

Formulation and Characterization of “Ready to Use” 1B4M-DTPA-rituximab for Lu-177 Labeling

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Abstract: Investigations for NHL treatment are oriented towards radiolabelled therapeutics. This research focuses on formulation and characterization of a new, ready to label immunoconjugate, 1B4M-DTPA-rituximab, which is suitable for labeling with Lu-177. The conjugation was performed using 20-fold molar excess of the bifunctional chelating agent, 1B4M-DTPA and subsequent lyophilization. The characterization of the (radio)immunoconjugate was performed using SE-HPLC, SDS-PAGE and MALDI-TOF-MS. The results show that the conjugation reaction yields immunoconjugates with average of 8.3 chelating groups per one rituximab molecule and the subsequent lyophilization did not affect the integrity, the structure, or radionuclide-binding properties. The radiolabelling with 555 GBq/mg Lu-177 resulted in over 95% radiochemical purity, which makes this agent a good candidate for further investigation of biological and pharmacological potency in NHL therapy.

Key words: Rituximab • 1B4M-DTPA • Lyophilization • Lu-177

INTRODUCTION

Non-Hodgkin’s lymphoma (NHL) is a form of blood cancer with origin in lymphatic system.

More than 90% of B-cell lymphoma cells express CD20 receptor who has proven to be an excellent target for the treatment of B-cell lymphoma [1]. Investigations for NHL therapy are mainly focused to development of antibody against CD20 antigens [2]. As a result, rituximab (chimeric antibody), was included in therapy [3, 4]. A step further in NHL therapy was the implementation of radiolabelled anti-CD20 monoclonal antibodies, ⁹⁰Y-ibritumomab (Zevalin) and ¹³¹I-tositumomab (Bexxar) [5-8]. Other rituximab radioimmunoconjugates are under investigation for use in NHL therapy. There are on-going studies investigating various radionuclides for rituximab labeling, such as ⁹⁰Y [9], ¹¹¹In [10, 11], ¹⁵³Sm [12] and ¹⁷⁷Lu [9, 13, 14]. The radionuclides can be attached to the antibody through different chelating agents and various derivatives of the acyclic agent diethylene triamine pentaacetic acid (DTPA) and the macrocyclic agent 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) are also under investigation [15].

Owing to its immunoglobulin protein nature, rituximab possesses very complex molecular structure and may go through a variety of chemical and physical

degradation processes. These processes include adsorption to surfaces, unfolding, aggregation, isomerization, oxidation or disulfide bond rearrangements [16-19]. The presence of water either as a reactant or a solvent, is critical for many routes of chemical degradation that lead to protein instability. Therefore, lowering the water content may provide resistance to chemical modification. A common approach of stabilization of therapeutic proteins is lyophilization in which retention of native protein structure in the dried solid and prevention of degradation during storage is achieved [20, 21].

The purpose of this investigation was to establish a technology for a “ready-to-use” lyophilized kit formulation of conjugated rituximab suitable for labeling with Lu-177, which meets the quality control criteria. As bifunctional chelating agent, a derivate of DTPA, 1B4M-DTPA (2-(4-isothiocyanatobenzyl)-6-methyl-diethylene-triaminepentaacetic acid) was used. Various protein characterization methods were used to determine the possible changes in physicochemical properties of rituximab conjugate, including size-exclusion HPLC (SE-HPLC), SDS-PAGE and MALDI-TOF-MS. The main goal of this investigation was characterization of the obtained immunocomplex and analysis of its physicochemical and radionuclide binding properties.

MATERIALS AND METHODS

Conjugation of Rituximab: 1B4M-DTPA was dissolved in 0.1 M PBS (pH 8.0) to final concentration of 10 mg/mL. Calculated amounts of 1B4M-DTPA required to give a 20-fold molar excess over the amount of rituximab were added to the purified rituximab in 0.1 M PBS (pH 8.0). The mixture was incubated overnight (4°C with gentle shaking). Purification of the conjugates was made with ultrafiltration (Ultracel®- 30K, Millipore, Ireland), by washing the immunoconjugates with 0.05 M ammonium acetate, pH 7.0.

Freeze Drying Procedure: The freeze dried process was performed using Labconco Free Zone Stoppering Tray Dryer, (USA) using a protocol described by Park *et al.*, in 2013 [22], modified to our experience. Briefly, the liquid immunoconjugates were filled in 10 mL type I glass tubing vials using a fill volume of 1 mL and were partially stoppered with corresponding lyophilization stoppers. Then, the vials were equally distributed on the shelves of the freeze-dryer, including empty vials with thermocouples on each shelf to monitor the temperature variation during the process. The samples were loaded at shelf temperature of 5°C and the temperature was decreased to -40°C at 0.40°C/min and held for 3 h. In order to achieve complete crystallization and to obtain the desired cake structure, an annealing step was included, at -15 °C, The freezing step was completed in 10 h. The primary drying was performed at temperature of -10°C for 25 h and the secondary drying at shelf temperature 25°C for 11 h. Upon finishing the process, the vials were stoppered and kept at 4°C until analysis.

