Approaches in evaluation of freeze-dried antibody conjugates

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INTRODUCTION

Antibodies, proteins and other biotechnological products are often challenging in terms of their in-solution stability. Various physical and chemical changes occur during their in-solution storage, leading to shorter shelf-life. Freeze drying is often proposed as a method of choice. During freeze-drying, antibodies and other protein pharmaceuticals can experience in-process changes that may reduce their physicochemical, biological and/or pharmacological properties. In this context, many attempts have been made to reduce these changes, both in optimization of the freeze-drying process and in optimization of solution formulation, adding various buffers, cryoprotectants, etc.
The presented experience was in freeze-drying of monoclonal antibody – rituximab, conjugated with three types of bifunctional chelating agents, *p*-SCN-Bn-DOTA, *p*-SCN-Bn-DTPA, and 1B4M-DTPA, and evaluation of possible changes in post-freeze-drying phase. An insight in possible changes in structure was made, using SDS-PAGE electrophoresis and FT-IR and Raman spectra pre- and post- freeze-drying process.

METHODS

Commercially available rituximab (Mabthera® , Roche) was conjugated with three bifunctional chelating agents, *p*-SCN-Bn-DOTA, *p*-SCN-Bn-DTPA, and 1B4M-DTPA (Macrocyclics Inc. USA). The conjugates were synthesized, purified, adjusted to concentration of 1mg/mL and freeze dried, using Labconco Free Zone Stoppering Tray Dryer. The protein integrity was assessed using SDS-PAGE in 5 µL of reconstituted samples and 1mg/mL purified, commercial rituximab (Mabthera®, Roche), using 12% bisacrylamide under reducing conditions. Visualization of the bands was enabled using Coomassie Brilliant Blue R-250 (Sigma). For comparison, Low molecular weight marker (Amersham GE Healthcare) was used. For determining protein structure, FT-IR spectroscopy was conducted on PARAGON 1000 (Perkin Elmer) spectrophotometer in the spectral range 2000–500 cm^{-1}. Attenuated Total Reflectance (ATR) spectra were acquired at a resolution of 4 cm^{-1}. The obtained data was processed with Grams_32 software (Thermo Scientific). Raman spectra (2000–400 cm^{-1}) were recorded on a micro-Raman multichannel spectrometer Horiba JobinYvon LabRam 300 Infinity, using He:Ne laser.

RESULTS AND DISCUSSION

Using SDS-PAGE electrophoresis we discovered migration pattern is characteristic of IgG antibodies: two bands in all three rituximab conjugates, that corresponded to ~50 kDa and the lower band to ~25 kDa. The lyophilization protocol used did not affect structure properties and caused no post-lyophilization modification, as shown in the reducing SDS-PAGE lane patterns, compared to commercially available rituximab sample (Fig. 1.).
Protein denaturation upon freeze-drying was monitored by IR spectroscopy, but Raman spectroscopy was also applied. The IR spectra of all three rituximab conjugates revealed higher percentage of β-sheet conformation (antiparallel and parallel) in the structure (strong band in the region between 1612 and 1640 cm^{-1}, followed by a weaker band around 1685 cm^{-1}), followed by α-helices (Bands at 1655 or 1656 cm^{-1}), as obtained in the band frequencies for amide I, II and III bands which are used as diagnostic bands. We observed that the freeze-dried rituximab conjugates regain their native conformation upon rehydration (reversible unfolding). Also, strong absorption bands below 1620 cm^{-1} can be correlated with aggregation, usually associated with the formation of new strong beta-sheet structures . With the lowest frequency band detected at 1620 cm^{-1} (in all samples analyzed), we concluded no obvious aggregation in all three freeze-dried antibody conjugates. (Fig. 2 and 3).

