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ORIGINAL ARTICLE

ELECTROPHORESIS AND RAMAN SPECTROSCOPY CHARACTERIZATION OF INTEGRITY AND SECONDARY STRUCTURE OF *p*-SCN-Bn-DTPA- AND *p*-SCN-Bn-1B4M-DTPA-CONJUGATED TRASTUZUMAB

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Abstract

Trastuzumab is a humanized monoclonal antibody approved for treatment of HER2 (human epidermal growth factor 2) positive breast cancer. Conjugation of trastuzumab offers a promising strategy of selective anticancer therapy. Trastuzumab-emtansin is a new generation of cytotoxic drug conjugated antibody with higher tumour selectivity and less toxicity of emtansin. Conjugates of trastuzumab with bifunctional chelators (BFCs) for further radiolabelling are a step ahead in the field of radiopharmacy for therapy and imaging of aggressive HER2 positive cancers. The purpose of this study is characterization of integrity and secondary structure of antibody in already formulated lyophilized conjugates with *p*-SCN-Bn-DTPA- and *p*-SCN-Bn-1B4M-DTPA- by applying SDS-PAGE electrophoresis and Raman spectroscopy. The results are positive and give an opportunity for further radiolabelling of freeze dried conjugates.

Rezumat

Trastuzumab este un anticorp monoclonal umanizat, aprobat pentru tratamentul cancerului de sân HER 2 (factorul de creștere epidermal uman 2) pozitiv. Conjugarea trastuzumabului oferă o strategie promițătoare de terapie selectivă împotriva acestei afecțiuni. Trastuzumab-emtansin este o nouă generație de anticorpi conjugați cu medicamente citotoxice, cu o selectivitate crescută asupra tumorii și o mai mică toxicitate datorită emtansinei. Conjugatele trastuzumabului cu chelatori bifuncționali (BFC) pentru radiomarcare ulterioară sunt un pas înainte în domeniul radiofarmaceutic pentru terapia și imagistica cancerelor HER 2 pozitive agresive. Scopul acestui studiu a fost caracterizarea integrității și a structurii secundare a anticorpului în conjugatele liofilizate, formulate cu *p*-SCN-Bn-DTPA- și *p*-SCN-Bn-1B4M-DTPA-, prin aplicarea electroforezei *SDS-PAGE* și a spectroscopiei RAMAN. Rezultatele sunt promițătoare și oferă o oportunitate pentru marcarea ulterioară a derivaților conjugați.

Keywords: trastuzumab, p-SCN-Bn-DTPA, *p*-SCN-Bn-1B4M-DTPA, electrophoresis, Raman spectroscopy

Introduction

HER2 receptor (Human epidermal growth factor 2) is a part of tyrosine kinase receptor family. Over-expression and amplification of HER2 has been associated with 20 - 30% of patients with primary breast cancer. This condition is correlated with aggressive and metastatic carcinoma with poor clinical prognosis [4, 17]. Due to the easy availability of the extracellular domain of HER2 and high affinity binding of trastuzumab to the receptor, Herceptin® was approved for treatment of aggressive HER2 positive cancers [11]. Trastuzumab, humanized monoclonal antibody, has shown significant improvement of clinical condition of patient, particularly in combined administration with other cytotoxic drugs [25]. The binding of naked monoclonal antibodies with cytotoxic drugs achieves higher tumour selectivity of drugs that are too toxic to be separately used. Conjugation of drugs with antibodies reduces the risk of drug resistance [7]. After many years of examinations in the field of HER2 positive cancers, a novel, more stable, conjugate of trastuzumab is formulated – trastuzumab-emtansine (T-DM1), which is approved for treatment of advanced metastatic breast cancer. The T-DM1 shows better activity and selectivity compared with naked antibody [22]. Besides the cytotoxic drug-trastuzumab conjugation, radiolabelling of the anti-body *via* BFCs is crucial for development of therapeutic and potent agents for molecular imaging and identification of metastatic lesions [2]. Significant

