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Research paper

Characterization of thermosensitive chitosan-based hydrogels by rheology and electron paramagnetic resonance spectroscopy

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Abstract

Chitosan, an amino-polysaccharide, has been proposed as a promising biopolymer for tissue repair and drug delivery. Chitosan solutions containing glycerol-2-phosphate (β-GP) have been described as injectable in situ gelling thermosensitive formulations, which undergo sol–gel transition at physiological pH and temperatures. This feature makes them suitable for the parenteral administration of drugs, especially for peptides and proteins. The aim of the present study was to get a deeper insight into the macro- and microstructure of chitosan/β-GP systems. In addition to oscillating rheology, electron paramagnetic resonance (EPR) spectroscopy was applied to examine the microviscosity and pH inside the gels depending on the β-GP concentration and to follow the loading and release of spin-labelled Insulin. All chitosan/β-GP solutions showed a physiological pH ranging from 6.6 to 6.8 that did not change during gelation, irrespective of the proportion of β-GP. The dynamics of the spin-labelled Insulin and its microviscosity inside the gels and during release were monitored by EPR spectroscopy. The results indicate that the Insulin was incorporated into the aqueous environment of the gel and was released in its native form. The in vitro drug release from the gels was governed by diffusion of drug from the gel matrix. A sustained release of Insulin was observed over a period of 2 weeks. Increasing the proportion of β-GP increased the amount of released Insulin and the velocity thereof.

Keywords: Chitosan; Hydrogel; Thermogelling; Glycerol phosphate; Rheology; EPR; ESR

1. Introduction

In the recent years an increasing number of thermosensitive hydrogels have been reported for various biomedical applications [1]. Especially systems which show sol–gel transition at body temperature have gained great interest, because they are generally liquid formulations that form a semi-solid depot after injection into the desired tissue or organ. Therefore no surgery is required for placement and withdrawal and they fill cavities or defects due to their flowing nature. Many new formulations have been proposed for drug delivery [2–4], tissue engineering [5,6] and cell encapsulation [7,8]. The most studied polymers are copolymers of poly(ethylene oxide) and poly(propylene oxide) (known as Poloxamers) and copolymers of *N*-isopropylacrylamide [9–11]. However the use of these systems is limited because they are not biodegradable. Block copolymers of poly(ethylene oxide) and poly(lactic acid) were proposed as alternative biodegradable materials, but the need of high injection temperature of about 45 °C excludes their use for delivering sensitive proteins or living cells [12].

Chitosan is an amino-polysaccharide obtained by alkaline deacetylation of chitin (exoskeleton of shrimps or crabs), with applications ranging from biomedical, food,

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drug and cosmetics [13]. It is a biocompatible and biodegradable pH-dependent cationic polymer [14,15]. Chitosan is soluble in acidic solution and phase separates at pH > 6 to form a hydrogel. The addition of β-GP to acidic chitosan solutions allows rising the pH to neutral without phase separation. These systems are thermosensitive and form hydrogels at temperatures around 37 °C and above [16]. The sol–gel transition of chitosan/β-GP systems has been studied mainly by rheology [17]. However, the knowledge concerning the microenvironment within the chitosan/β-GP gel is still very limited.

The non-invasive EPR method detects paramagnetic molecules in complex and non-transparent samples. Using nitroxides as spin probes or spin labels it is possible to quantify micropolarity and microviscosity at the molecular level [18–21]. The influence of microviscosity on the spectral shape is illustrated in Fig. 1. The three lines arise from the hyperfine interaction between the electron spin and the nuclear spin of nitrogen (^{14}N). The anisotropy of the hyperfine splitting is averaged in low viscous environments (water) and three lines of almost the same signal amplitude are observed. The anisotropy is less averaged in more viscous media (PEG 400). As a result, the line widths and the signal amplitudes of the three transitions are broader and unequal (e.g., the central line has higher signal amplitude compared to the third line due to its smaller line width). In the solid materials, the anisotropy is not averaged and a typical "powder spectrum" is recorded, which reflects the sum of all orientations of the nitroxide radical. Typical parameters that can be obtained from EPR spectra are the hyperfine coupling parameter ($2aN$) and rotational correlation time (τ_c). Magnetic interaction between the free electron and the nuclear spin ($N = 1$) of the nitrogen in the nitroxides results in a hyperfine splitting of three lines. The distance between the low and high field lines of the EPR spectrum ($2aN$) depends on polarity of nitroxide environment. In regions with high polarity (e.g., water) the distance between the outer lines of the EPR spectra is increased, and therefore the hyperfine coupling parameter, compared to region with low polarity (e.g., oil). The microviscosity, another parameter of the microenvironment, strongly influences the tumbling behaviour of the nitroxyl radicals. In low viscous media they tumble free, resulting in highly symmetric spectra with three narrow lines and rotational correlation times of the order of 0.01–0.1 ns. An increase in the viscosity decreases the molecular tumbling rate of the nitroxide and leads to an increase of the rotational correlation time τ_c. Due to the restricted motion a line broadening, combined with decrease in signal amplitude of the low and high field peaks, is observed (Fig. 1).

