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Research paper

Preliminary studies of the physical stability of a glucagon-like peptide-1 derivate in the presence of metal ions

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Abstract

The physical stability and the secondary structure of a glucagon-like peptide-1 derivative were investigated in the presence of the metal ions Al^{3+}, Zn^{2+}, Mg^{2+}, and K^{+}, known as possible leachables from container-closure systems. Metal ions were investigated in concentrations of 0–50 ppm. Test solutions of the peptide were exposed to elevated temperature (25 °C) and rotation (37 °C) for up to 4 weeks. The samples were examined by nephelometry, thioflavine T fluorescence, and Fourier-transform infrared spectroscopy. Readily prepared test solutions were examined by tryptophan fluorescence. The stability profiles were unchanged after addition of Mg^{2+} and K^{+} in 0–50 ppm concentrations. However, a concentration-dependent increase in thioflavine intensities was observed after addition of Al^{3+} and Zn^{2+}. The destabilising effect of Al^{3+} and Zn^{2+} was furthermore confirmed by FTIR as the secondary structure of the peptide changed from predominantly α-helix to a higher β-sheet content. Additionally Al^{3+} changed the secondary structure of the peptide using Trp fluorescence.

Keywords: GLP-1 derivate; Metal ions; Physical stability; Tryptophan fluorescence; Thioflavine T test; Fourier-transform infrared spectroscopy

1. Introduction

The shelf-life of protein pharmaceuticals in liquid form is often limited by their instability [1]. In all stages of protein drug development, protein aggregation is a common manifestation of physical instability. Several factors affect the stability of protein and peptide pharmaceuticals, e.g. pH, temperature, ion strength, metal ions, stress from processing, shaking, and shearing [1,2]. Insulin, hemoglobin, and recombinant human factor XIII are examples of proteins sensitive to shaking-induced aggregation [3–5].

Leachable chemicals in container-closure systems are generally of greater concern for liquid dosage forms than for solids [6]. Of particular concern are extractable materials in elastomers used for stoppers and seals for packaging. The rate of leaching is generally temperature- and pH-dependent. Leachables can be undesirable for a number of reasons: (1) these materials can affect chemical stability of the active drug either as reactants, as catalysts or by affecting the pH of the drug solution; (2) they can induce physical changes in the pharmaceutical formulation, such as precipitation; or (3) they can themselves be toxic [6].

Trace amounts of metal ions may leach from primary packaging during shelf-life influencing the physical and chemical stability of pharmaceutical proteins and peptides [7–9]. The control of leachables from container-closure systems may therefore present a challenge for the formulation and production of stable drug products as the shelf-life required for economic viability of a typical pharmaceutical product is 18–24 months [10].

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For formulations of proteins and peptides, metal ions may stabilise or destabilise the active pharmaceutical ingredient. Many proteins have distinct metal binding sites which may function to facilitate proper folding [12]. The presence of Zn^{2+} is known to stabilise insulin into hexamers [3] as well as Zn^{2+} facilitates dimerisation of human growth hormone (hGH) [11]. On the other hand, metal catalysed oxidation of proteins represents an important path of protein degradation; the presence of iron in a formulation of hGH, e.g. catalysed the oxidation of hGH [12]. Non-oxidative interactions have also been reported. The role of metal ions in neurodegenerative diseases has been widely investigated due to their involvement in the formation of fibrils [13]. Metal ions such as Al^{3+}, Co^{2+}, and Fe^{3+} have been shown to accelerate Factor VIII aggregation and inactivation [14], and small amounts of metal ions have likewise been demonstrated to induce structural changes and diminished physical stability in α-synuclein [15].

The ease of metal ions to stabilise or destabilise proteins and peptides is influenced by their chemical characteristics such as oxidation potentials. If the metal binding site or amino acid susceptible to oxidation is located in a narrow site of the protein, the size of the metal ion and also the hydrated radius may influence the tendency of the metal ion to bind or the tendency to induce oxidation [16,17].