radiopharmaceuticals, based on peptide and antibody, designed for diagnostic and therapeutically purpose involve different radioisotopes (^{99m}Tc/^{188}Re, ^{67}Ga, ^{177}Lu, ^{90}Y, ^{131}I, ^{18}F). Several studies have shown that already existing radiolabelled formulations, using beta and alfa emitters (^{90}Y, ^{86}Y, ^{177}Lu, ^{227}Th, ^{225}Ac) are potent against HER2 positive breast cancer [21, 28]. The most sensitive marker for detecting hypermetabolic sites and identification of metastatic lesions are: γ camera (planar scintigraphy), computed tomography and positron emission tomography (PET) [10]. Until now, the most investigated BFCs for successful labelling of proteins and antibodies are: DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), DTPA (diethylene triamine pentaacetic acid), TCMC (1,4,7,10-tetra-(2-carbamoyl methyl)-cyclododecane), HYNIC (succinimidyl-6-hydrazino-nicotinamide) and DTPA derivate 1B4M-DTPA (2-(4-isothiocyanatobenzyl)-6-methyl-diethylene-triamine-pentaacetic acid) [3].

The purpose of this study was the characterization of possible changes in secondary structure of antibody after freeze dried kit formulation with *p*-SCN-Bn-1B4M-DTPA (2-(4-isothiocyanatobenzyl)-6-methyl-diethylene-triaminepentaacetic acid) and *p*-SCN-Bn-DTPA (2-(4-izothiocyanatobenzyl)-diethylenetriaminepentaacetic acid) (Figure 1), by using SDS-PAGE electrophoresis under reducing conditions and Raman spectroscopy.

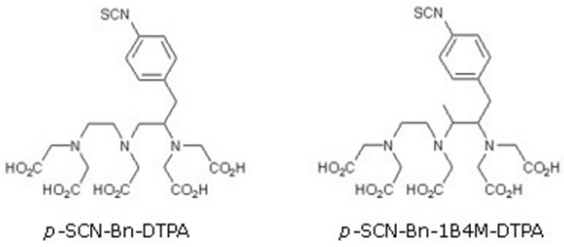

Figure 1.
Chemical structure of chelators for trastuzumab conjugation

Materials and Methods

Trastuzumab-BFCs conjugation. The chelators prepared as 10 mg/mL solutions, were mixed with purified antibody from Herceptin® (Hoffmann-La Roche, Basel, Switzerland), in 10 and 50-fold molar excess by gentle shaking for 18 hours at 4ºC. The purification of antibodies and conjugates were made with Amicon® Ultra-4, 30 kDa (Sigma Aldrich, Missouri, USA) in six cycles, using HuMax 4k (Human, Wiesbaden, Germany) centrifuge. The concentration of trastuzumab-conjugates was adjusted to 1 mg/mL using the 6715 UV/Vis Spectrophotometer Jenway® (Staffordshire, UK).

Process of freeze drying. Modified protocol from Gjorgieva Ackova *et al*. [8] was applied for freeze drying process, using Labconco Free Zone Stoppering Tray Dryer (Kansas City, Missouri, USA). The volume of 1 mL of the liquid immunoconjugates was transferred to type I glass vials and equally placed on the shelves after reaching the temperature of 4°C. The freezing step was performed at -40ºC at a rate of 1ºC/min and the hold time for 5 hours. The primary drying was performed at pressure of 0.133 mBar and temperature of -10ºC at a rate of 0.15ºC/min and held for 28 hours. For secondary drying the temperature was reached to 25ºC at a rate of 0.2ºC/min for 14 hours. After completing the whole process, the vials were closed and kept at 4°C in order to perform the following examinations.

SDS-PAGE for antibody integrity analysis. SDS-PAGE electrophoresis was performed with Enduro® Modular Vertical Gel Electrophoresis System (Labnet, Edison, NJ, USA), in reduced conditions (2-mercaptoethanol). According to the protocol of the manufacturer, 12% separating acrylamide/bisacrylamide gel and 4% stacking gel were prepared. To 30 µg of the sample with concentration of 1 mg/mL was added 10 µL of loading buffer. The mixtures were heated 5 min at dry bath at 98.5ºC and centrifuged for 5 min at 5000 rpm. 25 µL of every sample and 10 µL of SigmaMarker™, Wide Range, Molecular Weight (Saint Louis, MO, USA) were applied in wells of stacking gel. According to our experience, constant voltage at 150 V and approximately current at 23 mA have been used. Coomassie staining was performed after finishing the entire process. All used reagents were from Sigma-Aldrich (Missouri, USA). The prepared gel was scanned with Glite 900 BW Gel Scanner (PacificImage, Torrance, CA).

