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Short communication

Three spectroscopic techniques evaluated as a tool to study the effects of iodination of monoclonal antibodies, exemplified by rituximab

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ABSTRACT

Radioiodinated monoclonal antibodies have been used for radioimmunotherapeutic and radiodiagnostic purposes. Radioiodination of monoclonal antibodies may lead to deterioration of the immunoreactivity of the monoclonal antibody. Methods for the determination of the immunoreactivity, however, do not provide information about any structural changes of the radioconjugate which may influence the binding properties of the protein to the target antigen. Within this study we demonstrated the potential role of three alternative spectroscopic analytical techniques to characterize the structural changes emerging after iodination of rituximab. We conclude that techniques as liquid chromatography coupled to mass spectrometry, fluorescence emission spectrophotometry, and circular dichroism can provide valuable information about structural changes of a radiolabeled compound, e.g. during pharmaceutical development and for quality control.

1. Introduction

Radioiodinated monoclonal antibodies have been used for radioimmunotherapeutic and radiodiagnostic purposes [1–4]. Several methods are available for iodination of monoclonal antibodies. Methods using Iodogen for iodination of the protein are most frequently applied. In previous studies, it has been shown that the reaction conditions may have a large impact on the immunoreactivity of the resulting iodinated monoclonal antibody, which can be considered as a measure of protein integrity [5]. By using Iodogen as oxidizing agent, linkage of the iodine on the tyrosine residues *ortho* to the hydroxyl group of the phenolic moiety predominates when conducting the reaction at neutral pH (Fig. 1) [6]. For the optimal use of the radioconjugate, a high specific activity is required while the integrity, and thus the desired biological targeting, of the monoclonal antibody has to be preserved. High specific activity can be reached by using techniques which ensure relatively strong reaction conditions, but this may impair the integrity of the monoclonal antibody.

The most widely applied method for the determination of the immunoreactivity has been reported by Lindmo et al. [7]. They have described a method in which the immunoreactive fraction is determined by extrapolation from conditions representing infinite antigen excess. The extrapolation is performed in a double-inverse linear plot which is a modification of the Lineweaver–Burk plot. The inverse of the intercept of the *y*-axis then represents the immunoreactive fraction. The method provides information about the immunoreactivity of the monoclonal antibody, but does not provide insight into potential structural changes of the radioconjugate which may cause changes of the binding properties of the protein to a target antigen. Furthermore, this method is labor-intensive and time-consuming and, therefore, difficult to be used during pharmaceutical development and quality control.

Within this study we demonstrated the potential role of three alternative analytical techniques, liquid chromatography coupled to mass spectrometry (LC–MS), fluorescence emission and circular dichroism spectrometry, to characterize the structural changes during iodination of rituximab.

2. Materials and methods

Rituximab, an approved murine/humane chimeric anti-CD20 monoclonal antibody (Mabthera®, 10 mg/mL) was commercially obtained from Roche (Basle, Switzerland). 1,3,4,6-Tetrachloro-3α,6α-diphenylglycouril (Iodogen) originated from Sigma–Aldrich (St. Louis, MO, USA). 0.9% NaCl and phosphate buffered saline (PBS) was purchased from B. Braun Medical (Melsungen, Germany). Sodium iodide and ascorbic acid were from BUFA (Uitgeest, The Netherlands). 0.50 mol/L phosphate buffer and 0.05 mol/L NaOH were manufactured in-house (Department of Pharmacy

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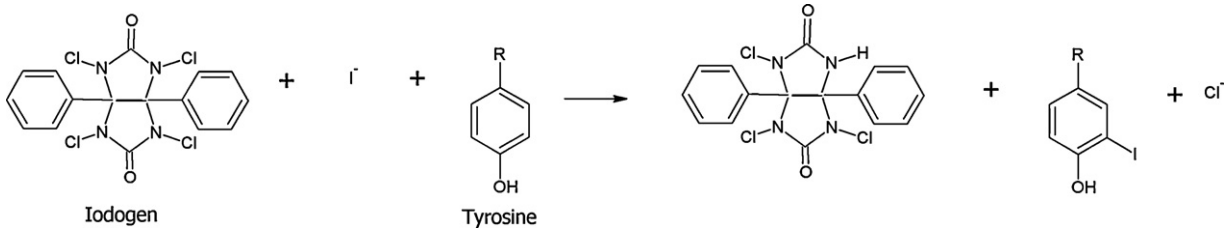

Fig. 1. Incorporation of Iodine into proteins and peptides. Application of Iodogen mainly leads to iodinated tyrosine moieties.