As metal ions are known both to stabilise and destabilise proteins and peptides it is of interest to elucidate how the specific metal ions influence stability of the glucagon-like peptide-1 (GLP-1) derivative as a liquid dosage form. The main indication of GLP-1 pharmaceuticals is for the treatment of type 2 diabetes. The aim of this study was to investigate the physical stability of a GLP-1 derivative in the presence of Al^{3+}, Zn^{2+}, Mg^{2+}, and K^{+} during accelerated stability studies. Metal ions were studied in concentrations of 5–50 ppm; concentrations that are more elevated compared to leachable concentrations from container-closure systems during shelf-life. Stress testing was performed at neutral pH during storage in siliconised 1.5 ml PenFill® cartridges containing a bromobutyl rubber plunger.

2. Materials and methods

2.1. Preparation of test solutions

GLP-1 derivative (Novo Nordisk, Bagsværd, Denmark) solutions were prepared in concentrations of 6.0 mg ml^{-1}. The solutions were buffered with disodium phosphate dihydrate (Merck, Darmstadt, Germany) and pH was adjusted to 7.40. Metal ions Zn^{2+} (Merck, Darmstadt, Germany), Al^{3+} (Sigma–Aldrich, Steinheim, Germany), Mg^{2+} (Merck, Darmstadt, Germany), and K^{+} (Merck, Darmstadt, Germany) were applied as chloride salts in concentration ranges from 0 to 50 ppm. Solutions were prepared aseptically. Single determinations were performed due to limited amount of active pharmaceutical ingredient. The studies presented here are therefore preliminary work.

2.2. Thioflavin T fluorescence

Thioflavine T (ThT) fluorescence spectroscopy was performed on a Perkin-Elmer luminescence spectrophotometer LS 50B with a Xenon discharge lamp as the light source. Spectra were obtained using the software FL WINLAB, version 2.1. Excitation was performed at 450 nm and emission obtained at 470–540 nm, with a scan speed of 60 nm min^{-1}. Test solutions with Al^{3+}, Zn^{2+}, Mg^{2+}, and K^{+} were prepared in concentrations of 0, 10, 25, and 50 ppm. ThT solution was prepared using ThT (Aldrich, Steinheim, Germany), tris(hydroxymethyl)aminomethane (Merck, Darmstadt, Germany), and NaCl (Merck, Darmstadt, Germany). Twenty millimolar ThT was added to the test solution and samples were thermostatted at 37 °C for 4–30 min prior to ThT fluorescence intensity measurements. A spectrum of an appropriate blank solution (without GLP-1 derivative) was subtracted from each spectrum. Data points represent one sample.

2.3. Nephelometry

Similar test solutions as investigated by ThT flouresence were investigated by nephelometry. The nephelometric measurements were performed on a Hach 2100 AN Turbidimeter with a 90° detector. A tungsten-filament lamp was used as light source. Software used was Hachlink 2000 version 2.0. Test items were carefully cleaned and examined for the presence of air prior to each measurement. Data points represent one sample.

2.4. Stress testing

Test solutions with Al^{3+} and Zn^{2+} were prepared in concentrations of 0, 5, 25 and 50 ppm. The solutions were rotated on a Heto Master Mix rotation apparatus in darkness at 37 °C. Solutions were rotated 4 h a day at 30 ± 2 rpm. During rotation testing, a glass bead was added to each cartridge in order to ensure physical stress through surface contacts. Samples applied to rotation test were evaluated by nephelometry. Solutions were investigated in the period of 0–28 days. Data points represent an average of five samples.

2.5. Fourier-transform infrared spectroscopy

Infrared spectra were acquired using a Bomem MB-104 Fourier-transform infrared (FTIR) spectrophotometer with BGRAMS/32 software using calcium fluoride windows. Test solutions of GLP-1 derivative (20 mg ml^{-1}) were prepared with Al^{3+} or Zn^{2+} in concentrations of 0, 5, 25, and 50 ppm as described above. Furthermore, stressed samples, stored at 37 °C for 3 weeks in order to induce fibrils, were investigated. Samples were analysed by recording 512 scans at 4 cm^{-1} resolution. Buffer and water vapor spectra were subtracted separately. The second derivative spectra were obtained using a Savitzky–Golay

algorithm with a second degree polynomial and 9-point smoothing. Spectra have been inverted and truncated to 1600–1700 cm^{-1}. Data points represent an average of three samples. Area-overlap calculations were performed according to Kendrick et al. [18].

