporated into tick tissues upon recognition of the sialic acid. That is why determining if the sialylated glycoproteins are recognized via sialic acid (glycan part) or the protein part was the main objective of this study. Thanks to using two different labels, where one (FTSC) labels the sialic acid, masking it and making it unrecognizable by the tick, and the other one (FITC) labels lysine residues in the protein chain, leaving sialic acid intact, it is possible to compare the metabolic fate of the two types of labeled proteins.

- Labeling of glycoproteins on protein part (amino acids) and glycan part (sialic acid).
- Cultivation of IRE/CTVM 19 tick cell lines with labeled glycoproteins.
- Detection of labeled glycoproteins in IRE/CTVM 19 tick cell lysates.

6. Materials and methods

6.1. Materials

6.1.1. Tick cell lines

The *Ixodes ricinus* embryo-derived cell line IRE/CTVM19 [47], supplied by the Pirbright Institute (www.pirbright.ac.uk), was grown in L-15 medium supplemented with 20% fetal calf serum, 10% tryptose phosphate broth, 1% glutamine (all PAA Laboratories, Pasching, Austria) at 28° C in flat-sided cell culture tubes (Nunc, Thermo Scientific, Waltham, MA, USA).Centrifuging cell lines to change the medium was always done at 400 x g for 10 mins at 4°C and all solutions used while working with cell lines were sterile filtered using sterile syringe filters before use.

6.1.2. Protein labeling

Four different proteins were used for this experiment and one control. Each of the 5 samples (4 proteins + 1 control) was labeled with two dyes (FITC and FTSC) resulting in 10 different samples (Table 2)

Samples	**Fluorescein-5-isothiocyanate (FITC) labeled**	**Fluorescein-5-Thiosemicarbazide (FTSC) labeled**
Fibrinogen (FIB)	F-FIB	FS-FIB
Fetuin (FET)	F-FET	FS-FET
Transferrin (TRA)	F-TRA	FS-TRA
Alpha 1-acid glycoprotein (AAG)	F-AAG	FS-AAG
Control (C)	F-C	FS-C

Table 2: Samples used for labeling.

Fibrinogen (FIB) 10 mg/mL in ddH_2O (Sigma)

Fetuin (FET) 10 mg/mL in ddH_2O (Sigma);

Transferrin (TRA) 10 mg/mL in ddH_2O (Sigma);

Human alpha 1-acid glycoprotein (AAG) 10 mg/mL in ddH_2O (Sigma)

Fluorescein-5-Thiosemicarbazide (FTSC) (Sigma) 1 mg/mL in DMSO

Fluorescein-5-isothiocyanate (FITC) (Thermo Fisher Scientific) 10 mg/mL in DMSO

10 mM $NaIO_4$/1 M acetic acid

1M NaOH

0.1M $NaHCO_3$ buffer (pH 8.3)

TRIS (pH 7)

1x PBS

Vivaspin® ultrafiltration spin columns 2mL

BCA Protein Assay Kit (Thermo Fisher Scientific)

6.1.3. SDS-PAGE and Western Blot

Bio-Rad Trans-Blot® Electrophoretic Transfer Cell

Bio-Rad Trans-Blot® Turbo™ Transfer System

Bio-Rad Mini-PROTEAN® TGX 12% Precast Gels

Bio-Rad Immun-Blot® PVDF Membrane

Bio-Rad Blot Absorbent Filter Paper

PageBlue staining solution

Electrode buffer 1x

25mM TRIS

192mM glycine

3.6mM SDS

Blotting buffer 1x25mM TRIS

192mM glycine

20% methanol

4x reducing sample buffer for SDS-PAGE (Fermentas, Thermo Fisher Scientific)

200mM TRIS

400mM DTT

8% SDS

0.4% bromophenol blue

40% glycerol

Protein marker

Protein Marker VI (10 –245) prestained (AppliChem)

Protein Marker III (6.5 – 200) unstained (AppliChem)

Blocking solution

1xPBS/5% nonfat dry milk

Washing solution

1xPBS/0.05% Tween 20

Horseradish peroxidase (HRP) developing buffer

Tris-Buffered Saline (20 mM Tris, 500 mM NaCl, pH 7.5)

1x TBS

DAB (3,3'-Diaminobenzidine tetrahydrochloride)

30% H_2O_2

Antibodies

Affinity Purified Anti-Fluorescein, made in goat (Vector Laboratories)

Affinity Purified Rabbt Anti-Goat IgG (H+L) - HRP (Vector Laboratories)

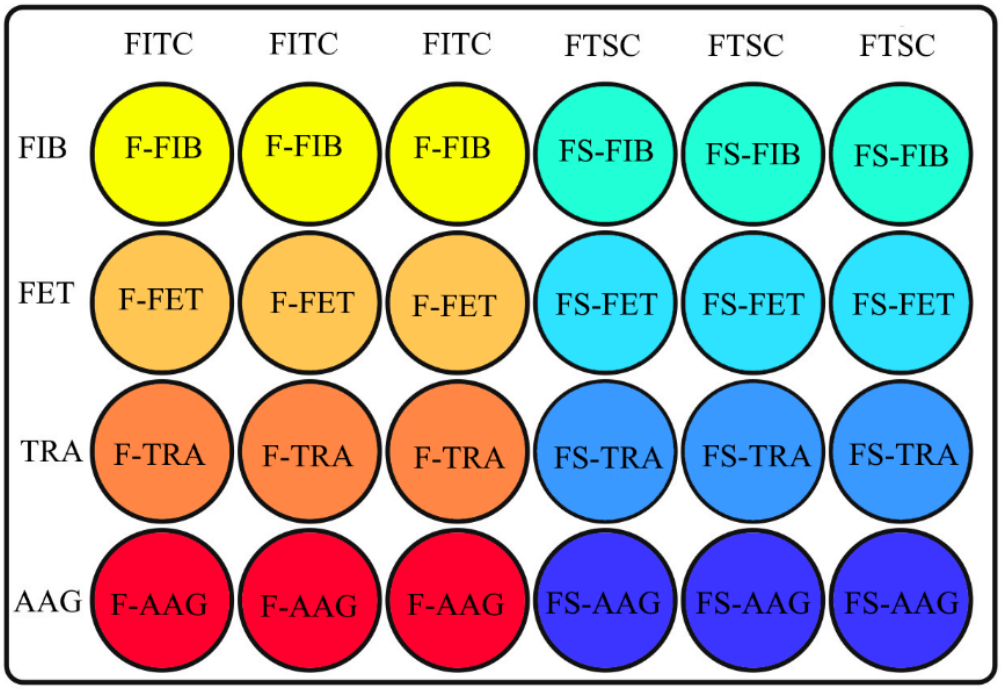

Figure 7: 24-well plate showing all samples for one timeframe. Each sample is used in triplicates.