& Pharmacology, The Netherlands Cancer Institute/Slotervaart Hospital, Amsterdam, The Netherlands) and all other chemicals used were from BUFA (Uitgeest, The Netherlands). Methanol, dichloromethane and acetonitrile for quality control, were obtained from Merck (Darmstadt, Germany). All other chemicals used for quality control experiments were of analytical grade and used without further purification.

2.1. Sample preparation

2.1.1. Sample preparation of unlabeled rituximab

Rituximab was isolated and purified from the commercially available Mabthera® solution. A PD-10 sephadex column (GE Healthcare, Diegem, Belgium) was equilibrated with 0.9% NaCl. 2 mL of the Mabthera® solution was subjected to the column and was eluted with 0.9% NaCl. Fractions were collected with rituximab eluting between 0.5 and 3.5 mL.

2.1.2. Preparation of the iodinated rituximab

To exclude the effect of radiolysis on the monoclonal antibody, 127-Iodine was used instead of radioactive 131-Iodine. The labeling procedure was simulated using a 127-Iodine concentration corresponding to a specific activity of 185 GBq/mg 131-Iodine in 0.05 M NaOH.

2.1.3. Rituximab iodinated according to the Iodogen coated vial method

20 mg rituximab was iodinated according to the Iodogen coated vial method [8] with an amount of 127-Iodine corresponding to 3700 MBq. A 10 mL glass vial was coated with 2 mg Iodogen by evaporating 2.0 mL dichloromethane containing 1 mg/mL Iodogen. Rituximab and 200 μL sodium iodide (0.116 mg/mL of 0.05 M NaOH) were added and the volume was adjusted to 10 mL with 0.9% NaCl. After 30 min incubation at room temperature with constant stirring, the reaction was terminated by transferring the reaction solution to another, non-coated, vial followed by sterile filtration of the solution. The product was stored at room temperature.

2.1.4. Rituximab iodinated according to the Iodogen coated mab method

20 mg rituximab was iodinated using the Iodogen coated mab method with an amount of 127-Iodine corresponding with 7400 MBq. The preparation was performed as described previously [8,9]. Briefly, 400 μL sodium iodide solution (0.116 mg/mL of 0.05 M NaOH) was added to a reaction vial with 2 mL rituximab (10 mg/mL) and the labeling reaction was initiated by addition of 70 μL of 1 mg/mL Iodogen in acetonitrile to the reaction vial. After 3 min, 1 mL of 50 mg/mL ascorbic acid was added. The reaction was completed after 10 min. Purification was performed using PD10 sephadex columns equilibrated and eluted with 5 mg/mL ascorbic acid in 0.9% NaCl. Fractions were collected with iodinated rituximab eluting between 0.5 and 3.5 mL. The product was stored at room temperature. Prior to each analysis, buffer exchange of the iodinated rituximab, using PD-10 sephadex columns, was performed as described earlier.

2.2. Liquid chromatography coupled to mass spectrometry

(Iodinated) rituximab was diluted to a concentration of 200 μg/mL in 10% (v/v) acetonitrile in water and was used immediately for analysis. The analysis was performed as described by Damen et al. [10] for the monoclonal antibody trastuzumab under identical chromatographic conditions. In brief, separation of the monoclonal antibody was performed using a high performance liquid chromatography (HPLC) system consisting of an 1100 series binary pump with mobile-phase degasser, column heater and a thermostated autosampler (Agilent Technologies Santa Clara, CA, USA). A POROS R2/10 2000 Å column was used at a flow rate of 0.2 mL/min. For the MS analysis, the eluent was directed to an API365 triple-quadruple mass spectrometer equipped with a turbo ion spray source (Sciex, Thornhill, ON, Canada).