Furthermore, special nitroxides permit the measurement of microacidity [22]. The principle is exemplified in Fig. 2(top). The pH-sensitivity of the nitroxide 4-amino-2,2,5,5-tetramethyl-3-imidazoline-1-oxyl (AT) is reflected in a smaller hyperfine splitting in acidic environments (see dashed line and the EPR spectra in Fig. 2, bottom). The calibration curve of the pH dependency gives the expected sigmoid shape (Fig. 3). As expected, the highest dependency occurs around the pK_a. Increasing differences between pH and pK_a decrease the response sensitivity. The curve shows that the pH range which can be assessed by AT ranges approximately from 4.3 to 7.9. It must be mentioned that other nitroxides with different pK_a values exist and therefore the measurement of more acidic and alkaline environments is possible. Unfortunately, in the

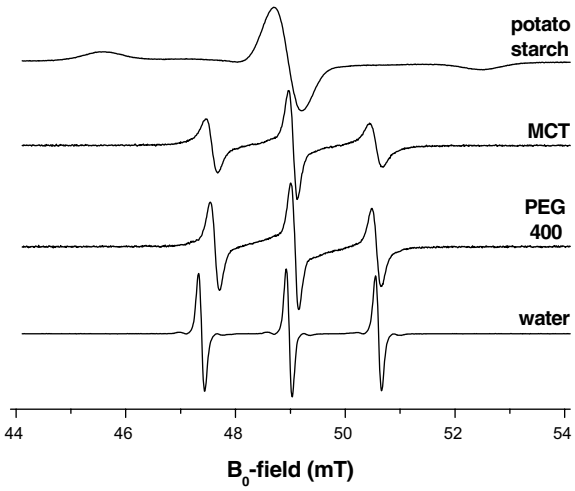

Fig. 1. EPR spectra of the nitroxide AT in environments with different viscosities: dry potato starch powder, middle chain triglycerides (MCT), PEG 400 and water.

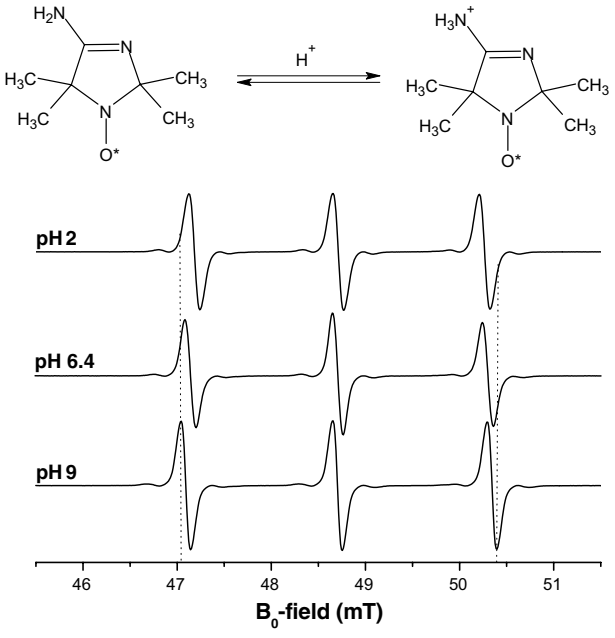

Fig. 2. Top: principle of the pH-sensitivity of the nitroxide AT. Protonation of the amino-group in position 4 results in a decrease of the spin density at the nitroxyl nitrogen, which is reflected by a decreased hyperfine splitting parameter $2aN$. Bottom: EPR spectra of the spin probe AT at different pH value. At pH values far away from pK_a of AT (6.1) the nitroxide is either fully protonated or non-protonated. The distance between the first and the third line (=$2aN$) is smaller for the protonated form.