6.2. Methods

6.2.1. Experimental setup

To find out whether sialic acid is a detection site for ticks to incorporate a protein, two different labels were used. One that binds specifically to glycans and under specific conditions to sialic acid (FTSC) (see above) and one that binds to residues in the polypeptide chain of proteins (FITC) (see above). By comparing the two it should be visible if one was incorporated into tick cells significantly more often than the other.

To achieve this, several proteins with high sialic acid content, that can be found abundantly in animal serum, were chosen (see Table 2). These proteins, once labeled, were mixed with the cultivation medium, added to tick cell lines and incubated for different time frames (3, 7, and 10 days). This was done to compare the metabolic fate of the labeled proteins over time, to make sure that proteins were really incorporated and not just present at stages of degradation. Also, not to overload the medium with too much protein of one kind, only 10% of the medium's protein concentration of each protein, was added to the medium. So, for example, the fibrinogen concentration in the medium was reported to be 0.4 mg/mL and only 10% of that concentration was added

as labeled proteins (see Table 3: Total amount of proteins used for incubation, half FTSC labeled and half for FITC labeled.).

A mix of proteins was used as a control, that underwent the same reaction procedures as the protein samples but excluding the fluorescent dye, to make sure no side reactions would influence the results.

The incubation took place in 24-well plates where each well contained a working volume of 1mL which was comprised of: 500uL of fresh medium, 200uL of tick cell line suspension, and 300uL of medium containing labeled proteins. Additionally, each sample was incubated in triplicates and each sample was incubated for 3 different timeframes. So, one 24-well plate could hold exactly the samples for one timeframe (Figure 7).

After labeling the proteins, they were transferred to a flow box were the appropriate amounts were mixed with fresh medium. Before adding the modified medium to the cell lines, the medium was sterile filtered using syringe filters to avoid contaminations.

Once a sample incubated for the specific amount of time the modified medium, that was used to incubate with, was removed and fresh unmodified medium was added. The well plate was placed back into the incubation chamber and left to incubate for another 7 days (for all 3 timeframes post-incubation was 7 days). This was done to flush the cell lines with unlabeled proteins to avoid unspecific detection.

After the 7 days post-incubation, the cells were collected and washed. The cells were then lysed, and the pellets removed. Finally, the protein concentration was measured, and the samples were frozen for later detection procedure.

For detection, SDS-PAGE followed by western blot followed by immunodetection and gel staining, was used.

6.2.2. Labeling

Sialic acid specific labeling using Fluorescein-5-Thiosemicarbazide

Sialic acid specific labeling was done following the previously discussed method of periodate oxidation to generate an aldehyde on sialic acid, followed by an oxime ligation with the fluorescent label (FTSC).

200 µg of protein in 100 µL ddH2O were mixed with 10 µL ice-cold 10 mM NaIO4 in 1M acetic acid and incubated 30 minutes in the dark at 0 °C (on ice). The pH was

neutralized using 1M NaOH and 50 µL of 1 mg/mL FTSC in DMSO was added. The mixture was incubated for 1 hour in the dark at 4 °C.

After incubation, the excess dye was removed and the protein solution was concentrated via ultrafiltration using 2 mL Vivaspin® ultrafiltration spin columns (9000 x g, 25-30 min, 4°C). The reaction buffer system was replaced with a 1x PBS buffer suitable for cell line incubation.

This method covalently binds a suitable tag to sialic acid, effectively masking it and allowing the metabolic fate of the sialic acid to be track (see above).

Protein labeling using Fluorescein-5-isothiocyanate

Protein aliquots (10 mg/mL) were mixed with 0.1M NaHCO3 buffer (pH 8.3) in a 1:1 ratio and a prepared (Fluorescein-5-isothiocyanate) FITC solution was added to have FITC in a 15-20-fold molar excess compared to the protein. The mixture was incubated on a shaker for 1 hour in the dark at room temperature. 15 µl TRIS (pH 7) per 100µg protein was used to stop the reaction and to bind excess FITC. Like in the procedure for FTSC labeling, ultrafiltration was used to remove excess reagents, to concentrate the solution, and to exchange the buffer system.

This method labels the amino acids of proteins, so the sialic acid moieties (glycans) remain untouched and we can compare the metabolic fate with the FTSC labeled proteins (see above).

6.2.3. Determining protein concentration for labeling

Each labeled protein was added to the medium in such amounts to represent 10% of the total concentration of that protein in the medium. As controls, a mix of all four proteins was used to rule out unwanted reactions with the reaction reagents. A total volume of 5.4 mL per labeled protein stock was prepared. (Table 3).

	Protein conc. in human serum [mg/mL]	Protein conc. in medium (20% BOFES) [mg/mL]	10% of medium proteins labeled [mg/mL]	Protein conc. per 0,3 mL (1 well)	Total amount of protein [mg]
Fibrinogen (FIB)	2	0.4	0.04	0.13	0.72
Fetuine (FET)	0.5	0.1	0.01	0.03	0.18
Transferrine (TRA)	2	0.4	0.04	0.13	0.72
AAG	0.45	0.09	0.009	0.03	0.162
Control (C)	4.95	0.99	0.099	0.17	1.782

Table 3: Total amount of proteins used for incubation, half FTSC labeled and half for FITC labeled.

6.2.4. Controls

As controls, a mix of all four proteins was treated the same way as the labeled proteins except no dye was used in the procedures. This was done to test against unwanted interreferences from reaction conditions.

6.2.5. Incubation of cell lines with protein mix

After each incubation period with labeled medium, the cell lines were washed once with 1xPBS, fresh medium was added and the cell lines were placed back into the incubation chamber for another 7 days with fresh unlabeled medium.

