Scientific paper

New Assay for Quantification of PEGylated Proteins During in Vitro Permeability Studies

Špela Jalen,[1,*] Vanja Smilović,[1] Katarina Fidler,[1] Barbara Podobnik,[1] Maja Marušić,[2] Radovan Komel,[2] Vladka Gaberc-Porekar[2] and Simona Jevševar[1]

[1] *Lek Pharmaceuticals d.d. (Sandoz Biopharmaceuticals), Kolodvorska 27, SI-1234 Mengeš, Slovenia*

[2] *National Institute of Chemistry, Hajdrihova 19, SI-1001 Ljubljana, Slovenia*

** Corresponding author: E-mail: spela.jalen@sandoz.com, Phone: +386 1 7297 927; Fax: + 386 1 7217 257*

Received: 15-01-2014

Abstract

One of the major challenges when analyzing very low amounts of PEGylated proteins is finding a sensitive analytical method. Immunoassays are most frequently used, however, conjugation can partially or completely mask protein epitopes, which can substantially lower the response and influence the quantitation range. Here we describe a novel assay that allows quantification of low amounts of PEGylated or differently conjugated proteins. The basic principle is similar to the classic sandwich ELISA but there are no antibodies used neither for capture nor for detection. Instead, Ni^{2+} chelation is exploited for capture and affinity between streptavidin and biotin for the detection step. The usefulness of the assay was proven in permeation studies (Caco-2 cell model) using diversely conjugated TNF-α protein. This approach could be extended to numerous other proteins eliminating the need to develop a separate assay for each protein/project.

Keywords: Immunoassay; PEGylation; His-tag; Biotin; TNF-α; Caco-2

1. Introduction

Conjugation of recombinant proteins has become a widely used technique for improving biomedical efficacy and physicochemical properties of therapeutic proteins. Currently, the most widespread and popular conjugation of recombinant proteins is probably PEGylation. It contributes important positive effects to proteins, such as reduced immunogenicity, increased solubility and prolonged elimination half-life.[1] Various PEGylated biopharmaceuticals have already been in use for at least a decade proving the applicability and safety of this technology.[2-9] In accordance with the successful achievements, it is expected that PEGylation will also be applied to other therapeutic proteins.[10,11]

Although therapeutic proteins have already been recognized as potent, safe and specific drugs, the administration is still very much limited to invasive, non-desirable routes, such as parenteral administration, where there are also some specific risks associated with this kind of administration for certain protein drugs.[12] In the last decade, many efforts have been made to achieve effective delivery of proteins and peptide drugs through various other routes of administrations to achieve successful therapeutic effects. Specifically, oral protein drug delivery as a patient-friendly choice of delivery has been extensively studied. The main obstacles for proteins to be delivered orally are enzymatic and acidic degradation in the stomach and low absorption of the protein through the intestinal barrier.[13] To overcome the obstacles, numerous approaches have been tested, such as chemical modification of the protein to improve stability and membrane permeability, use of enzyme inhibitors or absorption enhancers, as well as formulation vehicles and mucoadhesive polymeric systems. Among these approaches, protein modifications and use of mucoadhesive polymers seem to be most promising for protein and peptide drug delivery.[14]

In the late development phase of protein drugs *in vivo* PK/PD studies are necessary to evaluate potential toxicity, determine molecule's *in vivo* efficacy, and to select a dose for the first-in-human clinical trials.[15,16] During the

research and development of alternative protein drug delivery approaches, *in vitro* membrane permeability studies for the selected molecules are first performed. For this purpose the Caco-2 cell culture model, generally employed in permeation studies of small molecules, is also considered as a suitable tool for *in vitro* assessment of protein permeability.[17,18] Moreover, for *in vitro* and *in vivo* studies the analytical methods need to detect very low amounts of the tested substance in complex biological samples.

Since ELISA is by far the most sensitive analytical method for detecting proteins in complex mixtures, it is normally used as a quantitative assay. Independent of the type of conjugation (either PEGylation or other types of conjugation, such as sialylation, HESylation, and lipidization), protein surface is partially covered leading to weaker binding of antibodies in quantitative assays. Therefore, ELISA detection limits for conjugated proteins are generally significantly higher than for the unconjugated counterparts.[1,19,20]

To overcome this problem, commercially available competitive anti-PEG ELISA (Epitomics) seems to be a good solution. However, due to relatively high detection limit of this ELISA, it cannot be used for all applications. Recently, Cheng and colleagues introduced another anti-PEG ELISA with higher sensitivity when compared with traditional anti-interferon ELISA (specifically for Pegasys).[21] Anyway, the anti-PEG ELISA is restricted to PEGylated proteins and cannot be used for other types of protein conjugations, such as lipophilic modification or HESylation.