2.6. Tryptophan fluorescence

Intrinsic Tryptophan (Trp) fluorescence emission spectra of test solutions were acquired on a Perkin-Elmer Luminescence Spectrophotometer LS 50B with a Xenon discharge lamp as the light source. Spectra were subtracted using FL WINLAB software, version 2.1. Test solutions with Al^{3+}, Mg^{2+}, and K^{+} were prepared in concentrations of 0, 5, 10, 15, 20, 25, and 50 ppm. Intrinsic Trp fluorescence was measured after 1 h at 37 °C and samples were diluted to 0.1 mg ml^{-1} GLP-1 derivative prior to each measurement. The solutions were analysed with a sample volume of 4 ml. Excitation was performed at 295 nm to selectively excite Trp residues and emission was obtained at 305–450 nm with a scan speed of 200 nm min. Excitation and emission slits were set at 5.0 and 2.5 nm, respectively. A spectrum of an appropriate blank solution (without GLP-1 derivative) was subtracted. Data points represent one sample.

3. Results and discussion

The influence of the metal ions Al^{3+}, Zn^{2+}, Mg^{2+}, and K^{+} on physical stability of the GLP-1 derivative was investigated.

3.1. Preliminary stability study I: Accelerated stability study

Initially, a 4-week accelerated stability study was carried out on GLP-1 derivative test solutions using nephelometric measurements and ThT fluorescence. ThT fluorescence is a well-established method for evaluation of liquid protein and peptide formulations with regard to fibril content, as ThT binds specifically to fibrils, whereby the intensity of ThT fluorescence rises drastically [19–22]. Similarly nephelometry is a well-established method indicating change in physical appearance, normally due to aggregation. The GLP-1 derivative test solutions were stored in darkness at 25 °C for 4 weeks. The effects of the metal ions were investigated in concentrations of 0, 10, 25, and 50 ppm.

Nephelometric studies of the GLP-1 derivative test solutions did not show any significant development of aggregates (Fig. 1A). However, the ThT data showed an increase in the fluorescence intensity with an increase in concentration of Al^{3+} and Zn^{2+} (Fig. 1B), whereas the test solutions containing Mg^{2+} and K^{+} did not show a change in the fluorescence response (data not shown). The discrepancy between results of the nephelometric study and ThT data is related to the specificity of the analytical methods, i.e. ThT fluorescence is a sensitive method to detect fibrils.

For GLP-1 derivative test solutions containing Al^{3+} and Zn^{2+}, the ThT fluorescence intensity appeared to increase with metal ion concentration. Moreover, there appeared to be an initial increase in intensity, followed by a plateau after 3 days. This does not correspond to a typical fibrillation kinetics with a nucleation lag-time followed by a marked increase in fibrillation. The changes in the early phase were further scrutinised using ThT fluorescence (Fig. 2). Peptide solutions containing 50 ppm Al^{3+} or Zn^{2+} were monitored from 0 to 180 min. The results revealed that the fluorescence intensity increases steadily during the initial period to a ThT fluorescence intensity comparable to the levels reached in the 4-week stability study (Fig. 1B). The changes in fluorescence had no lag-time, but began immediately after preparation. The samples contaminated with metal ions displayed initial fluorescence intensities identical to those of the pure peptide samples; hence the changes must be due to changes in the peptide structure rather than the addition of metal ions per se. A similar observation for human α-synuclein, where fast changes in secondary structure occur on addition of metal ions, has been reported [15]. These results prompted further examinations on the conformation of the GLP-1 derivative in the presence of Al^{3+} and Zn^{2+}.

In order to further investigate the fibrillation tendency of the GLP-1 derivative, the accelerated stability study