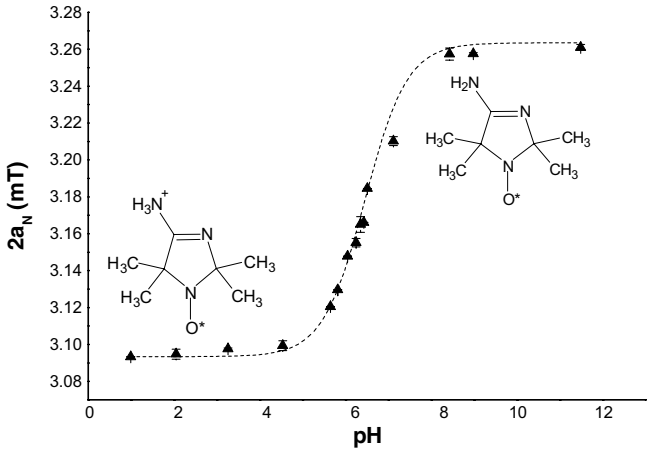

Fig. 3. pH-sensitivity of the nitroxide AT. The recorded EPR spectra (first derivative) were integrated and the distance between the first and the third peaks was determined as the measure of $2aN$. The pH-calibration curves were obtained by a sigmoid Boltzman fit to the values of $2aN$.

past EPR measurements of samples with high water content were limited to very small sizes (1–2 mm) due to the high dielectric loss. However, the recent development of low frequency EPR spectrometers did overcome this problem and enables EPR measurements of swollen tablets and even in vivo measurements on small mammals. Examples of EPR applications in drug delivery include the measurement of microacidity in microparticles [23], the in vitro detection of pH gradients in tablets [19], human skin [24] and degrading polymers [25] and the in vivo detection of pH values in biodegradable polymers [26]. A recent review summarises the applications of EPR spectroscopy and imaging in drug delivery [21].

The drug release rate from gels is determined by several parameters, including the size of the drug, the microviscosity and the mesh size of the polymer network [27]. Unfortunately, chitosan–β-GP systems have been characterized mainly by measurements of macroviscosity and no data on the molecular properties inside the gel are known so far. It was the aim of the current study to shed more light into the microviscosity and microacidity inside the thermogelling chitosan–β-GP, because these molecular parameters are important for an understanding an optimisation of these promising drug delivery systems.

2. Materials and methods

2.1. Materials

Chitosan (Chitoclear®FG95) having a deacetylation degree of 95% was obtained from Primex Siglufjordur, Iceland. Glycerol-2-phosphate disodium salt hydrate (β-GP) was purchased form Sigma Aldrich (Taufkirchen, Germany). EPR spin probe AT (4-amino-2,2,5,5-tetramethyl-3-imidazoline-1-oxyl) was given by Prof. Valery Khramtsov, Institute of Chemical Kinetics and Combustion, Novosibirsk, Russia. Spin-labelled Insulin was prepared as described in [28].

2.2. Preparation of the chitosan/β-glycerolphosphate solution

Chitosan was dissolved in 2 ml of 0.1 M hydrochloric acid. To the cooled chitosan solution, 0.5 ml chilled (5 °C) aqueous β-GP was carefully added dropwise to obtain a clear and homogeneous liquid solution in a final volume of 2.5 ml. The formulations contained 2.5% (m/V) chitosan and 6–16% (m/V) β-GP. Thermogelling of the systems was introduced by exposure to controlled temperatures in the range of 5–65 °C for 30 min. After 30 min, the samples were cooled to room temperature and finally stored in the refrigerator. The samples were inspected by eye to determine whether or not gelation did occur.

2.3. Rheological characterization

A strain controlled oscillating rheometer "Rheometrics Fluids Spectrometer RFSII" from Rheometrics, Inc., Piscataway, NJ, USA, was used at 25 °C. The rheometer was equipped with a cone – plate geometry ($d = 50$ mm, taper angle $\alpha = 0.0412$ rad $= 2.36°$) cone – plate distance 0.051 mm. The following samples were investigated: chitosan 2.5%/8% GP sol and gel; chitosan 2.5%/16% GP (sol and gel).