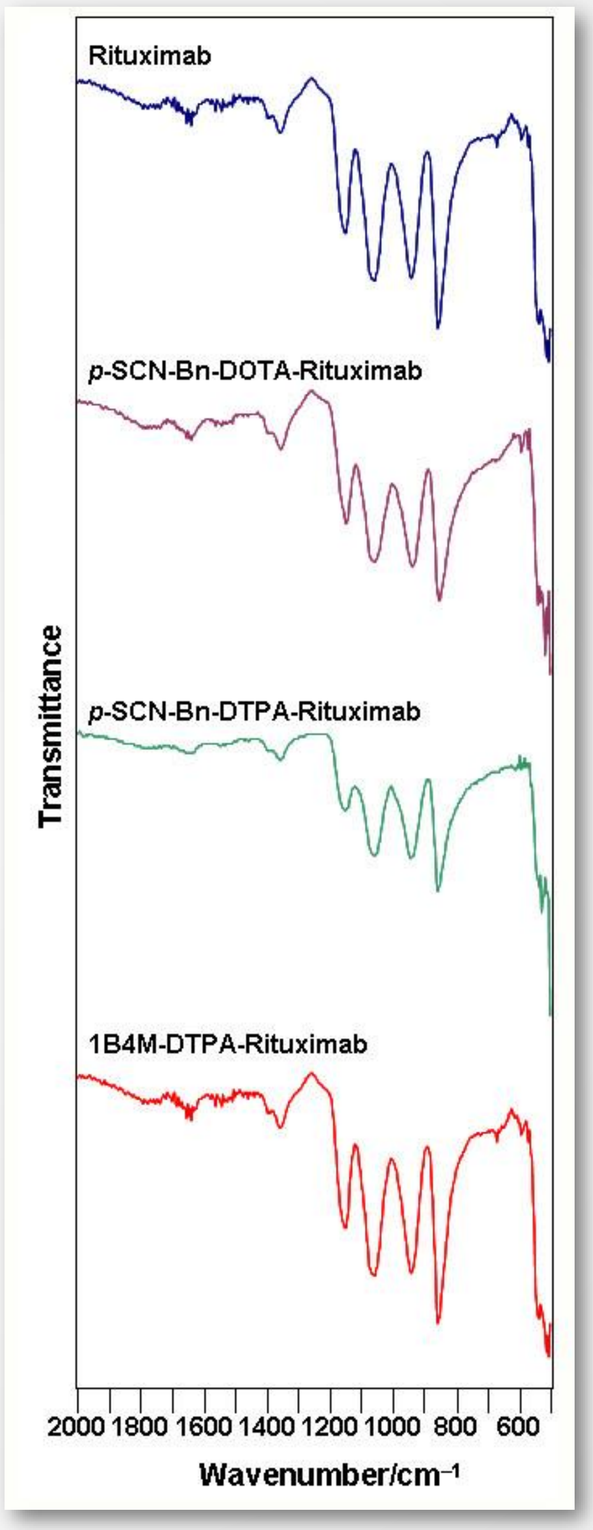

Fig. 2: ATR-IR spectra of freeze-dried rituximab, *p*-SCN-Bn-DOTA-rituximab, *p*-SCN-Bn-DTPA-rituximab and 1B4M-DTPA-rituximab.