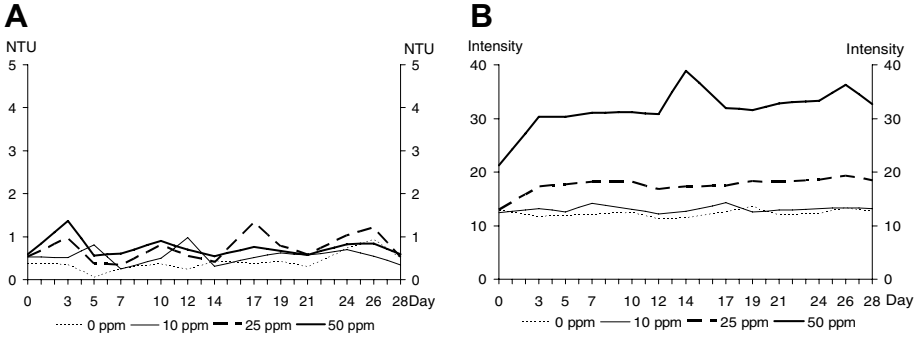

Fig. 1. (A) Nephelometric turbidity units (NTU) of test solutions (25 °C) with Zn^{2+}. Similar results for test solutions were seen with Al^{3+}, K^{+}, and Mg^{2+} (data not shown). (B) Thioflavin T fluorescence intensity of test solutions (25 °C) with Zn^{2+}. Similar results were seen for test solutions with Al^{3+} (data not shown).

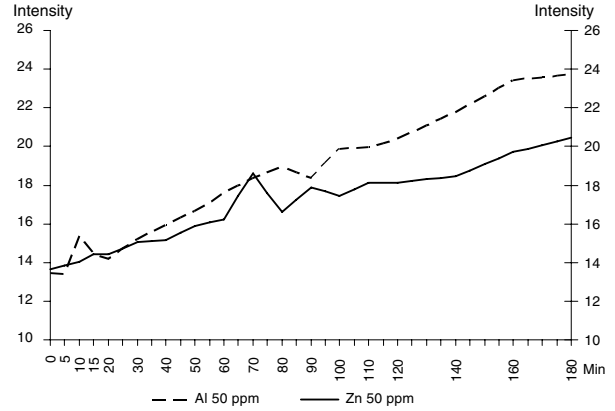

Fig. 2. Thioflavin T fluorescence intensity of non-agitated test solutions (25 °C) with Zn^{2+} and Al^{3+}.

was followed by stress testing under more severe conditions, in which samples containing Al^{3+} and Zn^{2+} were submitted to rotation at 37 °C. The cartridges submitted to rotation test were analysed by nephelometry. This analytical method was found applicable as a non-destructive method whereby aggregate formation was monitored on the same cartridge throughout the study period.

Nephelometric studies of rotated test solutions revealed typical fibrillation kinetics with a lag-time followed by a marked increase in turbidity. The lag time was not altered by the presence of Al^{3+} and Zn^{2+} or by an increase in concentration of the metal ions (Fig. 3). These findings did not correlate with the observations of the ThT fluorescence study where a concentration-dependent fibrillation was demonstrated for test solutions containing Al^{3+} and Zn^{2+}. Differentiated fibrillation kinetics of the rotated test solutions possibly did not occur due to severe test conditions i.e. mechanical stress and elevated temperature.

The findings of preliminary stability study I are summarised in Fig. 4. A decreased physical stability of test solutions containing Al^{3+} and Zn^{2+} was indicated only by the specificity of ThT to bind to fibrils. A similar differentiation

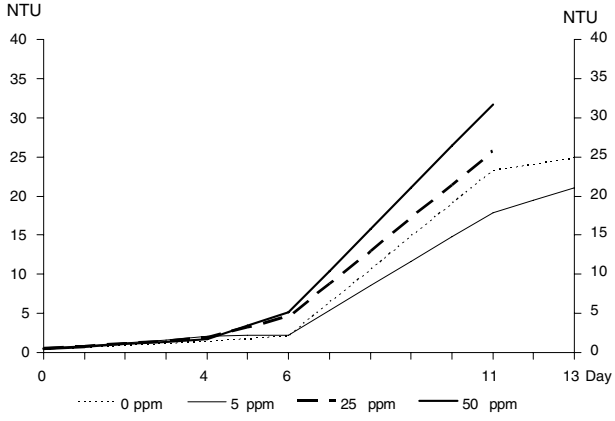

Fig. 3. Nephelometric turbidity units of rotated test solutions (37 °C) with Zn^{2+}. Similar results were seen for test solutions with Al^{3+} (data not shown).