When preparing the proteins for incubation, the medium was mixed with labeled protein stock solution to achieve the protein concentrations needed for each protein (see above). The resulting solution was sterile filtered using 0.22 µm sterile filters and mixed with the tick cell lines in one of the wells. Three 24-well plates were needed, and each plate corresponded to one of the timeframes (3,7, and 10 days) plus an additional plate for the three controls. After mixing the solutions in the wells the plate edges were wrapped in PARAFILM® stretch film to minimize evaporation.

6.2.6. Cell harvesting

After the 7 days of post incubation, the cell lines were centrifuged (400 x g, 10 min, 4°C) and washed three times with 1xPBS. Then, the lysis buffer was added, and the samples were sonicated for 15 minutes at 4 ° C. Then, the samples were vortexed for 15 minutes at RT and then centrifuged at 14000 x g for 5 minutes at 4°C. The

supernatant was pipetted off and the protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific) and frozen for later use.

6.2.7. SDS-PAGE

In principle, polyacrylamide gel electrophoresis (PAGE) is a technique to separate proteins based on their molecular weight. The gel is made of a polyacrylamide matrix which causes greater resistance to bigger bulkier molecules and less resistance to small molecules, which gives a separation over time. The use of sodium dodecyl sulfate (SDS) in the gel electrophoresis will largely eliminate structural and charge influences and separate proteins by polypeptide chain length alone.

The SDS-PAGE method is usually composed of several steps: **gel preparation**, **sample preparation**, **electrophoresis**, **protein staining** or **western blotting** and **analysis of** the generated **banding pattern**. Gel preparation was not needed because precast gels were used (details below).

6.2.8. Sample preparation

For SDS-PAGE it is needed for the sample to have somewhat similar concentrations of proteins, otherwise the generated bands are not comparable to each other. To achieve this, the protein concentration of the lysed samples was first measured using BCA assay and samples were diluted according to a set of dilution factors to get somewhat equal concentrations across the samples (Table 5).

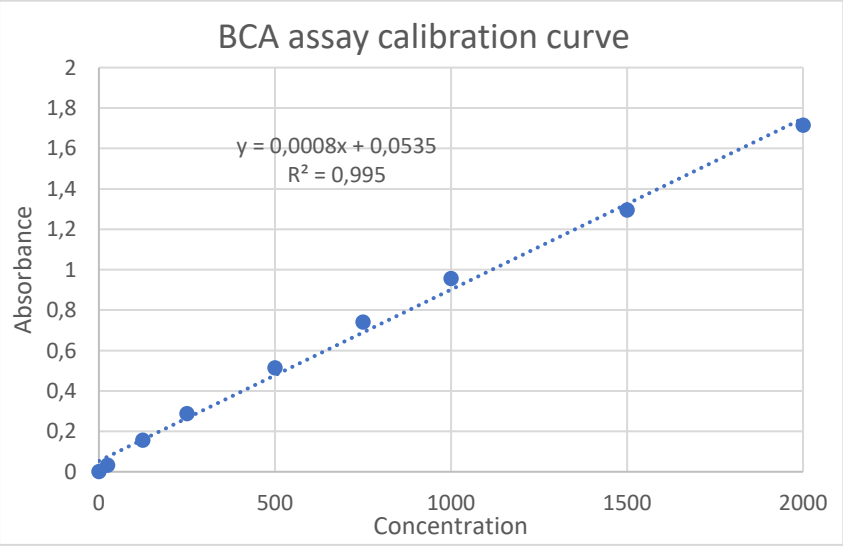

Figure 8: BCA assay calibration curve.

Reader Type:	Synergy H1
Plate Type	96 well plate
Read	Absorbance Endpoint
Wavelengths:	562
Read Speed:	Normal
Delay:	100 msec
Data Points:	8
Temperature:	25,4

Table 4: BCA assay software settings.

Measured protein concentration

Days of incubation	F-Fib	F-Fet	F-Tra	F-AAG	F-C	FS-Fib	FS-Fet	FS-Tra	FS-AAG	FS-C
3	214,6	231,1	100,3	113,3	121,5	172,8	206,9	192,8	190,4	296,4
7	330,6	355,9	786,4	329,4	308,2	474,9	555,0	279,9	349,4	111,5
10	521,4	553,2	447,8	480,8	133,9	533,2	529,0	507,3	676,9	142,1

I
I
V

dilution factor

	F-Fib	F-Fet	F-Tra	F-AAG	F-C	FS-Fib	FS-Fet	FS-Tra	FS-AAG	FS-C
3	1,0	1,9	1,0	1,0	1,0	1,0	1,0	1,0	1,0	1,9
7	1,9	1,9	4,6	1,9	1,9	3,2	3,2	1,9	1,9	1,0
10	3,2	3,2	3,2	3,2	1,0	3,2	3,2	3,2	4,6	1,0

I
I
V

final concentration

	F-Fib	F-Fet	F-Tra	F-AAG	F-C	FS-Fib	FS-Fet	FS-Tra	FS-AAG	FS-C
3	214,6	124,0	100,3	113,3	121,5	172,8	206,9	192,8	190,4	159,0
7	177,4	190,9	169,2	176,7	165,4	149,1	174,3	150,2	187,5	111,5
10	163,7	173,7	140,6	151,0	133,9	167,4	166,2	159,3	145,7	142,1

Table 5: BCA assay measured protein concentrations were diluted using a set of dilution factors to achieve a more comparable protein concentration across samples.

After diluting the samples, a 4x concentrated sample reducing buffer was added in the appropriate amounts to each sample followed by heating the samples to 75 °C for 5 minutes.

Six 10-well 12 % gels (2 gels per timeframe) were loaded with the prepared samples and a protein ladder (unstained protein ladder for gel staining, prestained for western blot). Electrophoreses was performed at 120 V for 70 minutes.

Gels were subsequently washed with ddH_2O and half the gels were prepared for Western blot, while the other half was put in a bath of PageBlue staining solution for 30 minutes under slight agitation.The stained gels were recorded using a digital imaging system

6.2.9. Western Blot

After SDS-PAGE, half of the gels (one of each timeframe) was prepared for western blot according to protocol [48]. The washed gels were placed in blotting buffer for 10 to 15 minutes under slight agitation. Polyvinylidene difluoride (PVDF) membranes

were placed in methanol for 5 minutes and then in blotting buffer for 5 minutes. The blotting apparatus was assembled by placing a gel on top of a membrane and then "sandwiching" the two between two filter papers. The stack was soaked in blotting buffer and placed on the electrode plate. Air bubbles were removed by gently rolling a glass rod over the stack. The electroblotting was performed using the machine supplied settings (100V, 750mA, 30 min).