Determination of the Immunoconjugate Concentration: The concentration of the antibody/immunoconjugate was determined with UV spectrophotometer (Jenway UV/VIS spectrophotometer 6715) at 280 nm, in triplicate, using the standard extinction coefficient for antibodies, 1.4 mL/mg/cm. The concentration of the lyophilized preparations was measured after complete reconstitution in 1 mL 0.9% NaCl.

Protein Integrity Test Using Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis (SDS-PAGE): Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed on the mini-gel system (GE Healthcare/Amersham Biosciences) according to Laemmli protocol [23]. About 5 µL of sample was mixed with 10 µL of sample buffer. The samples were boiled for 5 min at 95°C and approximately 5 µL of each preparation was

applied per lane in 12% bisacrylamide gel under reducing conditions. Coomassie staining (Coomassie Brilliant Blue R-250, Sigma) was performed according to the manufacturer's instructions. All chemicals used were reagent and HPLC grade. As a molecular marker, Low molecular weight marker (Amersham GE Healthcare) was used. For reproducibility, the electrophoresis was repeated in triplicates.

Protein Characterisation by MALDI-TOF MS: Matrix-Assisted Laser Desorption Ionization time-of-flight (MALDI-TOF) mass spectrometry was performed with Voyager-De MALDI-TOF (Applied Biosystems).

Both characterization of the conjugates and determination of the average number of chelator groups attached to each antibody molecule was performed by MALDI-TOF. A volume of 10 µL conjugated rituximab solution was diluted (1:10) with a matrix solution of 3,5-dimethoxy-4-hydroxycinnamic acid (10 mg/mL) to a final concentration of about 10 pmol/iL. 1-2 iL of the final solution was applied to the sample target prior insertion into the high vacuum chamber of the mass spectrometer. The results were recorded in acquisition mass range of 140-170 kDa.

Radiolabeling of the Freeze Dried Immunoconjugate with Lutetium-177: The radiolabeling of the freeze-dried immunoconjugate was performed after reconstitution in 0.9% NaCl, in the presence of acetate ions at pH 7.0 with Lutetium-177 with specific activity of 555 GBq/mg, at room temperature. The excess of Lutetium-177 was scavenged with DTPA (0.01M, pH 6.0). The radiolabeled immunoconjugates were obtained with high radiochemical yield. The radiochemical purity was determined using HPLC.

Size-exclusion HPLC: Obtained radioimmunoconjugate was characterized with SE-HPLC. Apparatus conditions were: column Zorbax Bio Series GF-250; sample volume: 20 iL; mobile phase: 0.9% NaCl flow rate: 1 mL/min; UV detection at 280 nm and radiometric detection.

RESULTS AND DISCUSSION

To assess the stability of rituximab conjugate, several different analytical methods were performed and protein characterization methods were used to determine possible changes of rituximab conjugate before and after lyophilization.

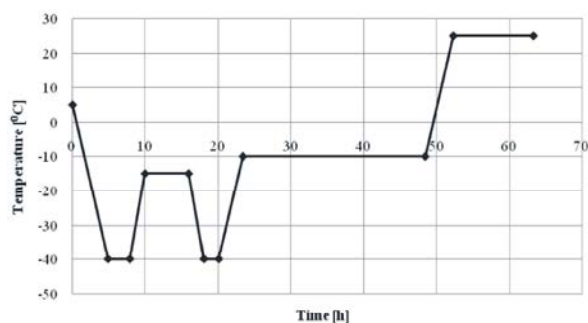


Fig. 1: Scheme of the applied lyophilization protocol.

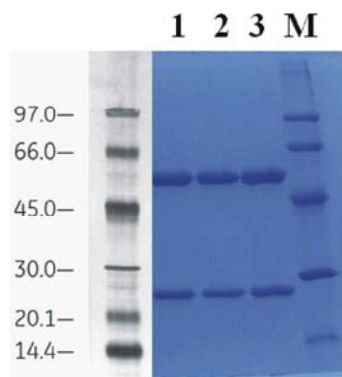


Fig. 2: Reducing SDS-PAGE lane patterns for 1B4M-DTPA-rituximab conjugate before lyophilization (1), 1B4M-DTPA-rituximab conjugate, after lyophilization (2); rituximab (3) (1 mg/mL), M is a low molecular weight marker;

During lyophilization, the freezing step is considered as important as the drying step, because of possible effects on the integrity of the protein. It is known that faster freezing creates ice crystals smaller, while slower freezing creates larger crystals, which in turn affects the primary drying process. However, it has been shown that rapid freezing may lead to formation of multiple aggregates in bovine and human IgG, other than slow freezing, because it leads to a higher percentage of protein denaturation [24, 25].