An amplitude test was carried out first at a radial frequency of $\omega = 6.28$ rad/s $= 1$ s^{-1} with a strain range from 0.1% to 100%. The results indicated that a deformation of 1% is within the linear viscoelastic range. Therefore, the following parameters were used for the frequency sweep tests: strain (γ): 1%, frequency range 0.01–100 rad/s.

2.4. pH and microviscosity determination by EPR spectroscopy

EPR spectra were obtained using an L-Band (1.3 GHz) spectrometer (Magnettech GmbH, Berlin, Germany) equipped with a re-entrant resonator. EPR parameters were set as follows: field centre 49 mT, scan range 10 mT, scan time 30 s, modulation amplitude 0.1 mT. The pH-sensitive nitroxide spin probe AT was incorporated into each sample (0.25 mM). AT was calibrated in citrate and phosphate buffer solutions in order to cover a range of between pH 1.0 and 12.0. The pH was determined by means of a glass electrode. Aliquots of 1 ml were taken and measured at 1.3 GHz. The recorded EPR spectra (first derivative) were integrated and the distance between the first and the third peaks was determined as the measure of $2aN$. The pH-calibration curves were obtained by a sigmoid Boltzman fit to the values of $2aN$. For the pH determination of the gels, AT was dissolved in the chitosan/β-GP solutions and spectra were recorded from the solutions before and after incubation at 37 °C for 1 h. All measurements were performed in triplicate, date are reported as means ± SEM. For the determination of the nitroxide

rotation correlation time and the microviscosity, the software EPRSIM from the "Jozef Stefan" Institute, Department of Solid State Physics, was used (http://www.ijs.si/ijs/dept/epr/erpsim.htm).

2.5. In vitro Insulin release studies

The spin-labelled Insulin was dissolved in the chitosan/β-GP solutions under stirring at room temperature, up to a final concentration of 0.2 mg/ml. Samples of 0.50 g were placed in Eppendorf cylinders and gelled at 37 °C for 1 h. The incorporation of the Insulin into gels was examined by EPR spectroscopy at 1.3 and 9.5 GHz. The gels were incubated with 1.5 ml of phosphate buffer pH 7.4 at 37 °C. At several time intervals 0.5 ml of the buffer was taken as a sample and replaced by new buffer solutions to assure sink conditions. The release was monitored by EPR spectroscopy at 9.1 GHz. Using an EPR spectrometer (Miniscope MS 200) from Magnettech (Berlin, Germany). The measurements were conducted with the following parameters: field centre 335 mT, scan range 10 mT, scan time 30 s, modulation amplitude 0.1 mT. All measurements were performed triplicate, data are reported as means ± SEM.

3. Results and discussion

No temperature induced gelation of chitosan/β-GP preparations was observable for β-GP concentrations below 6%. Higher β-GP concentrations lead to faster gelation. This result is in agreement with the finding by Leroux's group [29]. The systems described by Leroux did contain acetate and they were thermoreversible gels within a pH range from 6.6 to 6.9 and partially thermoreversible above pH values of 6.9 at concentrations ranging from 0.5% to 2% for chitosan and 2% to 8% for β-GP [30]. Our acetate free preparations did not show any thermoreversibility of gelation. Once the gelation did occur, the systems did not reliquidify after cooling, even at 5 °C. Increasing β-GP concentrations lead to a lowering of the gelation temperature from about 50 °C at 6% β-GP to 37 °C (16% β-GP).