6.2.10. Immunodetection

After the electroblotting, the PVDF membranes were removed from the blotting buffer and washed in ddH_2O. Since all labels were fluorescein based, I only needed one protocol for detection. First the washed membranes were placed in a blocking solution under gently agitation (5% nonfat milk/PBS, 1h, RT) to block unbound membrane sites. Followed by washing with PBS-T (3x, 10 min, RT) to remove excess blocking buffer.

Then the membranes were incubated with primary antibodies (anti-fluorescein, made in goat) (1:500 antibodies in blocking solution, 1h, RT), followed by another washing cycle with PBS-T.

Next, the membranes were incubated with my secondary antibodies (anti-goat HRP) (1:1000 antibodies in blocking solution, 1h, RT), followed by another washing cycle with PBS-T.

Finally, the freshly prepared HRP developing buffer was poured over the membranes which were then covered to reduce exposure to light and left until a clear signal with low background developed.

Membranes were then recorded using a digital imaging system.

7. Results

SDS-PAGE gels (Figure *9*, Figure 11, Figure 13) show an, more or less, even distribution of proteins in all samples, concentrations of loaded samples were kept relatively equal (Table 5).

Protein	kDa
Fibrinogen	340
Transferrin	80
Fetuin	64
AAG	40

Table 6: Molecular weight of labeled proteins.

Western blot membranes show a high concentration of fluorescent tags from cell lines incubated with Fibrinogen, Fetuin and Transferrin which were all labeled with FITC. This suggest that the sialylated proteins were incorporated into the tick cell lines. AAG did not show any or barely any signal for any tag (Figure 10, Figure 12, Figure 14). This could be related to a problem in the labeling procedure., but unfortunately, I couldn't verify that because all my labeled proteins were used during the experiment, due to high loses during the labeling procedure (ultrafiltration). A broad spectrum of bands was visible for the first three FITC labeled protein samples but the most prevalent signal was a 72kDa protein which was present in all samples. Interestingly, FTSC labeled Fibrinogen also shows strong signals in all three timeframes mainly a 72kDa and two 60-63kDa bands. The other three FTSC labeled proteins didn't have very strong signals except for the 72kDa band and a slight band of FTSC-Fetuin sample in the 245kDa region (Figure 10) There is a clear decrease in signal intensity over the three time frames, which is expected as a general turnover of proteins would get rid of older proteins over time (Figure 15) The bands that remained present the longest (17 days after the start of incubation) were some high molecular weight bands at 245kDa and higher but also at 135kDa, 100kDa, 75kDa and 60kDa.

3-Day incubation Polyacrylamide gel

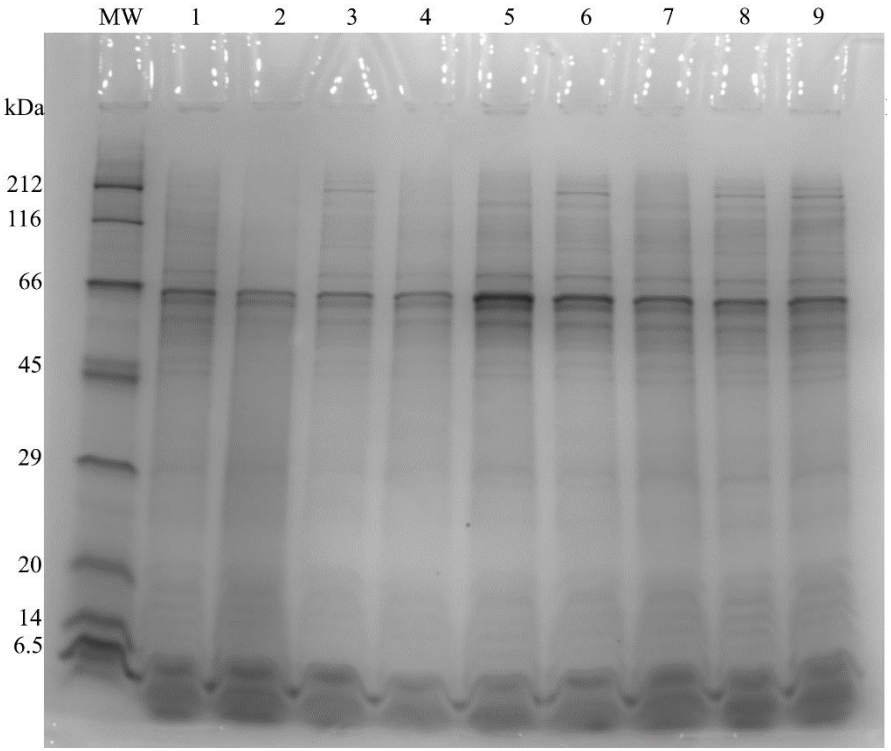

Figure 9: Polyacrylamide gel of cell line lysates of 3-Days incubation with labeled proteins:
MW: protein ladder (212-6.5 kDa)
1-4: lysates with 4 proteins labeled with FITC (F-Fib, F-Fet, F-Tra, F-AAG)
5: lysate with FTSC treated control
6-9: lysates with 4 proteins labeled with FTSC (FS-Fib, FS-Fet, FS-Tra, FS-AAG)
Key: Fib: Fibrinogen; Fet: Fetuin; Tra: Transferrin; AAG: alpha 1-acid glycoprotein.