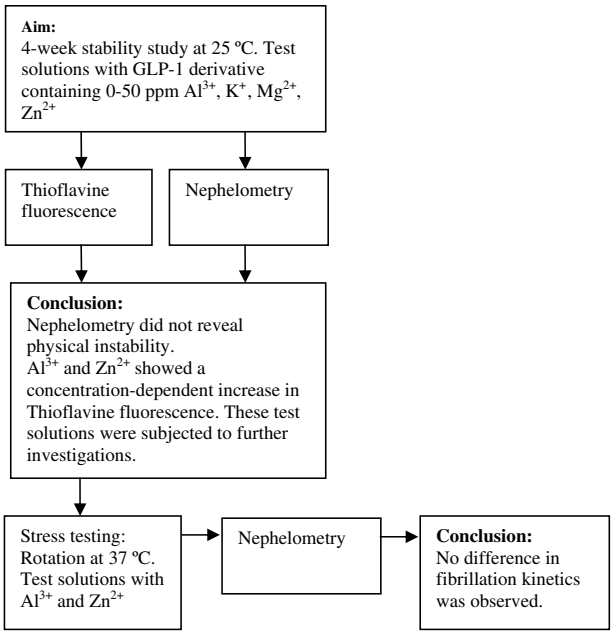

Fig. 4. Study design for preliminary stability study I.

of the fibrillation kinetics was not detected by nephelometry.

The difference in destabilising effect of the investigated metal ions, observed by ThT fluorescence, can be related to the chemical characteristics presented in Table 1. The observed destabilising effect of Al^{3+} and Zn^{2+} in preliminary stability study I can be explained by an oxidation mechanism and thus by the high standard oxidation potentials for these ions. However ion radii and hydrated radii do not seem to explain a destabilising effect of Al^{3+} and Zn^{2+} more than Mg^{2+} and K^{+}.

3.2. Preliminary stability study II: Changes in GLP-1 derivative structure in the presence of metal ions

The secondary structure of GLP-1 derivative with Al^{3+} and Zn^{2+} and a GLP-1 derivative reference was examined using FTIR. Following the secondary structure of GLP-1 derivative with Al^{3+}, Mg^{2+} and K^{+} was examined by Trp fluorescence spectroscopy.

The secondary structure of native GLP-1 in solution is reported to be predominantly α-helical; however, these studies were carried out in trifluoroethanol mixtures or in the presence of micelles [23,24]. The GLP-1 derivative

Table 1
Chemical characteristics of the investigated metal ions [16,17]

Ion	K^{+}	Mg^{2+}	Zn^{2+}	Al^{3+}
Standard oxidation potentials (V)	0.76	1.66	2.36	2.93
Ion radius (pm)	138	72	88	53
Hydrated radius (nm)	0.28–0.29[a]	0.41	0.41	0.40

[a] K^{+} forms only one coordination sphere, where Mg^{2+}, Zn^{2+}, and Al^{3+} form a second coordination sphere.

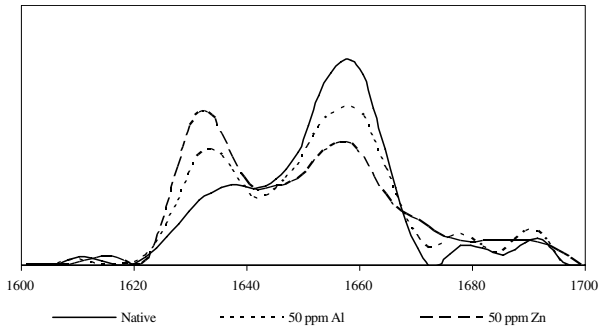

Fig. 5. Second derivative FTIR spectra of GLP-1 derivative with Al^{3+} and Zn^{2+}.

Table 2
Area-overlap of FTIR spectra of native GLP-1 derivative and GLP-1 derivative with metal ions

Area-overlap	Native	Average ± standard deviation	Significance
5 ppm Al^{3+}	0.929	0.939 ± 0.03	$P > 0.05$
25 ppm Al^{3+}	0.807	0.970 ± 0.01	$P < 0.05$
5 ppm Zn^{2+}	0.907	0.905 ± 0.05	$P > 0.05$
25 ppm Zn^{2+*}	0.780	0.915	Not applicable

Average ± standard deviation between samples is taken as a measure of the variation ($n = 3$, except * where $n = 2$).

displays similar structural elements, as demonstrated by FTIR (Fig. 5). The influence of Zn^{2+} and Al^{3+} ions on the secondary structure of the GLP-1 derivative was examined by FTIR (Fig. 5) and is reported as area-overlap calculations (Table 2). After physical stress, the GLP-1 derivative displayed a significant amount of β-sheet elements [25,26]. Readily prepared solutions of 5 ppm Zn^{2+} and Al^{3+} ions did not cause significant changes in the native structure of the peptide by FTIR (Table 2), whereas 25 and 50 ppm clearly induced formation of β-sheet (1630–1640 cm^{-1}) and loss of α-helical structural elements (1654–1660 cm^{-1}) (Fig. 5). The trend was identical for Al^{3+} and for Zn^{2+}, although the extents of the effects were different (Fig. 5).