In addition to anti-PEG ELISAs, various other novel immunoassays have been developed lately in order to increase specificity and sensitivity. Sakamoto and colleagues developed FLISA in order to detect low amount of ginsenosides.[22] Liberelle and colleagues developed ELISA based on E/K coiled-coil interactions showing a similar sensitivity to standard capturing antibody-based ELISA systems.[23] In another study, Ni-IDA was used for capture of the ZZ-his protein to achieve correctly oriented binding of monoclonal antibody to polystyrene plate.[24]

While studying the potential impact of protein conjugation on protein drug delivery through the intestinal barrier, we used a tumor necrosis factor alpha (TNF-α) analog as a model molecule. The protein was conjugated with different types of substances – including extensive PEGylation and labeling with biotin. To measure the quantity of the protein that crossed the intestinal barrier in an *in vitro* model (Caco-2), we developed a novel type of quantitation assay, similar to classic sandwich ELISA with the distinction that there are no antibodies used neither for capture nor for detection. Instead, immobilized metal affinity system (Ni-chelate) is used for capture of the histidine-tagged model protein and biotin-streptavidin system is employed for detection.

The intestinal barrier permeability studies on Caco-2 cells showed the applicability of the newly developed quantitation assay and confirmed that it is possible to overcome the sensitivity issues in the analysis of the protein with a partially or completely masked surface.

2. Experimental

2. 1. Materials

2. 1. 1. Model Molecule

His-tagged TNF-α was used as a model molecule. It is a TNF-α that has six amino-acid residues deleted from the N-terminus and seven histidine residues added to it (Swiss prot accession P01375, soluble TNF-α: residues 77–233; residues 77–82 were deleted and seven histidine residues were added to the N-terminus). His-tagged TNF-α was prepared as described previously (Fonda 2002), but in this study the cleavage of histidine tags was not performed. His-tagged TNF-α was further conjugated as described below (Methods).

2. 1. 2. Anti-HIS anti-Biotin Assay

Nunc-IMMOBILIZER, NI-CHELATE microtiter plates were purhased from Nunc (cat. # 436024), AMDEX Streptavidin-HRP was purchased from GE Healthcare (cat. # RPN4401). 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (cat. # T0440) and Stop reagent (cat. # S5814) were purchased from Sigma-Aldrich. PBS (10x) was purchased from Invitrogen and Tween 20 from Merck (cat. # 8.22184.0500).

2. 1. 3. Anti-TNF ELISA

Maxi-sorp microtiter plates were purhased from Nunc (cat. # 439454), BuPH Carbonate-bicarbonate buffer packs (0,2M Na-Carbonate-Bicarbonate Buffer, pH = 9.4) from Pierce (cat. # 28382). Bovine Serum Albumine (BSA) was purchased from Sigma-Aldrich (cat. # A7888). anti-human TNF antibodies (cat. # MAB610) and biotinylated anti-human TNF-α antibodies (cat. # BAF210) were both purchased from R&D Systems. Other reagents were the same as for anti-HIS anti-Biotin assay.

2. 1. 4. Protein Conjugations

Branched PEG-NHS reagents, NHS-PEG(4)-[PEG(12)-OMe]3 (MW = 2420.8 g/mol) and NHS-PEG (4)-[PEG(4)-OMe]3 (MW = 1363.54 g/mol) and a short PEG reagent with an –SH reactive group were purchased from Iris Biotech (cat. # PEG1840, # PEG2300 and #PEG1167). NHS-PEG4-Biotin (cat. # 21335, MW = 588 g/mol) and bifunctional PEG reagent with maleimide and -NHS reactive groups (cat. # 22105, MW = 601.6 g/mol) were purchased from Pierce (Thermo Scientific) as well as a short hydrophobic crosslinker Sulfo-EMCS (cat. #

22307), Sulfo-LC-SPDP (cat. # 21650, MW = 527.57 g/mol) and disulfide reducing gel TCEP (cat. # 77712). Amicon Ultra-15 centrifugal filter units for concentration of the conjugates were from Millipore (cat. #UFC901024).

2. 1. 5. Caco-2 Tests

Caco-2 cells were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). Minimum essential medium eagle (MEM; cat. # M5650), Dulbecco's modified eagle's medium (DMEM; cat. # D5921), heat inactivated FBS (cat. # F4135), L-glutamine (cat. # G-7513), non-essential amino acids (cat. # M7145) and trypsin (cat. # T4049) were purchased from Sigma-Aldrich and antibiotic/antimycotic solution (cat. # 15240) was obtained from Invitrogen. Permeability experiments were performed on Transwell® (Costar, cat. # 3460) polyester permeable supports (diameter, 12 mm; pore size 0.4 μm). Other reagents were purchased from Sigma-Aldrich (unless otherwise stated).

2. 2. Methods

2. 2. 1. Biotinylation of His-tagged TNF-a

First, His-tagged TNF-α was labeled with biotin using a Biotin-PEG4-NHS reagent (Pierce). Biotin-PEG4-NHS reagent was added in a threefold molar excess to H7dN6-TNF. The excess of the reagent was removed using gel permeation chromatography. The conjugate was designated as Biotin-His-TNF.