Raman spectroscopy to monitor protein secondary structure. Freeze dried samples were directly applied under the laser beam of the instrument. The room temperature Raman spectra (2000 - 100 cm^{-1}) were recorded on micro-Raman multichannel spectrometer Horiba Jobin Yvon LabRam 300 Infinity. The Raman effect was obtained using 632.8 nm from a He:Ne laser, without the use of the attenuation filter. An Olympus MPlanN confocal microscope with ×50 objective was selected and the spectral resolution was set to 4 cm^{-1}. A confocal hole of about 2 µm was used and the position on the sample surface was manipulated by a motorized *x-y* stage. The Raman shift was calibrated by using the Raman peak of silica located at 520.7 cm^{-1}. The acquisition time and the accumulation number were set to 20 s and 20 scans, respectively.

Results and Discussion

The most important goal of process of antibody-immunoconjugates formulation as therapeutic or diagnostic agents is to reduce the protein degradation. Antibodies are not stable in a liquid form and the process of freeze drying is one of the commonly approaches for the protein stabilization. The degradation

of the antibody during storage can be chemically (deamidation, isomerization, oxidation, fragmentation) and physically (denaturation, aggregation, surface adsorption) and may reduce the activity of the antibody. Lyophilized antibodies are more stable than liquid formulations. The process of lyophilisation can also damage the antibody and a proper formulation should maximize the activity of the antibody and prevent any changes in secondary structure [14, 19, 29]. One protocol to protect the antibody from stress temperature during the lyophilisation process is the addition of cryoprotectantes. In our case, we have been used 1% mannitol, 50 µL in each vial. Besides this role, the mannitol is a bulking agent and is important to obtain fluffy cakes for easy reconstitution.

In a process of preparation of four freeze dried kits of trastuzumab with *p*-SCN-Bn-1B4M-DTPA and *p*-SCN-Bn-DTPA in ratio 1:10 and 1:50 it is important to determine the physicochemical stability. Many instrumental techniques can be used to establish the possible changes and degradation in the protein after conjugation and freeze drying. As a part of this research we have been used SDS-PAGE under reducing condition and Raman spectroscopy in order to test the integrity, purity, possible aggregation and modifications in secondary protein structure of the trastuzumab. The four conjugated samples were reconstituted in concentration of 1 mg/mL before mixing with loading buffer. Purified trastuzumab in concentration of 1 mg/mL has been used as a control sample. The scanned gel with all five samples and the marker is shown in Figure 2.

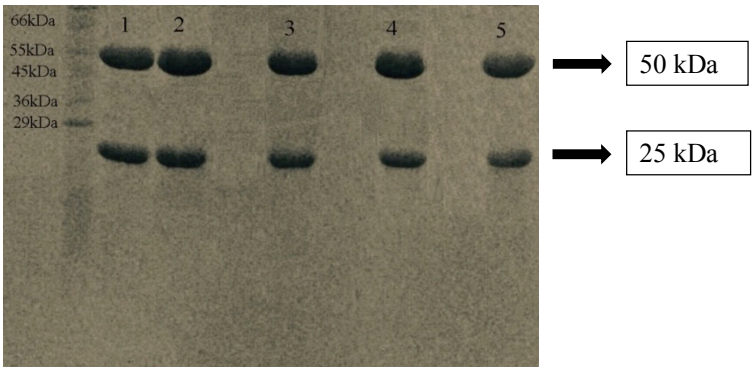

Figure 2.
Reducing SDS-PAGE of Trastuzumab 1 mg/mL (1); *p*-SCN-Bn-DTPA-Trastuzumab (1:10) (2); *p*-SCN-Bn-DTPA-Trastuzumab (1:50) (3); *p*-SCN-Bn-1B4M-DTPA-Trastuzumab (1:10) (4); *p*-SCN-Bn-1B4M-DTPA-Trastuzumab (1:50) (5)

The integrity testing of trastuzumab-immunoconjugates has been made by applying of SDS-PAGE in reducing or non-reducing conditions. Under reducing conditions, IgG1 antibodies were migrated as two bands a ~ 50 kDa and ~ 25 kDa (Mr of heavy and light chain), while under non-reducing conditions were observed only one band at ~ 150 kDa (Mr of whole antibody) [18]. Our obtained results have shown the fragments with the same molecular masses like already published results for other IgG1 monoclonal antibodies. In a presence of 2-mercaptoethanol, disulphide bones were reduced and trastuzumab-immunoconjugates were separated in two bands of fragments with molecular weight of 25 kDa for light chain and 50 kDa for heavy chain (proven by the used marker).