2.3. Circular dichroism

The analysis was performed on the day of the labeling (day 0), and one and seven days after the labeling after storage at room temperature (respectively day 1 and 7). Prior to each analysis, unlabeled rituximab, iodinated rituximab prepared according to the Iodogen coated vial method, and iodinated rituximab according to the Iodogen coated mab method were diluted with 0.9% NaCl to similar concentrations (0.5 mg/mL) as verified by measurement of the UV absorption at 280 nm (Eppendorf BioPhotometer, Hamburg, Germany). Far-ultraviolet circular dichroism (Far-UV CD) (195–260 nm) was performed at room temperature in a 0.02-cm quartz cuvette with a dual beam DSM 1000 CD spectroscopy instrument (On-Line Instrument Systems, Bogart, GA, USA). The substractive double-graded monochromator was equipped with a fixed disk, holographic gratins (2400 lines/mm, blaze wavelength 230) and 1.24 mm slits. Each measurement was the average of 5 repeated scans. Data were plotted over the range from 205 to 250 nm. 0.9% NaCl was used as buffer, whereas chloride ions interfere with data collection at wavelengths beneath 205 nm [11].

2.4. Fluorescence spectroscopy

Fluorescence emission spectra were recorded on a Fluorlog fluorimeter (Horiba, Kyoto, Japan). The analysis was performed immediately after iodination (day 0), on day 1 and day 7 to monitor the stability. Prior to each analysis, the samples were diluted with 0.9% NaCl to similar concentrations (0.05 mg/mL) as confirmed by UV spectrometry. The excitation slits were set at 5 nm. Measurements were executed a room temperature in a quartz cuvette (1 cm path length). The excitation wavelength was set at 280 nm and emission scans were performed at a range of 300–450 nm.