The rheological characterization did show significant differences between the sol and thermogelled systems. The sol systems did show low values (0.5–35 Pa) of the storage (G′) and the loss (G″) modulus (Fig. 4). The loss and the storage moduli are comparable, leading to a phase angle of about 45° (tan delta around 1, see Fig. 5). The values of the storage and the loss moduli and the phase angle are typical for low viscous systems with a low degree of elasticity. The viscosity of the gelled systems is much higher compared to the sol systems of the same composition (Fig. 4). A considerable increase of the loss modulus G″ is noticed, which is higher for the gel system with 16% β-GP. However, the increase in the storage modulus is much higher compared to the increase of the loss modulus for both systems, indicating the formation of a strong gel network with high elasticity (Fig. 4). The storage modulus of the 16% β-GP gel exceeds 1000 Pa, the storage modulus of the 8% β-GP system remains slightly below this value, indicating that a higher β-GP amount leads to stronger gels. The high elasticity is also reflected in very low values of tan delta, which are around 0.1 and below (Fig. 5). In conclusion, oscillating rheology proved the formation of strong elastic gels by the thermogelling of the chitosan–β-GP systems. The gel formation is visible in a strong

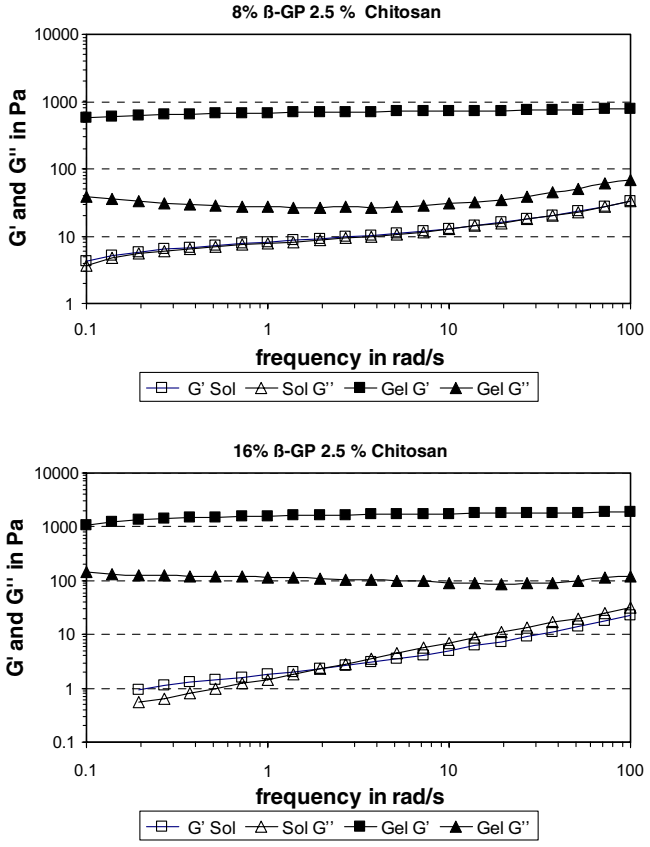

Fig. 4. Frequency dependence of storage (G′) and loss (G″) moduli of chitosan–β-glycerolphosphate mixtures before and after thermogelling. Top: samples composed of 2.5% chitosan and 8% β-GP. Bottom: samples composed of 2.5% chitosan and 16% β-GP.

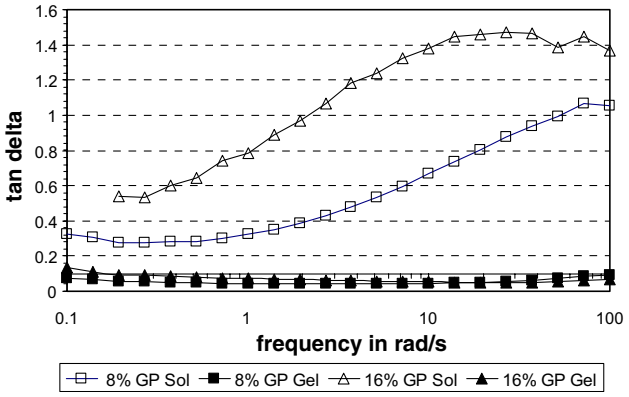

Fig. 5. Tangents of the phase angle delta of chitosan–β-glycerolphosphate mixtures before and after thermogelling.

increase (>2 orders of magnitude) of the storage modulus G′ and a decrease of the phase angle. Differences between 8% and 16% β-GP containing systems are detectable, but small compared to the temperature induced gelling effect.