The same intensity and the same Mr of the fragments of freeze dried BFCs-trastuzumab conjugates with the fragments of commercial unmodified trastuzumab was indicated that no changes occurred in the structure of the antibody. The results have shown that the formulation of freeze dried kits is successful, without any degradation, denaturation, fragmentation and aggregation of trastuzumab.

Acceptable electrophoretic results give the opportunity for further investigations. Possible destruction of antibody native structure can be identifying with various instrumental techniques. Raman and infrared (IR) spectrometers are suitable for studies of protein pharmaceuticals. IR spectroscopy is an oldest experimental method for characterization of structural features of proteins that requires minimal sample preparation and high speed of getting the spectra. On the other hand, Raman spectroscopy is a suitable method for determination of any folding, assembly and protein aggregation. It is a newer technique which can be used for measurement of aqueous solutions and protein therapeutics at very low concentration of 1 mg/mL [13, 27, 30, 31]. In this study, Raman spectroscopy was used for examination of secondary structure of trastuzumab-immunoconjugates and pure antibody as a comparison (Figure 3). This spectroscopy is a scattering technique and the produced signal is unique for every atom or molecule [13]. Trastuzumab, like other IgG1 antibodies, has molecular weight of ~ 150 kDa and structure mostly arranged of beta sheets, several beta turns, small part of alpha helix

and disordered structure [12]. Raman spectra provide two specific bands for secondary structure of the polypeptide backbone, known as amide bands and are related with a peptide linkage between amino acids in the proteins. Amide I band is referred to different stretching vibration of C=O bond.

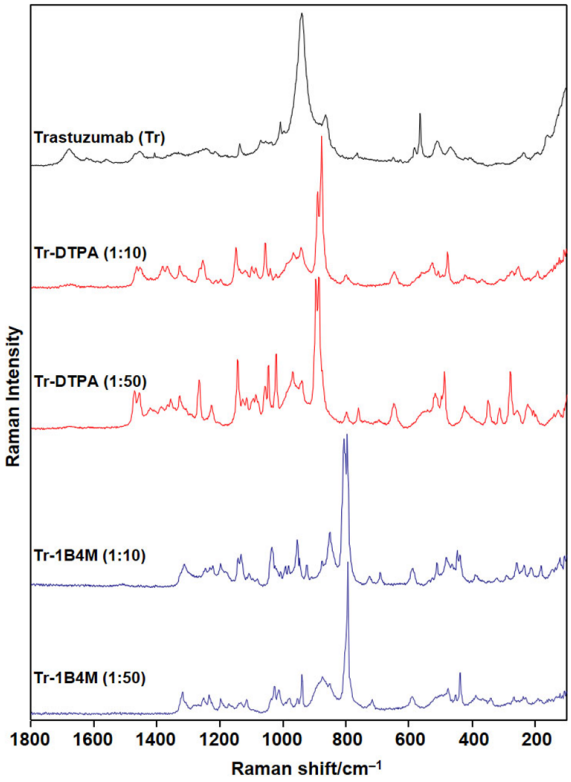

Figure 3.

Raman spectra of Trastuzumab 1 mg/mL; *p*-SCN-Bn-DTPA-Trastuzumab (1:10); *p*-SCN-Bn-DTPA-Trastuzumab (1:50); *p*-SCN-Bn-1B4M-DTPA-Trastuzumab (1:10); *p*-SCN-Bn-1B4M-DTPA-Trastuzumab (1:50)