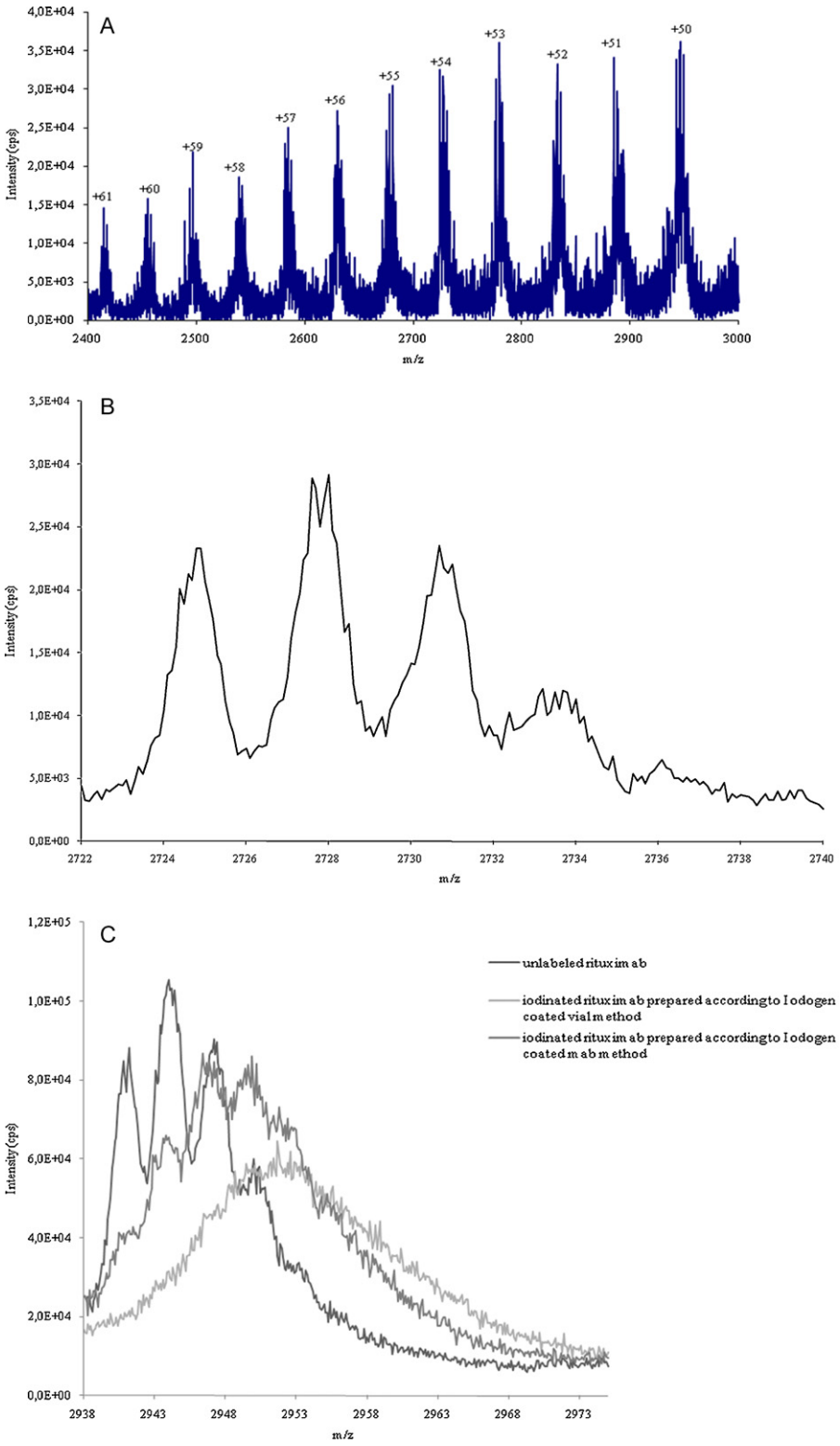

Fig. 2. (A) Positive mass spectrum from *m*/*z* 2400–3000 of unlabeled rituximab. (B) Positive mass spectrum from *m*/*z* 2722–2740 of unlabeled rituximab (charge state +54). (C) Positive mass spectrum from *m*/*z* 2938–2975 of unlabeled rituximab and iodinated rituximab according to the two iodination methods (charge state +50).

3. Results

3.1. Liquid chromatography coupled to mass spectrometry

Chromatographic analyses of (iodinated) rituximab resulted in a single peak with a retention time of 5.5 min (not shown). Subsequently, MS spectra were taken of rituximab, and iodinated rituximab prepared according to the two methods. Fig. 2A shows a full spectrum scan of unlabeled rituximab across the range from *m*/*z* 2400–3000. The different peaks correspond to the different charge states of rituximab, as indicated in Fig. 2A. Fig. 2B depicts the zoomed spectrum of unlabeled rituximab for the +54 charge

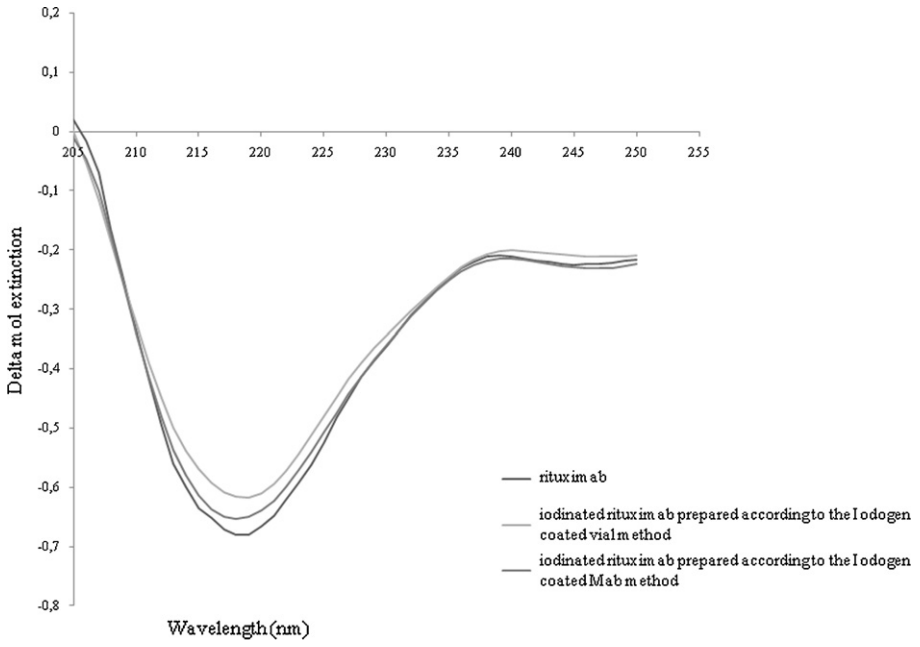

Fig. 3. Far-UV CD spectroscopy.

state. Four distinctive peaks, corresponding to a mass of 147,088; 147,250; 147,414 and 147,558 Da respectively, were observed due to glycosylation heterogeneity. Fig. 2C depicts the zoomed spectra (+50 charge state) of unlabeled rituximab and iodinated rituximab prepared according to the two methods. The spectra of iodinated rituximab showed a shift to higher m/z values which indicates an increase in mass of the molecule. In the spectrum of iodinated rituximab according to the Iodogen coated mab method, four peaks were still observed but with less resolution. The shift in m/z values corresponded with a mass difference of 361 Da between unlabeled and iodinated rituximab. The line representing iodinated rituximab according to the Iodogen coated vial method showed a larger shift in m/z values with a mass difference of 495 Da. The four distinctive peaks converged into a single broad peak.