The viscosity at the molecular level (=microviscosity) of the sol and gel systems was explored by EPR spectroscopy in the following experiments. The EPR spectra of the hydrophilic, low molecular weight nitroxide AT have very similar shapes before and after gelation (Fig. 6). The spectral shape indicates a high mobility of the AT molecule in a low viscous environment irrespective of the gel formation. A quantitative assessment of the rotational correlation time τ_c of the nitroxide AT was performed in different environments (Fig. 7). The mobility of the nitroxide AT is very close to the AT mobility in pure water or buffer systems and lower compared to PEG 400 and MCT. There are virtually no differences in the local mobility of the nitroxide between ungelled and gelled chitosan–β-GP systems, although the macroviscosity differs several orders of magnitude (compare Fig. 7 to Fig. 4.) This finding clearly indicates that the pore size of the chitosan/β-GP gel is large enough to have no influence on the local mobility of low molecular weight compounds. The dramatic change of macroviscosity due to temperature induced gel formation is not reflected in microviscosity.

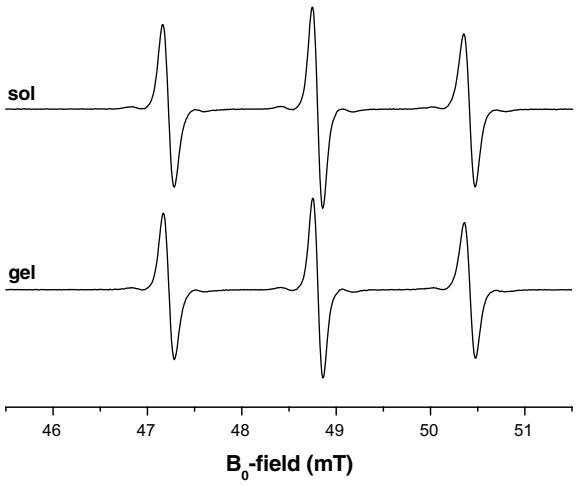

Fig. 6. EPR spectra of AT loaded chitosan/β-GP solutions before and after gelation (2.5% chitosan 8% and β-GP).

AT has a pK_a value of 6.1 and permits the measurement of pH values around pH 6.1 ± 1.8 pH units. As with pH sensitive dyes, the accuracy is highest at the pK_a and decreases with increasing differences between pH and pK_a values. The $2aN$ values of the nitroxide AT in β-GP solutions are around 3.26 mT. These values indicate the fully deprotonated state of the probe. The conclusion from the EPR measurement is that the pH of the β-GP solution is 7.9 or higher. No exact pH can be measured due to the large difference between the pK_a of AT (6.1) and the alkaline pH. The EPR results are in agreement with the pH determination by a glass electrode, which gave pH values between 9.1 and 9.2

Mixing of the acidic chitosan (pH values around 1.8) and alkaline β-GP solution resulted in pH values between 6.62 and 6.78. These pH values are in close proximity to the pK_a of the probe and therefore, the accuracy of the EPR-pH measurement is high (see error bars in Fig. 8). Two findings emerge from the experiments: firstly, it is clearly evident that despite the ratio of the protonated chitosan to the alkaline β-GP changing by a factor of 2.66 between the 6% and 16% β-GP solutions, there was no significant impact of the chitosan/β-GP ratio on the

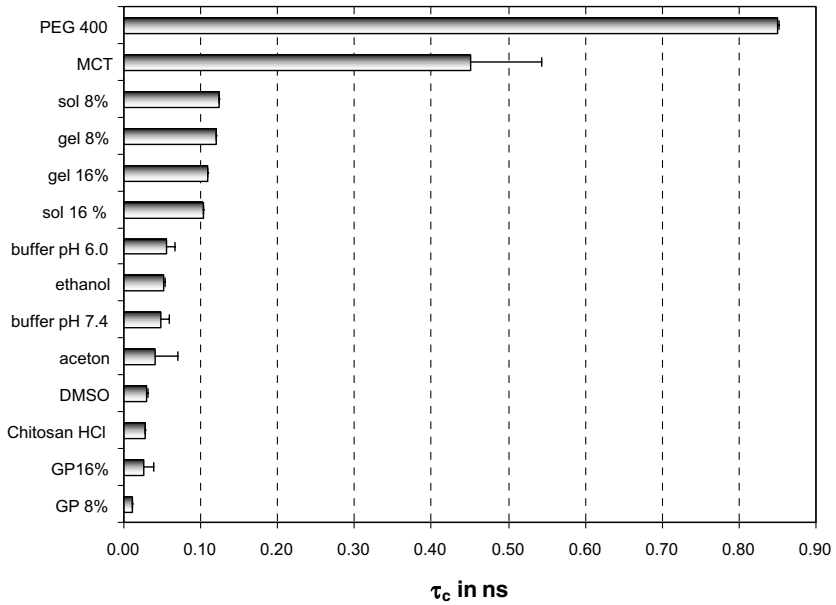

Fig. 7. Rotation correlation time τ_c (in ns) of the nitroxide AT in different environments. Higher correlation times indicate lower tumbling rates due to higher microviscosities.