2. 2. 2. PEGylation of Biotinylated His-tagged TNF-a

PEGylation of biotinylated His-tagged TNF-α was prepared using two different approaches: (1) PEGylation using branched PEG reagents and (2) conjugation using small PEG reagent and lipophilic molecule.

1) PEGylation using two different types of branched PEG-NHS reagents (NHS-PEG(4)-[PEG(12)-OMe]$_3$ and NHS-PEG(4)-[PEG(4)-OMe]$_3$). In both cases, 72-molar excess of branched-PEG-NHS reagent (either NHS-PEG(4)-[PEG(12)-OMe]$_3$ or NHS-PEG(4)-[PEG(4)-OMe]$_3$) was added to Biotin-His-TNF. Excess of the reagent was removed using gel permeation chromatography. The conjugate prepared using larger PEG reagent NHS-PEG(4)-[PEG(12)-OMe]$_3$ was designated as PEG12-Biotin-His-TNF, the conjugate prepared using smaller branched PEG reagent NHS-PEG(4)-[PEG(4)-OMe]$_3$ was designated as PEG4-Biotin-His-TNF.
2) Biotin-His-TNF was conjugated using a small PEG reagent (MeO-PEG6-SH, 600 Da) and a short lipophilic spacer (activated hexanoic acid, Sulfo-EMCS) in one step – 72-molar excess of Sulfo-EMCS reagent and 216-molar excess of MeO-PEG(7)-SH reagent were added to Biotin-His-TNF. Excess of the reagents was removed using gel permeation chromatography. The conjugate was designated as PEG-C6-Biotin-His-TNF.

The last conjugate, designated C6-PEG-Biotin-His-TNF, was prepared.as follows. First, the solution of 108-molar excess of Sulfo-LC-SPDP was incubated overnight at 20 °C to assure complete hydrolysis of reactive Sulfo-NHS group. The disulfide bond in hydrolyzed Sulfo-LC-SPDP was reduced using appropriate quantity of TCEP. A 36-molar excess of Mal-PEG6-NHS was incubated 2h/20 °C with Biotin-His-TNF, afterwards reduced and hydrolyzed Sulfo-LC-SPDP was added to the solution. The solution was then incubated overnight at room temperature. Excess of the reagents was removed using gel permeation chromatography.

2. 2. 3. Anti-His anti-Biotin Assay

The Ni-Chelate mictrotiter plate was washed 4x using PBS-T buffer (PBS + 0.05% Tween 20). 150 μL of standards, blanks and samples were added to the microtiter plate in duplicates. The plate was sealed and incubated for 3h at RT without shaking. Then, it was washed 4x with PBS-T buffer. 150 μL of AMDEX streptavidin-HRP (1:4000) in PBS-T buffer was added to the plate. The plate was sealed, incubated for 2 h and washed 4x with PBS-T buffer. 150 μL of ready-to-use TMB substrate solution was added to the plate. After approx. 20 min the reaction was stopped with 150 μL of TMB stop solution. Absorbance at 450 nm and 600 nm (for correction) was measured using a Biotek Synergy HT plate reader. Detection limit of anti-His anti-Biotin assay was determined as the protein amount corresponding to background absorbance plus threefold standard deviation.

2. 2. 4. Anti-TNF ELISA

Anti-TNF ELISA was performed for basic conjugate His-tagged TNF-α following an in-house procedure using commercially available antibodies.

2. 2. 5. Caco-2 test

Caco-2 tests were performed as previously described[18] with the difference, that experiments were performed in HBSS buffer and 0.02% NLS was used as a permeation enhancer.[25]

3. Results and Discussion

To improve permeability through the intestinal barrier, two approaches to modify the biotin-labeled model protein molecule (His-tagged TNF) were used: (1) exten-