If the polypeptide backbone is predominantly α helix the Amide I band would appear at ~ 1655 cm^{-1}. Monoclonal antibodies and the other molecules that contain primarily β-sheet have shown characteristic Amide I band at ~ 1670 cm^{-1} [15, 31]. In the spectra of pure trastuzumab (1676 cm^{-1}) and the other four immunoconjugates: (1:10) *p*-SCN-Bn-DTPA- (1674 cm^{-1}), (1:50) *p*-SCN-Bn-DTPA- (1676 cm^{-1}), (1:10) *p*-SCN-Bn-1B4M-DTPA- (1676 cm^{-1}), (1:50) *p*-SCN-Bn-1B4M-DTPA- (1678 cm^{-1}), the characteristic Amide I band were detected, which has shown the retention of α-helix in all samples. Amide III band, associated with coupled C-N stretching and N-H bending vibrations of the peptide group, is also important for determination of protein secondary structure (β-sheet structure) and was registered between 1230 cm^{-1} and 1300 cm^{-1}. In α-helix arranged proteins this band occurs at interval between 1300 cm^{-1} and 1340 cm^{-1}. Monoclonal antibodies primarily contain β-barrel structure and provide Amide III band at ~ 1245 cm^{-1} [1, 20, 24, 32]. The presence of Amide III band in the collected spectra of trastuzumab-immunoconjugates in comparison with Raman spectra of pure trastuzumab (1245 cm^{-1}), (1:10) *p*-SCN-Bn-DTPA- (1255 cm^{-1}), (1:50) *p*-SCN-Bn-DTPA- (1227 cm^{-1}), (1:10) *p*-SCN-Bn-1B4M-DTPA- (1226 cm^{-1}), (1:50) *p*-SCN-Bn-1B4M-DTPA- (1235 cm^{-1}), has shown no modification of the antibody secondary structure and the retention of β-sheet structure. Raman bands of aromatic amino acids in proteins are clearly visible in Raman spectra, but can be covered with amide bands of other side chain groups. The presence of well-defined tryptophan (trp), phenylalanine (phe) and tyrosine (tyr) bands (400-4000 cm^{-1}) are in accordance with the previously published data [5, 6, 9, 16, 23, 26, 31] (pure trastuzumab (**trp**-765 cm^{-1}, 865 cm^{-1}, 1332 cm^{-1}, **phe**-1010 cm^{-1}, **tyr**-650 cm^{-1}), (1:10) *p*-SCN-Bn-DTPA- (**trp**-764 cm^{-1}, 878 cm^{-1}, 1328 cm^{-1}, **phe**-1056 cm^{-1}, **tyr**-647 cm^{-1}), (1:50) *p*-SCN-Bn-DTPA- (**trp**-762 cm^{-1}, 886 cm^{-1}, 1328 cm^{-1}, **phe**-1022 cm^{-1}, **tyr**-648 cm^{-1}), (1:10) *p*-SCN-Bn-1B4M-DTPA- (**trp**-762 cm^{-1}, 879 cm^{-1}, 1328 cm^{-1}, **phe**-1022 cm^{-1}, **tyr**-645 cm^{-1}), (1:50) *p*-SCN-Bn-1B4M-DTPA- (**trp**-757 cm^{-1}, 878 cm^{-1}, 1328 cm^{-1}, **phe**-1040 cm^{-1}, **tyr**-648 cm^{-1})). The majority of the proteins have shown Raman bands of free sulfhydryl and disulphide bonds. According to already published results [9, 31] in our samples, pure trastuzumab (471cm^{-1}, 510 cm^{-1}), (1:10) *p*-SCN-Bn-DTPA- (479 cm^{-1}, 528 cm^{-1}), (1:50) *p*-SCN-Bn-DTPA- (425 cm^{-1}, 489 cm^{-1}, 519 cm^{-1}), (1:10) *p*-SCN-Bn-1B4M-DTPA- (479 cm^{-1}, 488 cm^{-1}, 527 cm^{-1}), (1:50) *p*-SCN-Bn-1B4M-DTPA- (479 cm^{-1}, 496 cm^{-1}, 521 cm^{-1}) were detected disulphide bands in region between 540 and 400 cm^{-1}. No significant variations in Raman spectra of immunoconjgates supported by positive SDS-PAGE results have shown unchanged protein structure, without any aggregation after process of conjugation and lyophilisation.

Conclusions

The obtained results have undoubtedly confirmed no integrity change and no physicochemical and structural modifications of trastuzumab after *p*-SCN-Bn-DTPA- and *p*-SCN-Bn-1B4M-DTPA- conjugation in the process of freeze drying. The lyophilized kit formulations, due to increased stability allow prolonged storage and extension of the shelf life of the antibody. An appearance of two bands of fragments (25 kDa for light chain and 50 kDa for heavy chain) on electrophoretic gels revealed that no degradation of the antibody took place. The obtained results from the collected Raman spectra (especially the presence of Amide I and III bands) also demonstrate retained secondary β-sheet structure. Therefore, this work

supports the opportunity for further determination of the number of the chelators attached to the antibody with Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometer (MALDI-TOF-MS) and development of radiopharmaceuticals for imaging and treatment of HER2 positive breast tumours.

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