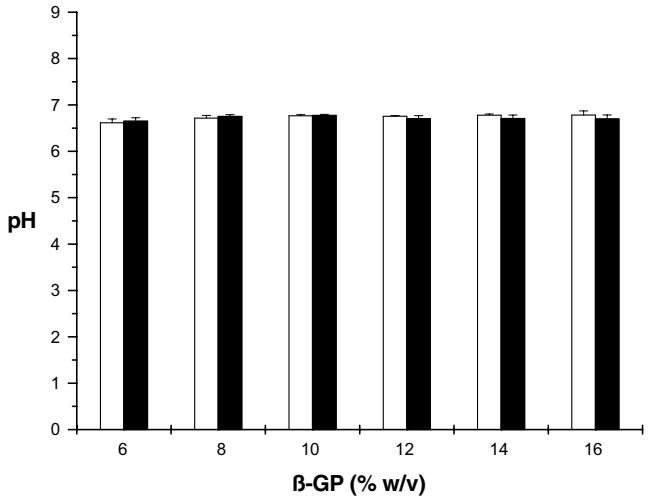

Fig. 8. Influence of the β-GP content on the pH of the chitosan/β-GP solutions and gels (White bars – sol, black bars – gel).

internal pH. Secondly, the pH values measured by EPR before and after thermogelling of the samples show that the gelation formation process does not change the microacidity inside the sample. The pH remains all the time within a very narrow range (supported by one-way ANOVA test followed by TURKEY'S test). The chitosan and β-GP solutions and gels act as a buffering system. The large stability of the pH within a physiological range makes the system very attractive for protein delivery.

In a further step we explored the impact of the chitosan/β-GP gel on protein status and release rate. Insulin was chosen as a model protein. Insulin was spin labelled according to established procedures [29]. The EPR spectra of spin-Insulin are shown in Fig. 9. The spectral shape indicates a high mobility in buffer, which is reflected in rotation correlation times of less than 0.2 ns (see Table 1). However, lower Insulin concentrations showed the lowest rotation correlation times and therefore the highest mobility. The hyperfine splitting parameter is independent from the Insulin concentration, indicating no differences in polarity of the microenvironment for the spin-labelled Insulin.

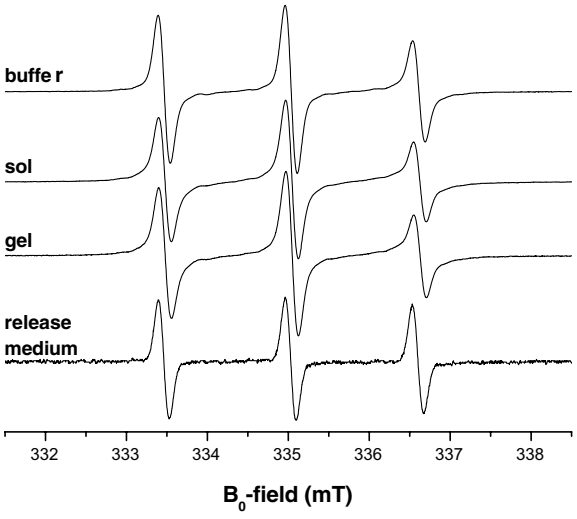

Fig. 9. EPR spectra of spin-labelled Insulin incorporated in different environment: phosphate buffer (conc.: 0.2 mg/ml), chitosan/β-GP solution, chitosan/β-GP gel (conc.: 0.2 mg/ml, 2.5% chitosan 8% and β-GP), and released spin-labelled insulin in phosphate buffer (conc. approx. 0.005 mg/ml).