3.2. Circular dichroism

Far-UV CD represents the peptide bond absorption and information on the secondary structure of rituximab can be obtained [12]. Fig. 3 shows Far-UV CD spectra of on day 0 of unlabeled rituximab, iodinated rituximab according to the Iodogen coated vial method, and iodinated rituximab according to the Iodogen coated mab method. The spectra of iodinated rituximab were comparable to the spectrum of the unlabeled rituximab. At 218 nm a peak minimum is visible. A variation in peak intensity was observed. Unlabeled rituximab showed a larger negative elipticity compared to the two iodinated forms whereby iodinated rituximab prepared according to the Iodogen coated vial method showed less intensity. Isobestic points at 209 nm and 234 nm were seen. At day 1 and 7, the spectra remained constant (data not shown).

3.3. Fluorescence spectroscopy

The emission spectra of unlabeled rituximab, iodinated rituximab prepared according to the Iodogen coated vial method, and iodinated rituximab prepared according to the Iodogen coated mab method are depicted in Fig. 4A. Unlabeled rituximab showed a higher emission intensity than iodinated rituximab prepared according to both iodination methods. Fig. 4B shows the emission of (iodinated) rituximab at $\lambda = 350$ nm ($\lambda = 280$ nm for excitation) at day 0, day 1, and day 7. For iodinated rituximab prepared according to the Iodogen coated vial method, a low intensity at day 0 which increases in time was observed. This is also observed for iodinated rituximab prepared according to the Iodogen coated mab method but with a higher intensity at day 0. For unlabeled rituximab, the emission intensity remains constant during the 7-day observation period.

4. Discussion

Radioiodinated monoclonal antibodies are used as imaging agents [3] and as radioimmunotherapeutic agents [13,14]. The chemistry of radioiodination of monoclonal antibodies has been studied extensively and numerous methods are available for radioiodination. Among the several methods of iodination, the Iodogen procedure is still the most frequently used method. By using Iodogen as oxidizing agent, linkage of the iodine on the tyrosine residues *ortho* to the hydroxyl group of the phenol moiety predominates when conducting the reaction at neutral pH.

Previous studies already have shown that the method of radioiodination influences the immunoreactivity of rituximab [8,9]. Even though less iodine atoms are incorporated when applying the Iodogen coated vial method, this method results in a lower immunoreactivity compared to the Iodogen coated mab method where twice as much iodine is applied. Therefore the immunoreactivity is proved to be not only dependent on the specific activity of the product.

The immunoreactivity is an important biological feature as it illustrates the binding capacity of the radiolabeled compound. The most widely used method to determine the immunoreactivity of a radiolabeled antibody is described by Lindmo et al. [7]. The method determines the immunoreactivity, but it does not give further explanation about any change of binding capacity of the radioiodinated analyte. Also, the method is laborious and time consuming. In this study, radioiodinated rituximab was used for further, structural, characterization. For this, three spectroscopic methods of analysis were evaluated which may give further insight into any structural changes following iodination.