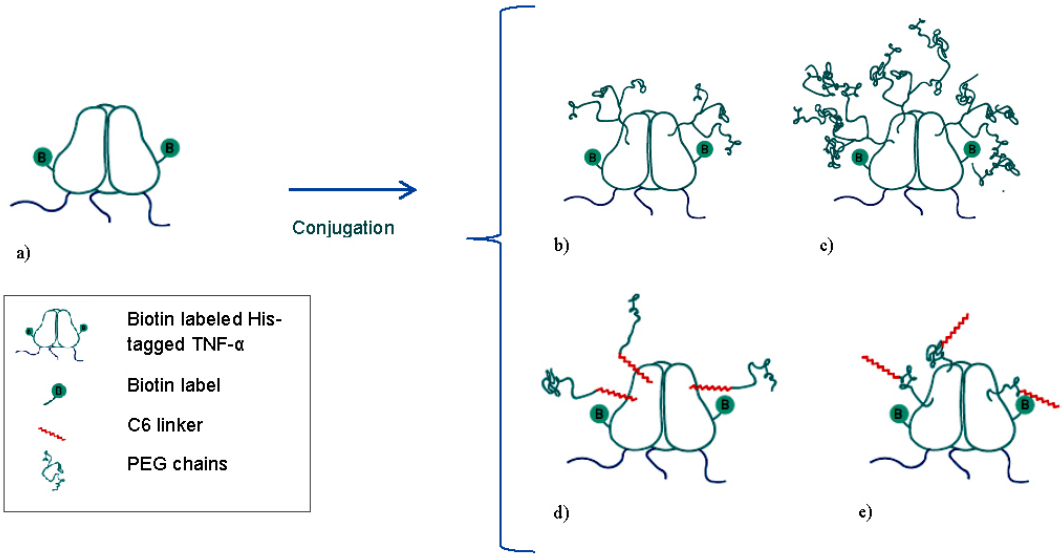


Figure 1: Schematic representation of differently PEGylated Biotin-His-TNF molecules. (a) Biotin labeled His-tagged TNF-α (Biotin-His-TNF) that was used for all further conjugations (Biotin randomly attached to the lysines); (b) Biotin labeled His-tagged TNF-α PEGylated with a small branched PEG reagent, PEG4-Biotin-His-TNF (1.4 kDa PEG chains), and (c) Biotin labeled His-tagged TNF-α PEGylated with a larger branched PEG reagent, PEG12-Biotin-His-TNF (2.4 kDa PEG chains). Hydrophobizated conjugates are presented in (d) PEG-C6-Biotin-His-TNF and (e) C6- PEG-Biotin-His-TNF.

sive PEGylation using two different types of branched PEG reagents with longer and shorter PEG branches, and (2) a combination of PEGylation reaction using small PEG reagents and hydrophobization with a short acyl chain (C_6) to increase lipophilicity. Conjugates are schematically represented in Figure 1. For extensive PEGylation, branched PEG reagents were used, somehow mimicking small dendrimer structures because dendrimers are known to easily penetrate through biological membranes and also through the intestinal barrier.[26] The other approach aiming at increased lipophilicity of the Biotin-His-TNF was used, since the lipophilization has already been proved to facilitate transfer of the therapeutic drugs across the mucosal barrier.[14,27,28]

Although nowadays various types of conjugation reactions are a generally recognized tool for improving properties of pharmaceutical proteins, detection of PEGylated and otherwise modified proteins, necessary for *in vitro* and *in vivo* studies during the protein drug development, still represents a challenge. Due to partially or completely covered protein epitopes traditional ELISA assays are usually not sensitive enough for detecting low amounts of conjugated proteins in complex biological mixtures (e.g. serum samples in PK studies), or during *in vitro* membrane permeation assays (e.g. Caco-2 cell model). For PEGylated proteins a commercially available competitive anti-PEG ELISA could be a solution, however, due to sensitivity issues it is as well not appropriate for analyzing low amounts of highly PEGylated proteins. On the other hand, usefulness of sandwich anti-PEG ELISA for detection of PEGylated interferon was proved,[21] however, it cannot be used for detection of proteins modified with short PEG chains. In addition, sialylated, lipophilizated or HESylated proteins cannot be detected either.

3. 1. Anti-His anti-Biotin Assay

To overcome these obstacles, we developed a new assay appropriate for all histidine rich (bearing either natural histidine patches or engineered histidine tags) and biotin-labeled proteins. The newly developed quantitation assay does not utilize antibodies at all. Since all the conjugates contained histidine tags, Ni^{2+} – His interaction is utilized for capture exploiting the adsorption properties of proteins based on the coordination between an immobilized metal ion and electron donor groups from the protein surface, mostly side chains of histidine residues. Ni^{2+} ion has 6 positions available for formation of coordinative bonds with exposed histidine residues of the protein and chelator molecules, exhibiting very high affinity for histidine-rich proteins.[29,30]

On the other hand all conjugates were labeled with biotin. Therefore, affinity between streptavidin and biotin, which is one of the strongest non-covalent interactions in nature, was employed for detection. This combination resulted in a very simple and sensitive assay which can be used for a variety of different histidine rich proteins labeled with biotin. The newly developed quantitation assay was designated as anti-His anti-Biotin assay (schematically presented in Figure 2). The novel assay is very specific due to strong streptavidin-biotin and Ni^{2+}-His tag inte-