3-Day incubation immunoblot membrane

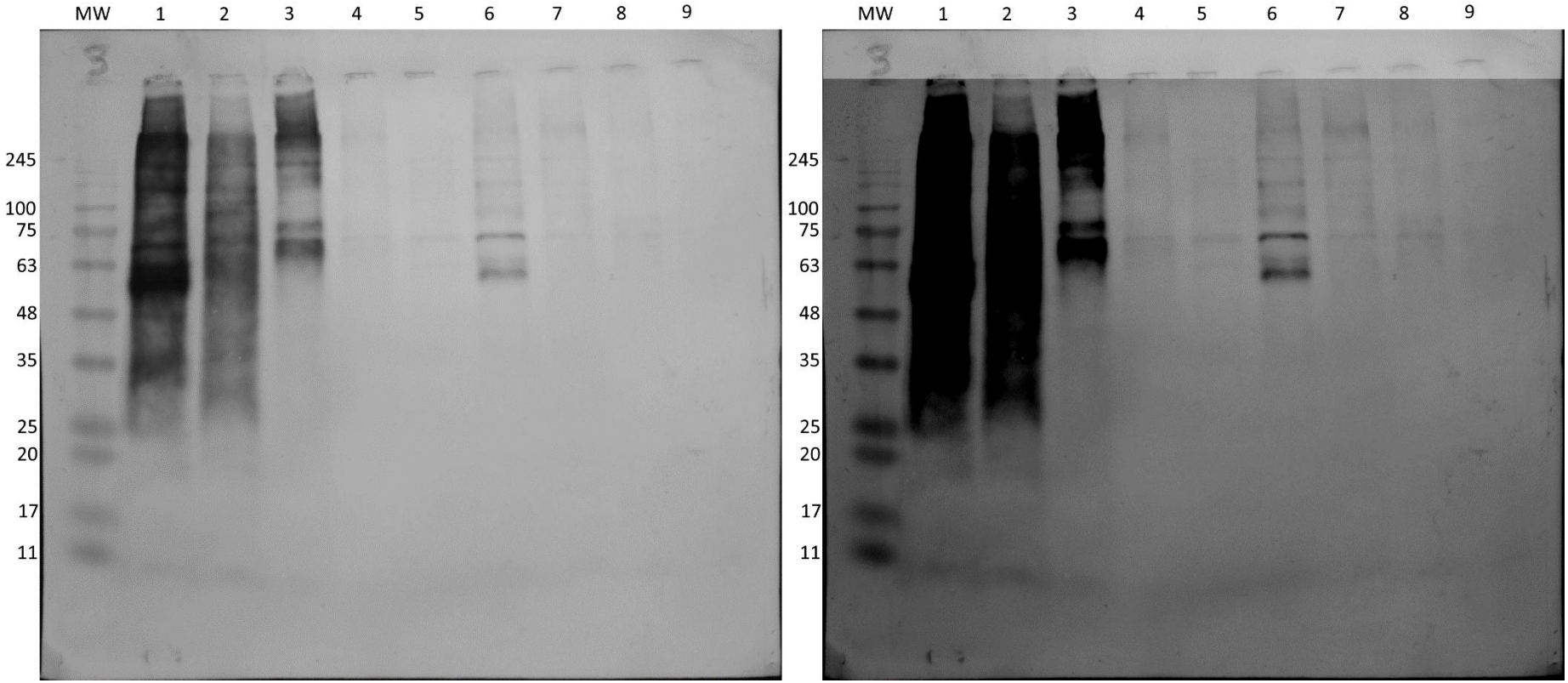

Figure 10: Immunoblot membrane of cell line lysates of 3 day incubation with labeled proteins
MW: protein ladder (245-11 kDa)
1-4: lysates with 4 proteins labeled with FITC (F-Fib, F-Fet, F-Tra, F-AAG)
5: lysate with FTSC treated control
6-9: lysates with 4 proteins labeled with FTSC (FS-Fib, FS-Fet, FS-Tra, FS-AAG)
Key: Fib: Fibrinogen; Fet: Fetuin; Tra: Transferrin; AAG: alpha 1-acid glycoprotein.

7-Day incubation Polyacrylamide Gels

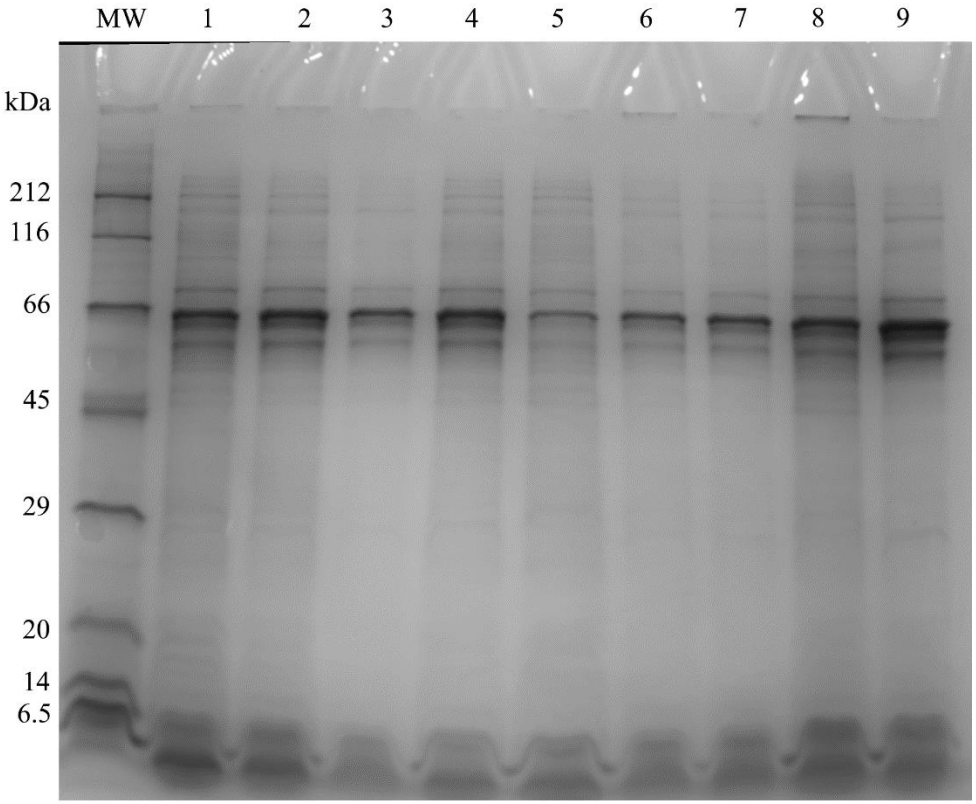

Figure 11: Polyacrylamide gel of cell line lysates of 7-Days incubation with labeled proteins:
MW: protein ladder (212-6.5 kDa)
1-4: lysates with 4 proteins labeled with FITC (F-Fib, F-Fet, F-Tra, F-AAG)
5: lysate with FTSC treated control
6-9: lysates with 4 proteins labeled with FTSC (FS-Fib, FS-Fet, FS-Tra, FS-AAG)
Key: Fib: Fibrinogen; Fet: Fetuin; Tra: Transferrin; AAG: alpha 1-acid glycoprotein..