Instability of the protein containing pharmaceutical formulation is a common problem because of the aggregate formation. Aggregates can be present in liquid or solid form, from which they can be easily dissolved or they can stay in not dissolved form [26]. Many strategies are available for improve formulation development of anticancer drugs [27]. Our previous research showed that the protocol employed (Fig. 1) showed minimal or no changes in the immunoconjugate upon lyophilization, using a formulation without added cryoprotectants. Therefore, this lyophilization protocol was employed in the preparation of the kit formulation.

In order to assess the integrity of the protein and purity after conjugation and lyophilization, gel electrophoresis was performed on SDS-PAGE gels using 12% bisacrylamide gel. The loaded samples were rituximab (Commercial sample diluted to 1 mg/mL), conjugated liquid rituximab-1B4M-DTPA and reconstituted lyophilizate, both in concentration of 1 mg/mL. Figure 2 shows the SDS-PAGE patterns for rituximab-1B4M-DTPA conjugates, compared to unconjugated rituximab as control sample.

1B4M-DTPA-rituximab conjugates (Before and after lyophilization) were resolved in two distinct Mw species that migrated in two bands (upper band at ~50 kDa and lower band at ~25 kDa), a migration behavior typical for IgG antibodies composed of two identical subunits, each composed by two polypeptide chains: two heavy and two light chains, linked via disulfide bonds. The obtained fragments correspond to molecular masses of rituximab heavy and light chain given at the corresponding literature [28].

As it is shown in Figure 2, the reducing SDS-PAGE patterns for rituximab and 1B4M-DTPA -rituximab immunoconjugates were with very similar intensity. The reducing SDS-PAGE results, compared to the commercially available rituximab sample, showed no clear indication for antibody degradation. At the same time, no other degradation entities were detected using this technique. Almost identical results were obtained after one month integrity test on 1B4M-DTPA-rituximab immunoconjugate products stored at 4°C (Results not shown).

It is known that the active ester groups of the bifunctional chelating agent react with the neutral form of the antibody amine groups, forming thiourea-type bonds with the α -amino residues of lysine [14]. The structure of the used chelating agent for conjugation of rituximab is shown in Figure 3.

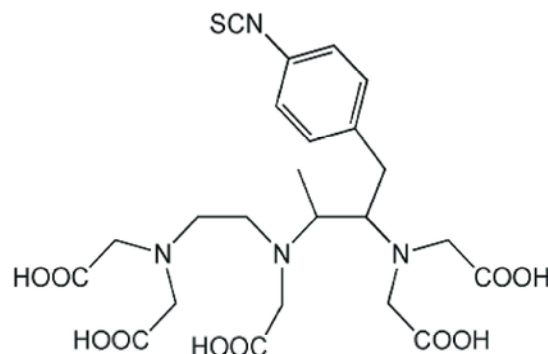


Fig. 3: Structure of used 1B4M-DTPA for rituximab conjugation

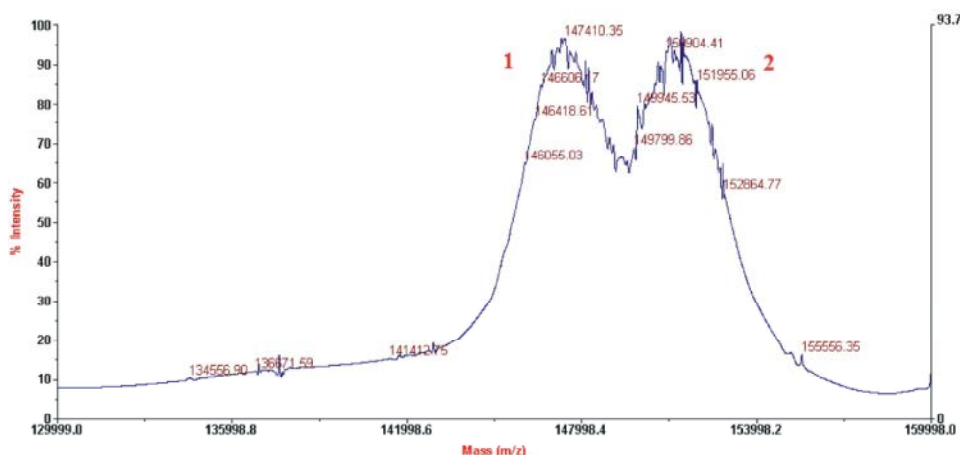


Fig. 4: MALDI-TOF results for 1B4M-DTPA-rituximab conjugate.