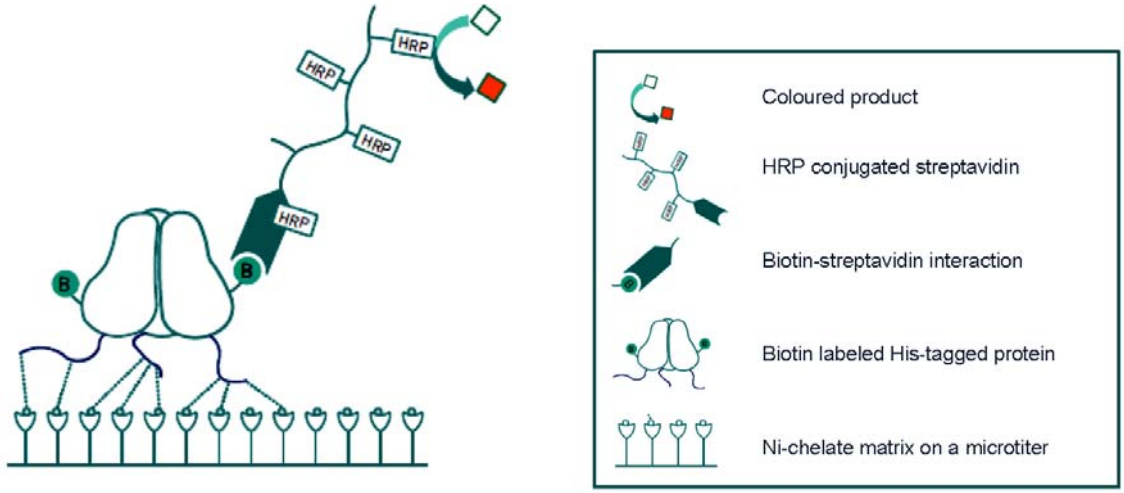


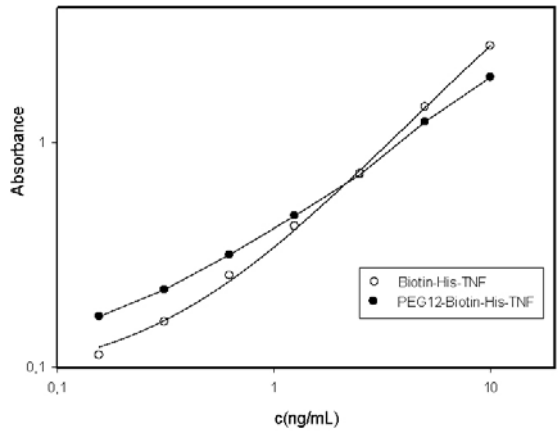
Figure 2: Schematic representation of anti-His anti-Biotin assay. Ni-Chelate is bound to the ELISA plate surface. Using very strong interaction between Ni^{2+} and His-tag on the protein, protein is captured to the plate. Detection is performed using HRP-streptavidine conjugate, which interacts strongly with Biotin attached to the protein.

ractions. On the other hand, no particular interference of PEG chain groups of different lengths with Ni^{2+} or chelator molecule could be expected.[31]

The limit of detection (LOD) of the new anti-His anti-Biotin assay, defined as the protein amount corresponding to background absorbance plus threefold standard deviation, was determined for each conjugate. LOD for conjugates was determined to be 40 – 130 pg/mL (Figure 3). LOD values for extensively PEGylated conjugates are higher than for basic conjugate, still low enough to evaluate permeate concentrations in Caco-2 experiments. As expected, the lowest LOD values were observed for the basic conjugate Biotin-His-TNF (40 pg/mL) and PEG4-Biotin-His-TNF conjugate (40 pg/mL). Conjugates with lipophilic molecules attached (PEG-C6-Biotin-His-TNF and C6-PEG-Biotin-His-TNF) are also readily detectable (LODs 50 and 60 pg/mL, respectively), whereas conjugate PEG12-Biotin-His-TNF, with longer PEG chains, has a somewhat higher LOD (130 pg/mL), but can still be readily measured in Caco-2 assay.

In contrast to classic anti-TNF ELISA, sensitivity of anti-His anti-Biotin ELISA is high enough to detect very low amounts of extensively PEGylated TNF conjugates in Caco-2 permeation assay. The LOD value of extensively PEGylated conjugate obtained by anti-TNF ELISA was around four times higher than LOD obtained by the newly developed anti-His anti-Biotin ELISA (500 pg/mL compared to 130 pg/mL). In addition, when the LOD values of the novel ELISA assay were compared with the commercially available competitive anti-PEG ELISA, the latter showed substantially higher LOD value (estimated to 8 ng/ml for extensively PEGylated TNF-α), which is absolutely not appropriate for detection of the selected conjugates in Caco-2 experiments. The novel ELISA assay is four times more sensitive in comparison to the classic anti-TNF ELISA and has two orders of magnitude higher

a)



b)

Conjugate	LOD value
Biotin-His-TNF	40 pg/ml
PEG4-Biotin-His-TNF	40 pg/ml
PEG12-Biotin-His-TNF	130 pg/ml
PEG-C6-Biotin-His-TNF	50 pg/ml
C6-PEG-Biotin-His-TNF	60 pg/ml

Figure 3: (a) Examples of standard curves for PEG12-Biotin-His-TNF and Biotin-His-TNF. (b) LOD values of anti-His anti-Biotin ELISA for each of the conjugates.