7-Day incubation membrane

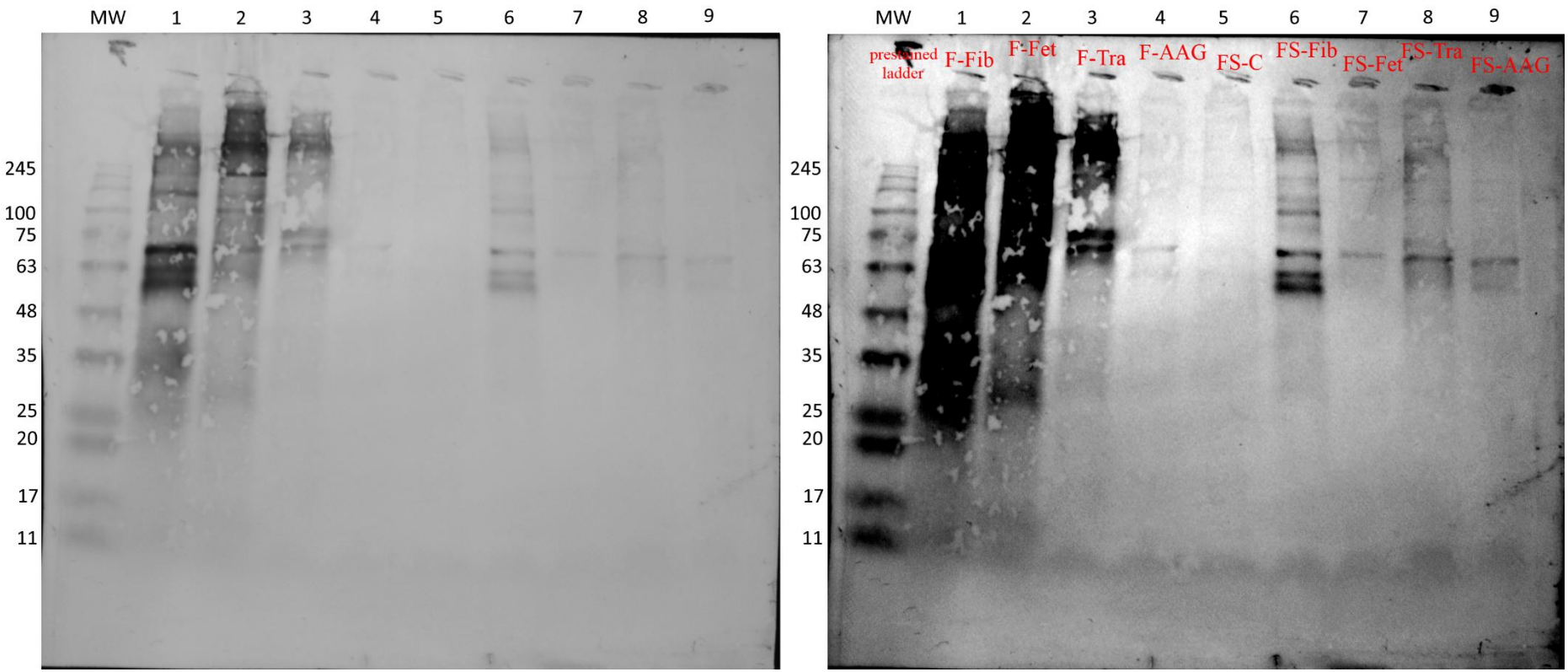

Figure 12: Western Blot membrane of lysates of 3-Day incubation

MW: protein ladder (245-11 kDa)
1-4: lysates with 4 proteins labeled with FITC (F-Fib, F-Fet, F-Tra, F-AAG)
5: lysate with FTSC treated control
6-9: lysates with 4 proteins labeled with FTSC (FS-Fib, FS-Fet, FS-Tra, FS-AAG)
Key: Fib: Fibrinogen; Fet: Fetuin; Tra: Transferrin; AAG: alpha 1-acid glycoprotein.

10-Day incubation Polyacrylamid gel.