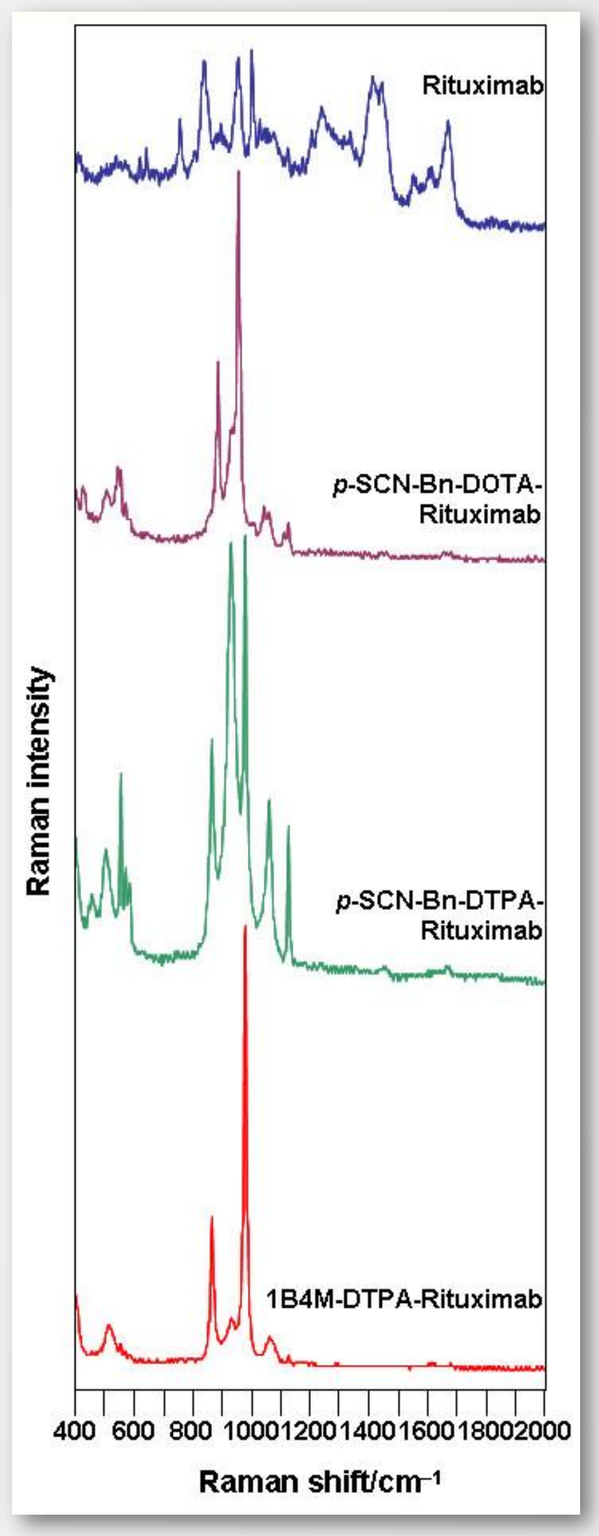

Fig. 3: Raman spectra of freeze-dried rituximab, *p*-SCN-Bn-DOTA-rituximab, *p*-SCN-Bn-DTPA-rituximab and 1B4M-DTPA-rituximab .

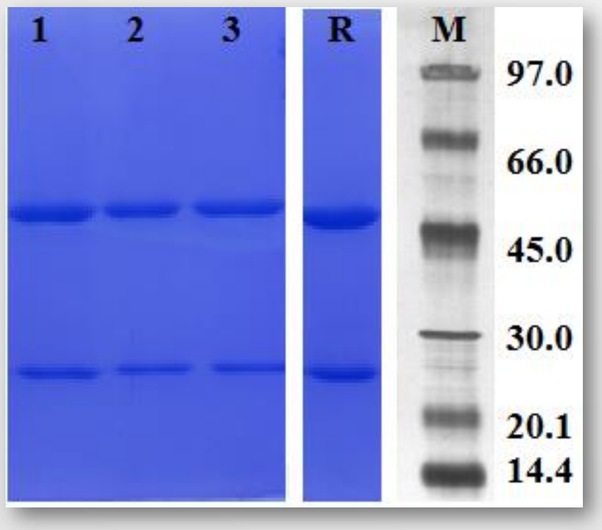

Fig. 1: Reducing SDS-PAGE lane patterns for 1) DTPA-rituximab conjugate, after lyophilization, 2) DOTA-rituximab conjugate, after lyophilization, 3) 1B4M-DTPA-rituximab conjugate, after lyophilization, R) rituximab 1 mg/mL, M) molecular marker

CONCLUSION

Among the many techniques available for evaluation of the structure and stability of freeze-dried antibody conjugates, SDS-PAGE electrophoresis, FT-IR and Raman spectroscopy can be employed in assessing structural properties as well as in determination of stability of these potential drug candidates.

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