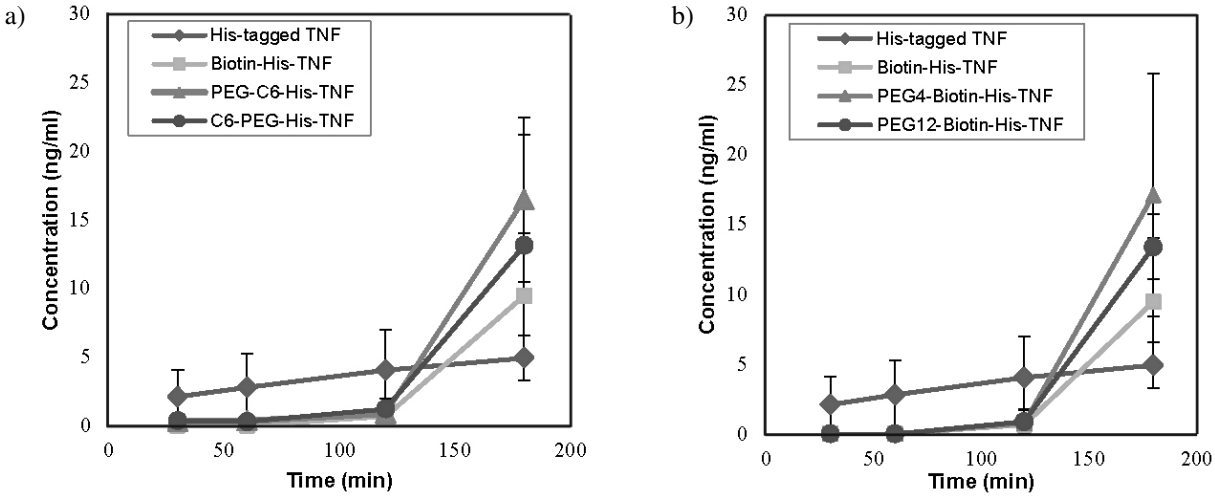
sensitivity than commercially available anti-PEG ELISA, being essential for measuring very low amounts of PEGylated proteins in permeability studies.

3. 2. Caco-2 Tests

All conjugates as well as the basic TNF-α analog (His-tagged TNF-α) were tested *in vitro* using a Caco-2 cell permeability model. In each Caco-2 experiment, His-tagged TNF-α was used as a reference and its concentration on the basolateral site of the Caco-2 monolayer was measured using an in-house anti-TNF ELISA. For all conjugates, the newly developed anti-His anti-Biotin assay was used, since the sensitivity of anti-TNF ELISA was substantially lowered due to conjugation reactions and consequently partially masked surface of the TNF-α. From the obtained concentrations at the basolateral site, the permeation coefficients (P_{app} values) were calculated, using the procedure described in the recent paper of Marušić et al.[18] Results are presented in Figure 4. Transepithelial electric resistance (TEER) was monitored during and after the Caco-2 test as described earlier[18] to assure that substances in the test or handling do not affect the integrity of the cell monolayer.

All conjugates showed higher permeation rates as the His-tagged TNF-α analog, which could be described as 3–6 times higher permeation coefficients P_{app}. However, there are clear differences in permeation coefficients observed for different kinds of conjugates. The highest P_{app} values were observed for the conjugate PEGylated with small branched PEG reagent (PEG4-Biotin-His-TNF) and for the conjugate bearing C6 lipophilic spacer between protein and PEG chains (PEG-C6-Biotin-His-TNF). These two conjugates showed approximately 6-times higher permeability than the His-tagged TNF-α. In both cases, the P_{app} values are 1.2×10^{-8} cm/s as compared to a P_{app} of 2.1×10^{-9} cm/s for the His-tagged TNF. The lowest permeability increase was observed for the basic conjugate, Biotin-His-TNF (3-times higher than His-tagged TNF-α analog).

While for PEGylated and hydrophobic conjugates higher permeability was expected, in the case of the basic conjugate (Biotin-His-TNF) the enhanced permeation (in comparison to His-tagged TNF-α) could be explained by biotinylation itself. Chae and colleagues have already shown that biotinylation and especially biotin-PEGylation of Glucagon-like peptide 1 increased permeation (absorption) of the conjugate into bloodstream from rat intestine (oral application).[32] Nevertheless, biotinylation (or combined PEGylation and biotinylation) as a promising ap-



c)

Conjugate	P_{app}(cm/s)	Relative P_{app}
His-tagged TNF	2.1×10^{-9}	1
Biotin-His-TNF	6.8×10^{-9}	3.2
PEG4-Biotin-His-TNF	1.2×10^{-9}	5.7
PEG12-Biotin-His-TNF	9.6×10^{-9}	4.6
PEG-C6-Biotin-His-TNF	1.2×10^{-9}	5.7
C6-PEG-Biotin-His-TNF	9.2×10^{-9}	4.4

Figure 4: Overview of the permeability study results for the model molecule (His-tagged TNF) and its conjugates. Graphical representation of the permeability study results: (a) lipophilic conjugates, (b) PEGylated conjugates. For comparison, His-tagged TNF and basic conjugate Biotin-His-TNF are included on both graphs. (c) Calculated permeability coefficients (P*app*) for each conjugate. Relative P*app* is calculated relatively to the His-tagged TNF.

proach for permeation enhancement has so far never been shown on a complex structure, such as a protein.