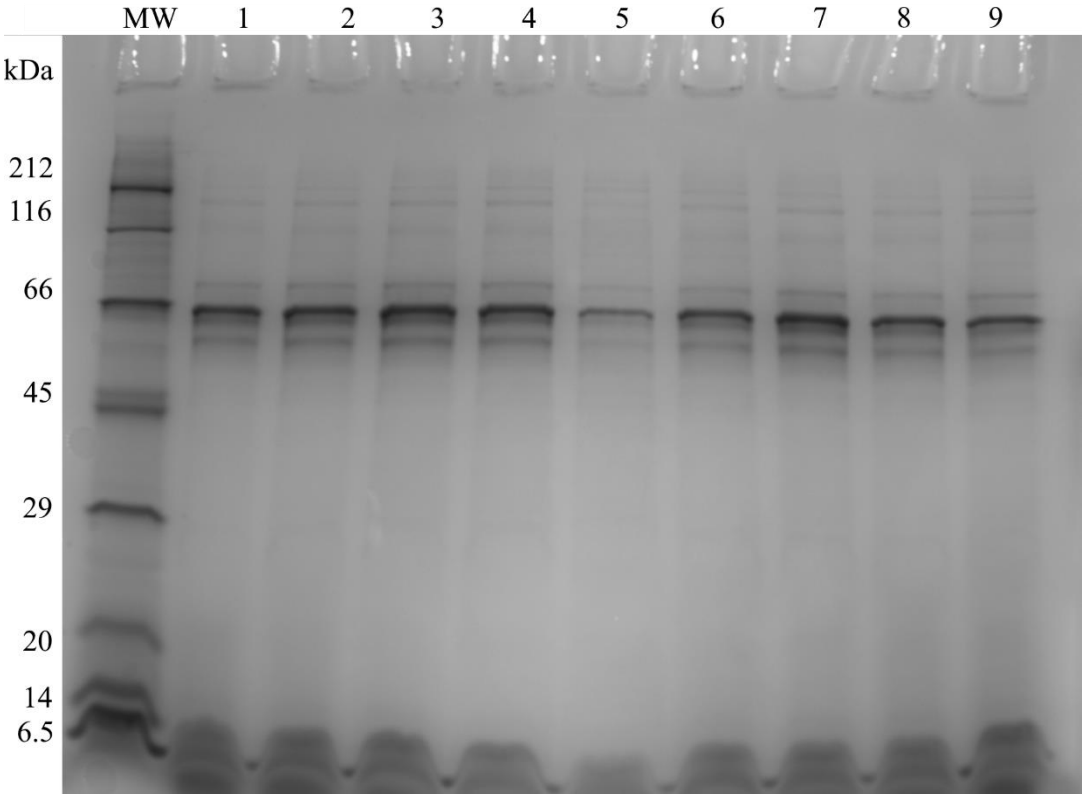

Figure 13: Polyacrylamide gel of cell line lysates of 7-Days incubation with labeled proteins:
MW: protein ladder (212-6.5 kDa)
1-4: lysates with 4 proteins labeled with FITC (F-Fib, F-Fet, F-Tra, F-AAG)
5: lysate with FTSC treated control
6-9: lysates with 4 proteins labeled with FTSC (FS-Fib, FS-Fet, FS-Tra, FS-AAG)
Key: Fib: Fibrinogen; Fet: Fetuin; Tra: Transferrin; AAG: alpha 1-acid glycoprotein.

10-Day incubation membrane

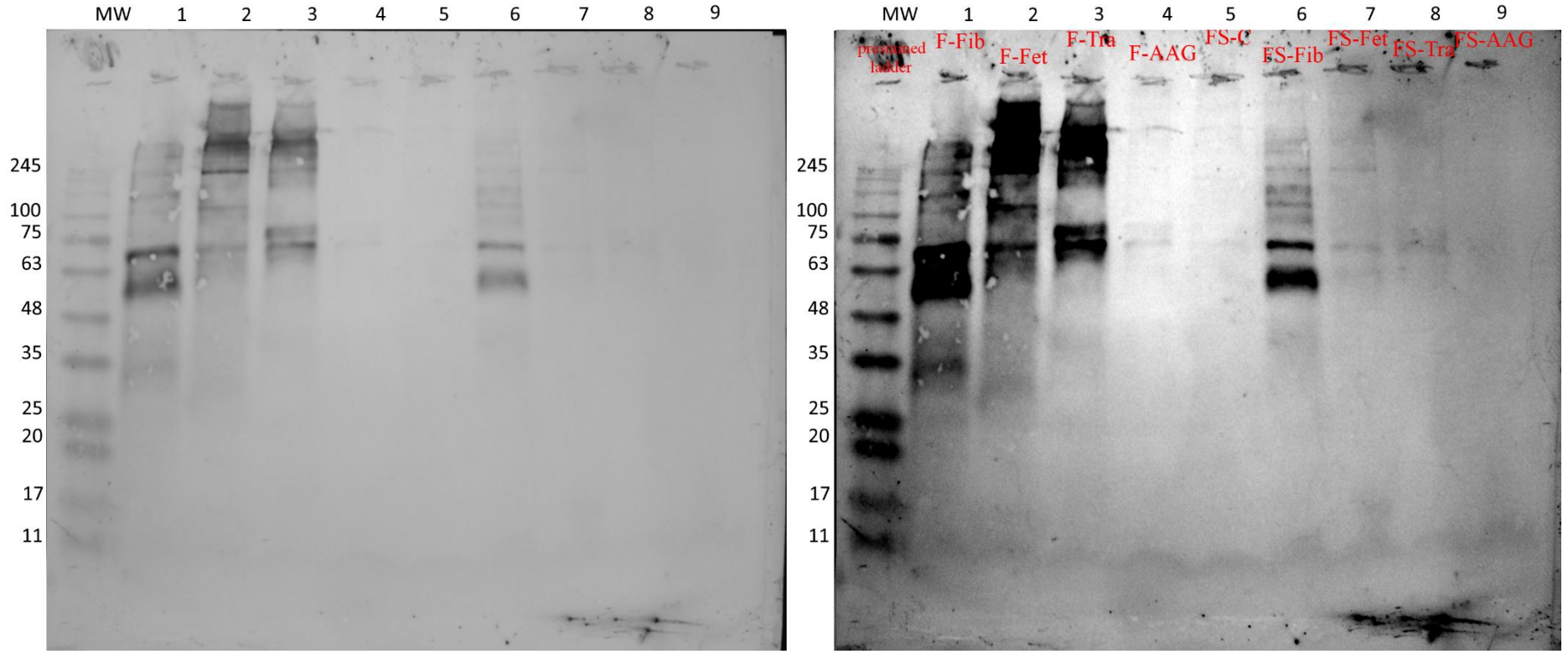

Figure 14: Polyacrylamide gel of cell line lysates of 7-Days incubation with labeled proteins:
MW: protein ladder (245-11 kDa)
1-4: lysates with 4 proteins labeled with FITC (F-Fib, F-Fet, F-Tra, F-AAG)
5: lysate with FTSC treated control
6-9: lysates with 4 proteins labeled with FTSC (FS-Fib, FS-Fet, FS-Tra, FS-AAG)
Key: Fib: Fibrinogen; Fet: Fetuin; Tra: Transferrin; AAG: alpha 1-acid glycoprotein.