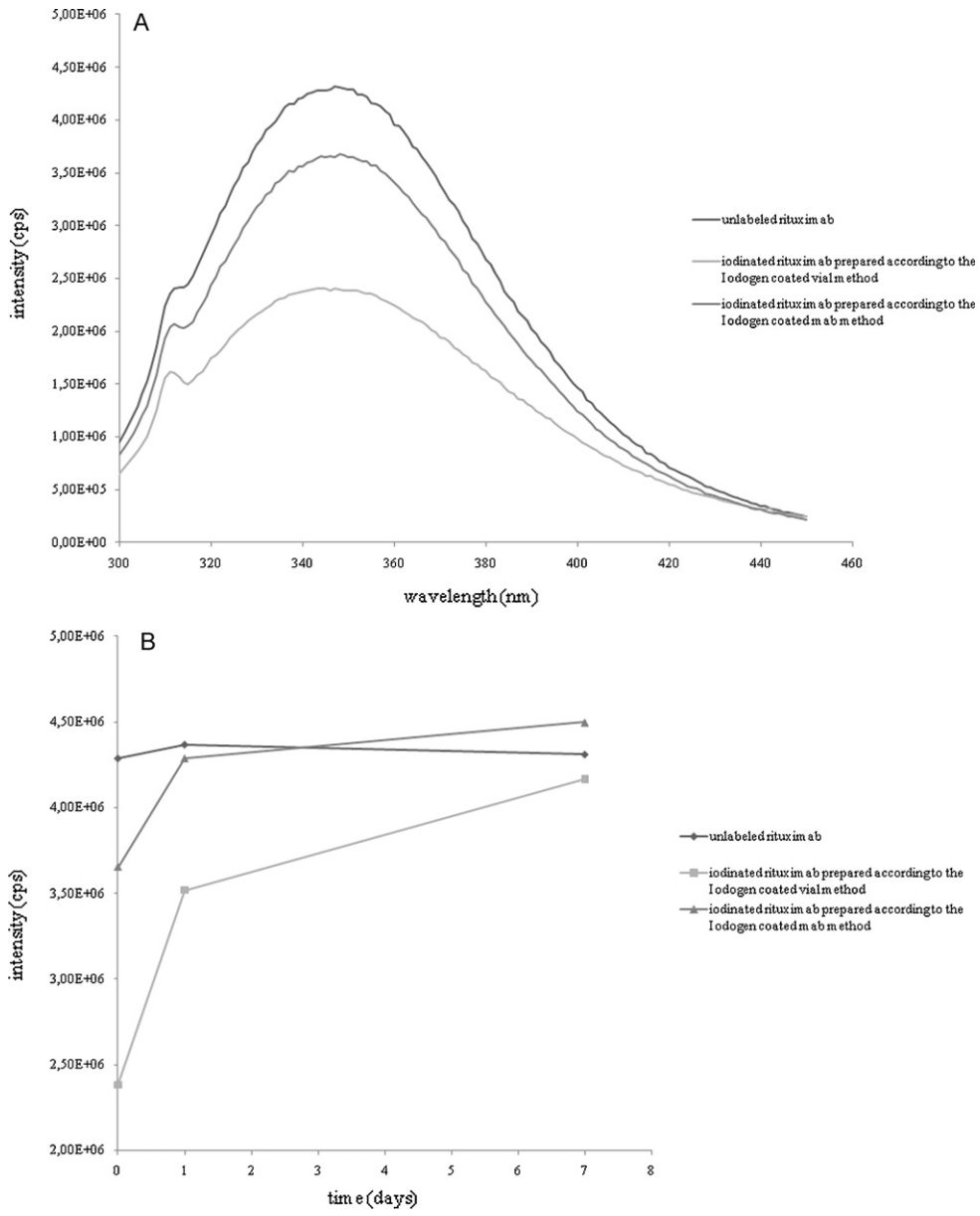

Fig. 4. (A) Fluorescence spectra of unlabeled and iodinated rituximab immediately after iodination at λ = 280 nm for excitation. (B) Emission of (iodinated) rituximab at λ = 350 nm measured immediately after iodination and storage for 1 and 7 days at room temperature.

By using liquid chromatography coupled online with mass spectrometry a protein specific charge envelope is generated and structural changes in the molecule can be detected [10]. When zoomed in a single charge state of unlabeled rituximab, four distinctive peaks were observed. These peaks correspond to four different glycosylation forms with mass differences corresponding with the mass of one hexose residue [10]. Compared to unlabeled rituximab, both spectra of iodinated rituximab showed a shift to higher m/z values which indicates an increase in the mass of the molecule. The spectrum of iodinated rituximab prepared according to the Iodogen coated mab method resulted in a shift of 361 Da. The spectrum of iodinated rituximab prepared according to the Iodogen coated vial method showed a shift of 495 Da. Based on the amount of Iodine used in both methods, an average of 1–2 atoms iodine per molecule rituximab could be incorporated. This corresponds to a maximum mass increase of 254 Da. The increases in the mass of both iodinated forms could thus not only be attributed to the addition of iodine atoms as the observed mass differences were larger than 254 Da.