Interestingly, all conjugates prepared showed different permeation kinetics in comparison with His-tagged TNF-α. There is a lag phase up to 120 min, where only little of the conjugates successfully penetrate the Caco-2 barrier, and afterwards the concentration on the basolateral site significantly increases for all conjugates (Figure 4). Potential explanation could be, that biotinylation promotes receptor-mediated transcytosis,[33] where initial lag phase could be expected.

4. Conclusions

To detect low amounts of extensively conjugated proteins, a new quantitation assay was developed. During the development, very good sensitivity of the anti-His anti-Biotin assay was shown with LOD values of variously PEGylated TNF-α ranging from 40 pg/mL to 130 pg/mL. The applicability of the assay was proven within Caco-2 cell permeation studies, where five different protein conjugates were tested.

Anti-His anti-Biotin assay could be potentially used for numerous other proteins, such as Lys26-Biotin-Lys34-(Biotin-PEG)- Glucagon-like peptide 1,[32] as the only prerequisite is that the protein has a histidine-rich patch and a biotin label. The development of a new immunoassay for each protein/project separately could therefore be omitted, which could speed up the research substantially. In addition, an unified analytical method for different types of modifications is highly recommended when comparing the results.

Furthermore, biocompatibility of our approach, from histidine tags, which have already been used in medical applications,[34,35] to biotin labeling (biotin being an essential vitamin and micronutrient for normal cellular functions in the human body) makes a good basis for using the same method in early *in vitro* studies as well as later in *in vivo* studies on animals and possibly even in clinical studies.

5. Acknowledgment

We acknowledge Mrs. T. Tomšič for her excellent technical support in preparation of TNF analog (His-tagged TNF-α). This work was supported by the Slovenian Research Agency and by the European Commission under the Sixth Framework Programme.

6. Abbrevations

TNF-α: Tumor necrosis factor alpha; PK study: pharmacokinetic study; PD study: Pharmacodynamic study; ELISA: Enzyme-linked immunosorbent assays; FLISA: fluorescence-linked immunosorbent assays; HRP: horseradish peroxidase; FBS: fetal bovine serum; LOD: limit of detection; TEER: Transepithelial electric resistance; PEG: polyethylene glycol; P*app*: permeation coefficient; PBS: phosphate buffered saline

7. References

1. V. Gaberc-Porekar, I. Zore, B. Podobnik, V. Menart, *Current Opinion in Drug Discovery and Development.* **2008**, *11*, 242–250
2. C. Booth, H. B. Gaspar, *Biologics.* **2009**, *3*, 349–358
3. G. R. Foster, *Drugs.* **2010**, *70*, 147–165
4. B. B. Yang, M. A. Savin, M. Green, *Chemotherapy.* **2012**, *58*, 387–398
5. S. Pradhananga, I. Wilkinson, R. J. Ross, *J. Mol. Endocrinol.* **2002**, *29*, 11–14
6. W. Sulowicz, F. Locatelli, J. P. Ryckelynck, J. Balla, B. Csiky, K. Harris, P. Ehrhard, U. Beyer, *Clin. J. Am. Soc. Nephrol.* **2007**, *2*, 637–646
7. W. Kaushik, R. J. Moots, *Expert Opinion on Biological Therapy.* **2005**, *5*, 601–606
8. W. J. Sandborn, B. G. Feagan, S. Stoinov, P. J. Honiball, P. Rutgeerts, D. Mason, R. Bloomfield, S. Schreiber, I. Study, *New England Journal of Medicine.* **2007**, *357*, 228–238
9. R. L. George, Jr., J. S. Sundy, *Drugs Today (Barc.).* **2012**, *48*, 441–449
10. S. Jevsevar, M. Kunstelj, V. G. Porekar, *Biotechnology Journal.* **2010**, *5*, 113–128
11. R. E. Kontermann, *Current Opinion in Biotechnology.* **2011**, *22*, 868–876
12. M. Werle, A. Makhlof, H. Takeuchi, *Recent Patents on Drug Delivery and Formulation.* **2009**, *3*, 94–104
13. S. Gupta, A. Jain, M. Chakraborty, J. K. Sahni, J. Ali, S. Dang, *Drug Delivery.* **2013**, *20*, 237–246
14. J. Shaji, V. Patole, *Indian Journal of Pharmaceutical Sciences.* **2008**, *70*, 269–277
15. J. A. Cavagnaro, *Nature Reviews.* **2002**, *Drug Discovery. 1*, 469–475
16. J. Tibbitts, J. A. Cavagnaro, C. A. Haller, B. Marafino, P. A. Andrews, J. T. Sullivan, *Regulatory Toxicology & Pharmacology.* **2010**, *58*, 243–251
17. A. C. Foss, N. A. Peppas, *European Journal of Pharmaceutics and Biopharmaceutics.* **2004**, *57*, 447–455
18. M. Marusic, T. Zupancic, G. Hribar, R. Komel, G. Anderluh, S. Caserman, *New Biotechnology.* **2013**, *30*, 507–515
19. P. Bailon, W. Berthold, *Pharmaceutical Science and Technology Today.* **1998**, *1*, 352–356
20. I. Mahmood, M. D. Green, *Clin. Pharmacokinet.* **2005**, *44*, 331–347
21. T.-C. Cheng, K.-H. Chuang, M. Chen, H.-E. Wang, S.-C. Tzou, Y.-C. Su, Chuang C.-H., C.-H. Kao, B.-M. Chen, L.-S. Chang, S. R. Roffler, T.-L. Cheng, *Bioconjugate Chemistry.* **2013**, *24*, 1408–1413