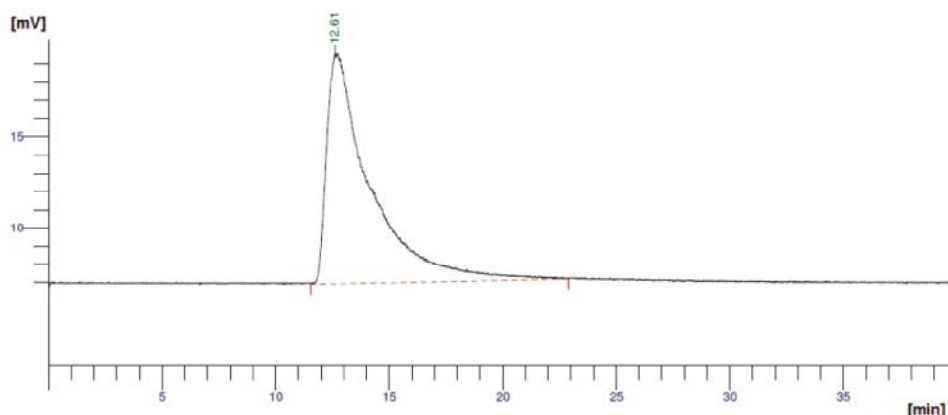


Fig. 5: SE-HPLC of ¹⁷⁷Lu-1B4M-DTPA-rituximab

In order to obtain good radiolabeling yield, it is important that enough chelator molecules are conjugated to the antibody. This is achieved by using a molar excess of the chelator during the conjugation reaction. Therefore, one of the most important properties of the immunoconjugates is the average number of conjugated chelator molecules. Determination of the average number of 1B4M-DTPA molecule attached to each antibody molecule was performed by MALDI-TOF MS. This is a rapid and sensitive technique for characterization of peptides and proteins and it is used successfully in biochemical and biotechnological areas for analysis of therapeutic proteins, peptides, glycoproteins, complex carbohydrates and oligonucleotides. Many studies comparing mass spectra of intact antibodies conjugated with chelating agents (DTPA, macrocycle 12N4) or with drugs (Calicheamicin, methotrexate, mitoxantrone) with the corresponding unconjugated antibodies, exist [29, 30]. MALDI-TOF results for 1B4M-DTPA-rituximab conjugate, after lyophilization (Shown on Figure 4), show presence of two major peaks corresponding to a Mw of

146848 Da (Unconjugated mAb) and 151506 Da (Conjugated mAb), which corresponds to an average of 8.3 groups of 1B4M-DTPA per molecule of rituximab. No structural changes in terms of appearance of additional peaks were observed.

There are studies where 4.25 ± 1.04 DOTA-SCN molecules were attached to each antibody molecule [9] and five DOTA molecules were conjugated to MORAb-003, with no apparent loss of immunoreactivity [31]. These results are found to be sufficient for prompt subsequent labelling with radioisotope. Our results of average of 8.3 groups of 1B4M-DTPA per molecule of rituximab, pointed that this number can be increased using different experimental conditions, as molar ratios for conjugation, for example. Highly substituted anti-tumor antibody leads to formation of immunoconjugates with high specific activity which is a valuable option for radioimmunotherapy [9].

Radiolabeling of the immunoconjugate was performed after reconstitution of the lyophilized preparation with 0.9 % saline. Representative SE-HPLC

profile of the radiolabeled immunoconjugate is shown in Figure 5. In the chromatogram obtained, a presence of only one peak is registered, that corresponds to the radiolabeled antibody. The radiochemical purity of the immunoconjugate is over 95%, which meets quality criteria.

This study was conducted to evaluate the physicochemical properties of the obtained lyophilized formulation of rituximab-1B4M-DTPA conjugate. Based on the results, we can conclude that conjugation and lyophilization process did not affect structure properties and caused no post-lyophilization modifications, giving a good base for inclusion of this kit formulation in further investigations.

CONCLUSION

The results of this study concluded that the conjugation reaction in 20-fold molar excess of bifunctional chelating agent, yields immunoconjugates that were lyophilized in a manner that did not affect its integrity, the structure or radionuclide-binding properties. Furthermore, radiolabeling of this lyophilized (kit) formulation of 1B4M-DTPA-rituximab conjugate, having approximate 8 molecules of chelator per antibody molecule with ^{177}Lu results in radioimmunoconjugates with good radiochemical yield and high radiochemical purity, with no visible aggregation. Still, further experiments are needed in order to develop a promising radiopharmaceutical for the treatment of non-Hodgkin's lymphoma.

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