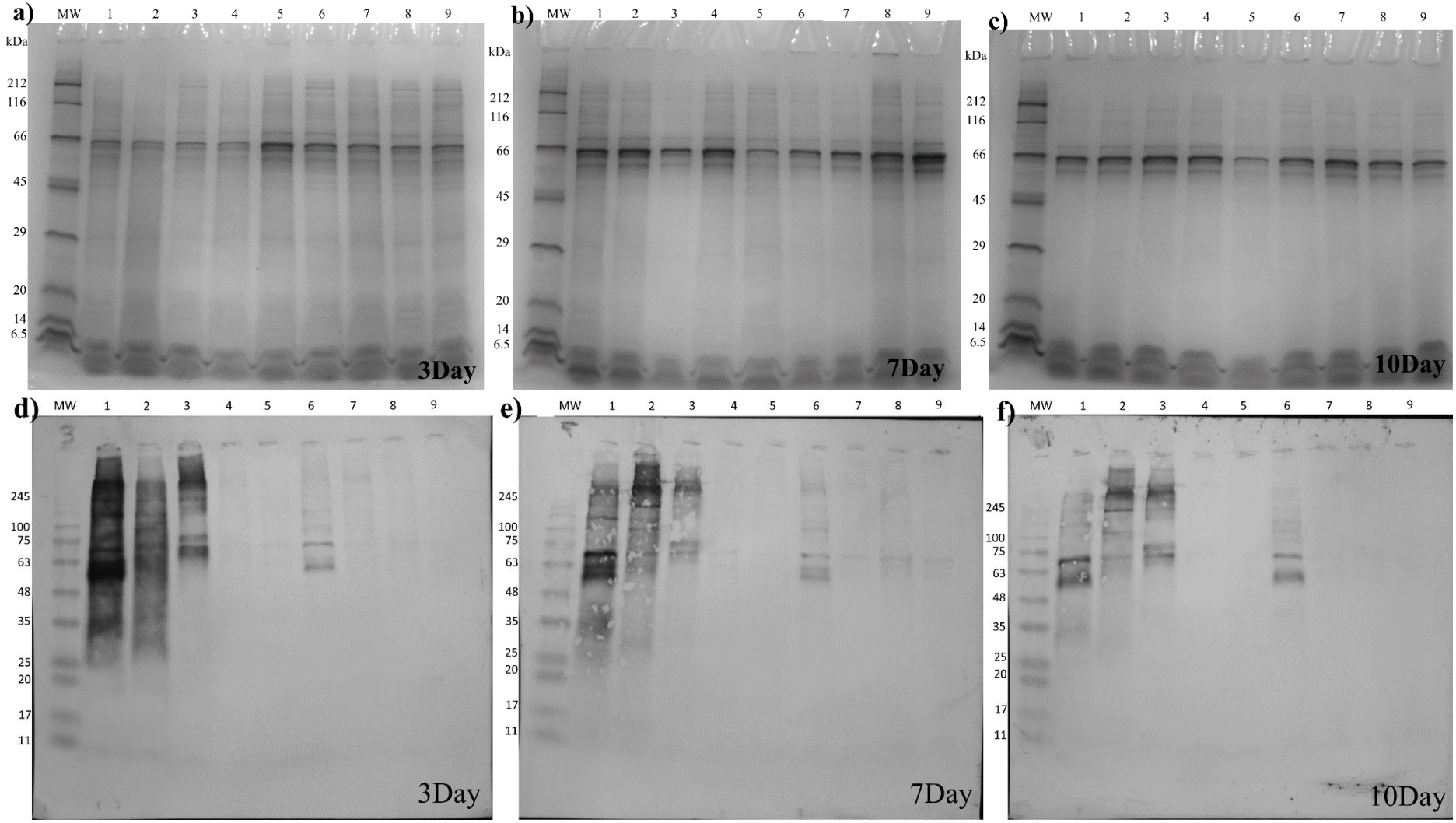

Figure 15: side by side comparison of the 3 timeframes. Protein concentrations were kept relatively constant (see Table 5) for all 9 samples as can be seen in gels a-c. Signal strength of the labeled proteins decreased over time as can be seen in membranes d-f.

8. Discussion

Sialic acid is usually found in vertebrate at the terminal position of O- or N-glycans but studies have shown that it can also be found in certain insects and other invertebrates [49]. Structural confirmation of Neu5Ac and Neu5Gc was revealed using mass spectrometry in tick salivary glands and ovaries [37]. Via quantitation of total sialic acid and comparison to biorthogonal labeled sialylated molecules, a host origin of sialylated glycoproteins was proposed [38]. Even though there is an evidence for tick protein sialylation, it is unlikely a major pathway. However the presence of sialyltransferase genes makes a sialic acid transfer to proteins possible [37]. The tick organism could use sialylated glycoproteins to combat and inhibit the host immune defenses [40], [41]. It was also shown that undigested complete host originated glycoproteins were present in the saliva of the tick during feeding [37]

In this work, Neu5Ac is proposed as a recognition motif for the uptake and incorporation of sialylated glycoproteins into tick organism. In the immunoblots it is shown that cell lines, incubated with glycoproteins with intact sialic acid end groups (FITC), recycled and incorporated those proteins into the cell lines showing a broad distribution of bands but. Largely it seems the cell lines retained or recycled the labeled glycoproteins when sialic acid was not masked and largely discarded it when it was masked. The unexpectedly high signal for FTSC labeled fibrinogen could be because the labeled fibrinogen was detected and incorporated for another reason than its sialic acid, maybe a different glycans serves a similar function as a detection motif. It is also possible that by its overall size in comparison to the other three proteins (Table 6) it will contain orders of magnitude more sialic acid on its surface so a higher unspecific signal is expected. The side by side comparison of all three timeframes clearly shows a reduction in labeled protein concentration.

This work was the result of many optimization experiments for labeled protein incubation of tick cell lines. The labeling procedure, especially for the sialic acid proved to be tricky, as other labels like aminooxy-biotin did not produce reliable sialic acid specific results, or Alexa Fluor 488, which works extremely well and highly specific but at a high cost for large samples. In the end FTSC was used as it turned out reliable, easy to handle, and more cost efficient. Also, trials for labeling full blood serum were conducted but did not turn out well with the proposed labeling method, as the ultrafiltration step precipitated lots of proteins and caused unpredictable losses. *In vitro*

feeding of ticks was also attempted but fell short because of reliability reason (heatwaves causing tick shortages, ticks not feeding in the artificial feeding chambers, or timing issues with the fresh blood, etc.). Later, *in vitro* feeding and whole serum labeling were successfully used to detect sialic acid in tick egg and larva and tick cell lines using click chemistry [50].

9. Conclusion

The importance of sialic acid as the recognition motif of host glycoproteins by ticks in the model organism I. ricinus IRE / CTVM 19 derived embryo cells were examined by glycoprotein labeling with two different molecules (Fluorescein-5-isothiocyanate and Fluorescein-5-thiosemicarbazide). Based on the results, we believe that sialic acid, or glycan part of glycoproteins, is responsible for the recognition of these molecules by the tick cells.

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