22. S. Sakamoto, Y. Tanizaki, B. Pongkitwitoon, H. Tanaka, S. Morimoto, *Protein Expression and Purification.* **2011**, *77*, 124–130
23. B. Liberelle, L. Bartholin, C. Boucher, F. Murschel, M. Jolicoeur, Y. Durocher, A. Merzouki, C. G. De, *Journal of Immunological Methods.* **2010**, *362*, 161–167
24. H. M. Yang, S. J. Liang, J. B. Tang, Y. Chen, Y. Z. Cheng, *Analytical Biochemistry.* **2013**, *432*, 134–138
25. K. Whitehead, N. Karr, S. Mitragotri, *Pharmaceutical Research.* **2008**, *25*, 1782–1788
26. Y. Y. Cheng, Z. H. Xu, M. L. Ma, T. W. Xu, *Journal of Pharmaceutical Sciences.* **2008**, *97*, 123–143
27. M. J. Hackett, J. L. Zaro, W. C. Shen, P. C. Guley, M. J. Cho, *Advanced Drug Delivery Reviews* **2013**, *65*, 1331–1339
28. N. Kocevar, N. Obermajer, S. Kreft, *Chemical Biology and Drug Design.* **2008**, *72*, 217–224
29. V. Gaberc-Porekar, V. Menart, *Journal of Biochemical and Biophysical Methods.* **2001**, *49*, 335–360
30. L. R. Paborsky, K. E. Dunn, C. S. Gibbs, J. P. Dougherty, *Analytical Biochemistry.* **1996**, *234*, 60–65
31. A. Mero, T. Ishino, I. Chaiken, F. M. Veronese, G. Pasut, *Pharmaceutical Reasearch.* **2011,** *28*, 2412–21
32. S. Y. Chae, C. H. Jin, H. J. Shin, Y. S. Youn, S. Lee, K. C. Lee, *Bioconjugate Chemistry.* **2008**, *19*, 334–341
33. M. Morishita, N. A. Peppas, *Drug Discovery Today.* **2006**, *11*, 905–910
34. N. Ferrer-Miralles, J. L. Corchero, P. Kumar, J. A. Cedano, K. C. Gupta, E. Vazquez, *Microbial cell factories.* **2011**, *10*, 101
35. J. Malliaros, C. Quinn, F. H. Arnold, M. J. Pearse, D. P. Drane, T. J. Stewart, R. I. Macfarlan, *Vaccine.* **2004**, *22*, 3968–3975

Povzetek

Pri analizi zelo nizkih količin PEGiliranih proteinov predstavlja enega glavnih izzivov izbor primerno občutljive analitske metode. Najpogosteje uporabljamo imunološke metode, vendar lahko konjugacija delno ali popolnoma prekrije proteinske epitope, kar lahko bistveno vpliva na občutljivost metode. Tukaj opisujemo novo metodo, ki omogoča kvantitativno določitev nizkih količin PEGiliranih ali drugače konjugiranih proteinov. Osnovni princip je podoben klasični metodi ELISA, vendar ne uporabljamo protiteles, niti za vezavo niti za detekcijo. Namesto tega za vezavo proteina izkoriščamo kelatno vezavo na Ni^{2+}, za detekcijo pa afiniteto med streptavidinom in biotinom. Uporabnost metode smo potrdili v študijah prehajanja celičnega enosloja (model Caco-2), pri čemer smo uporabili različno konjugiran protein TNF-α. Opisani pristop bi lahko razširili na številne druge proteine in se tako izognili razvoju metod za vsak protein/projekt posebej.