Next to the shift in m/z values, in the spectrum of iodinated rituximab prepared according to the Iodogen coated mab method, the four distinctive peaks are observed but with less intensity. In the spectrum of iodinated rituximab prepared according to the Iodogen coated vial method, the four peaks are converged into one single broad peak.

Most likely, incubation with the oxidizing agent Iodogen also resulted in heterogeneous oxidation of the amino acid moieties of rituximab which results in a mass increase. Heterogeneous oxidation may explain the observed increase in mass as well as the converging of the four distinctive glycosylation peaks into a single broad peak, which subsequently attribute to the loss of immunoreactivity. We have shown earlier that immunoreactivity depends on the iodination method. The Iodogen coated vial method resulted in a larger mass difference, but it also resulted in an immunoreactivity of only 30%. Iodinated rituximab, prepared according to the Iodogen coated mab method, yielded a lower mass difference and in an immunoreactivity of 70% [8]. In addition, in the spectra of rituximab iodinated according to the Iodogen coated mab

method, the four glycosylation peaks were preserved, though with less resolution. Compared to the Iodogen coated vial method, the Iodogen coated mab method is a relative mild reaction procedure. Firstly, a shorter incubation time is required. Secondly, less Iodogen is needed to obtain a high labeling yield. These reaction conditions may lead to a more conserved monoclonal antibody than when applying the Iodogen coated vial method.

Next to the immunoreactivity, the biological clearance and activity of rituximab can be affected by heterogenous oxidation. Rituximab, an IgG molecule, is consists of two identical Fab regions, and a Fc-region which is glycosylated through asparagine residue 297. The Fc-region expresses interaction sites for ligands that activate clearance mechanisms [17,18]. A correlation was found between Fc-region glycosylation and the binding to these ligands [19]. A structural change in the glycan moiety could therefore also cause a change in biological clearance and immunogenity of rituximab.

Far-UV CD is used to map the secondary structural stability of a protein. The peak minimum seen at 218 nm suggests a high degree of β-sheet structure [20]. The spectra of the iodinated rituximab, prepared according to the two methods, show less intensity suggesting the presence of less stable β-sheets. LC–MS data already showed that iodination of rituximab results in an increase in mass and structural changes due to the incorporation of iodine atoms and heterogeneous oxidation of amino acids moieties. Although the differences in CD spectra are subtle, the far-UV CD data supports these results by showing an alteration in secondary structure of the monoclonal antibody after iodination. In addition, the secondary structure remains intact after iodination for at least 7 days.

Fluorescence emission spectrophotometry is a simple procedure. Changes in the intensity of protein fluorescence emission spectra are widely used as indicators for alterations in the conformations of proteins [21]. At an excitation wavelength of 280 nm, both tryptophan and tyrosine contribute to intrinsic protein fluorescence. Tryptophan dominates fluorescence among the aromatic amino acids. Tryptophan fluorescence is highly dependent on the polarity of the environment of the fluorophore. At the excitation wavelength of 295 nm, only tryptophan emission is observed [22]. Because of the significant role of tyrosine in the iodination process, it was to perform this study with an excitation wavelength of 280 nm. Decreased fluorescence intensity was observed for the iodinated rituximab solutions. Iodinated rituximab according the Iodogen coated vial method exhibited a larger decrease in intensity. The decrease in intensity can be assigned to quenching. Large halogens such as Iodine are known for their quenching characteristics [23]. In time, the spectra showed higher emission intensity. Previous study has shown the instability of the product during 24 h after labeling [8]. Loss of incorporated iodine would result in less quenching and thus in a higher fluorescence intensity. Hence, fluorescence emission spectrophotometry can be a tool for rapid stability monitoring.

5. Conclusions

For development of radiolabeling procedures of monoclonal antibodies for diagnostic and therapeutic properties, thorough investigation of the integrity of the radioconjugate is of importance. Next to the determination of the immunoreactivity, structural characterization is essential. Techniques as liquid chromatography coupled to mass spectrometry, circular dichroism and fluorescence emission spectrophotometry can provide valuable information about structural changes and stability of a radiolabeled compound, both during pharmaceutical development as for quality control.

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