FTIR confirmed a structural change for Zn^{2+} and Al^{3+} in a concentration-dependent manner as observed in preliminary study I. The effect on the secondary structure was further investigated using Trp fluorescence. Test solutions with Al^{3+}, Mg^{2+} and K^{+} were investigated in order to confirm the results obtained in preliminary stability study I, i.e. no destabilising effect of Mg^{2+} and K^{+}. Changes in the fluorescence optimum of Trp are related to the environment of the Trp and hence to the physical structure of the protein. In general, a shift of the Trp fluorescence towards shorter wavelengths indicates that the surroundings of the Trp are changed towards a more hydrophobic environment [21,22]. This can indicate a change of the α-helix to a β-sheet conformation [21], to aggregates [14], or it can indicate a transition from a random coil structure to a partly folded protein [15]. These processes can imply an increased propensity for aggregation and fibrillation. The emission maximum of a Trp exposed to an aqueous solvent is typically approximately 350 nm, whereas Trp in non-polar environments will exhibit lower emission maxima down to 308 nm [22,27].

Fluorescence of the single Trp in the GLP-1 derivative was measured in freshly prepared GLP-1 derivative solutions containing from 0 to 50 ppm Al^{3+}. The data show a distinct blue shift from 349 to 336 nm (Table 3). In comparison, addition of Mg^{2+} and K^{+} did not induce changes in Trp fluorescence optima (Table 3), and thus confirmed the observations from preliminary stability study I.

The findings of preliminary study II are summarised in Fig. 6. A change in secondary structure of the GLP-1 derivative with Al^{3+} and Zn^{2+} was observed by FTIR. The change in secondary structure was also confirmed by Trp fluorescence for test solutions with Al^{3+}. These changes were concentration-dependent. Mg^{2+} and K^{+} did not induce changes in secondary structure of the GLP-1 derivative. Thus the changes in secondary structure observed by

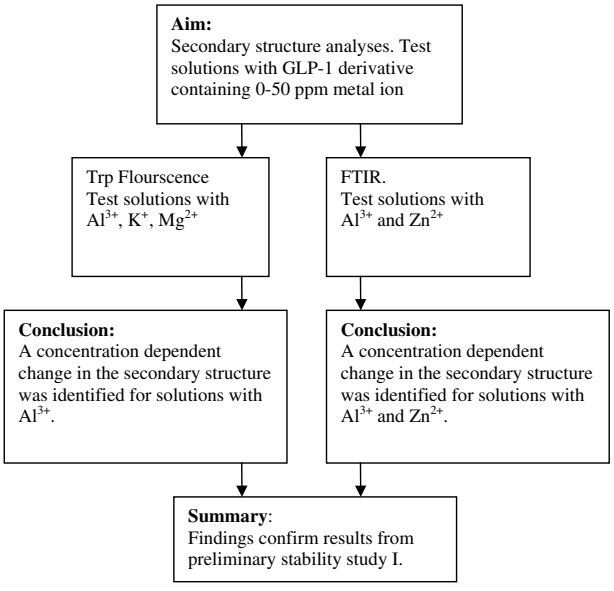

Fig. 6. Study design for preliminary stability study II.

Table 3
Trp fluorescence of test solutions with Al^{3+}, Mg^{2+}, and K^{+}

Metal ion/concentration	0 ppm	5 ppm	10 ppm	15 ppm	20 ppm	25 ppm	50 ppm
Al^{3+}	349	348	348	348	345	343	336
Mg^{2+}	349	349	349	349	349	349	347
K^{+}	349	349	350	349	350	350	349

Results are shown as Trp maxima (nm).

FTIR and Trp fluorescence confirmed the findings from preliminary study I for all investigated test solutions.

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