The EPR spectra of spin-labelled Insulin in the chitosan/β-GP solution and gel show also high mobility (Fig. 9). Quantitative analysis of the EPR spectra yields that the gelation has no impact on the molecular mobility of Insulin, the EPR spectra of sol and gel are superimposible. This finding indicates that Insulin, although larger compared to the nitroxide AT, is not hindered in its mobility by the formation of the gel network. However, it was found that the β-GP concentration impacts the molecular mobility in a sense that higher concentrations showed a slower tumbling rate and a higher rotational correlation time. This result agrees with the general knowledge that Insulin aggregates at higher concentrations [30]. The rotation correlation of spin-labelled Insulin is (although still very fast) more than doubled in 8% β-GP systems compared to 16% β-GP preparations. It means that the formulation with the higher β-GP content, higher gel strength and a higher macroviscosity has – in contrast to the expectations – a lower microviscosity inside the gel network. The EPR spectra of the released Insulin overlap with their spectral shape to the buffer system with a low Insulin concentration (compare Tables 2 and 1), suggesting that the spin-labelled Insulin is released without denaturation (Fig. 9).

Table 1
Influence of the Insulin concentration on the EPR spectral parameters of spin-labelled Insulin

Insulin concentration in mg/ml	Rotation correlation time τ_c (ns)	Hyperfine splitting a_N (mT)
2	0.164	1.5958
0.5	0.142	1.5942
0.21	0.096	1.5943
0.0089	0.029	1.5897

High rotation correlation times indicate a slow tumbling rate and low mobility. High hyperfine splitting values indicate a polar environment. The parameters were obtained by fitting of the spectra with the program EPRSIM. Typical standard derivations are below 5%, in most cases below 1%.

Table 2
EPR parameters of spin-labelled Insulin in chitosan/β-GP sol and gels

Sample		Rotation correlation time τ_c (ns)	Hyperfine splitting a_N (mT)
8% β-GP	Sol	0.288	1.5964
	Gel	0.281	1.5959
16% β-GP	Sol	0.128	1.5950
	Gel	0.112	1.5995
Released insulin in buffer		0.029	1.5893

High rotation correlation times indicate a slow tumbling rate and low mobility. High hyperfine splitting values indicate a polar environment. The parameters were obtained by fitting of the spectra with the program EPRSIM. Typical standard derivations are below 5%, in most cases below 1%.

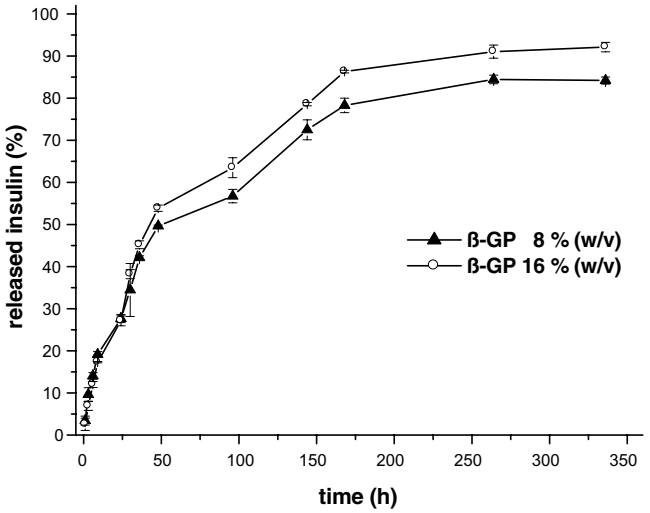

Fig. 10. In vitro release kinetics of spin-labelled Insulin from chitosan/β-GP gels with different β-GP content.

In order to investigate the impact of the β-GP content on the Insulin release rate, chitosan/β-GP gels with a high (16%) and low (8%) β-GP concentration were selected. Clearly, a controlled release was achieved despite the high mobility of Insulin within the gel (Fig. 10). The Insulin release rate did not differ between the formulations at early time points (up to 50% release within 48 h). However, in the following time a faster release from the gel with high β-GP (16%) content was observed. The faster release rate corresponds with the higher mobility of Insulin in the 16% β-GP system (Table 2). It is also clearly evident that the release is not linear, but more typical for diffusion controlled systems. Furthermore, the release is incomplete, about 15% of Insulin remain in the gel in case of the 8% β-GP and 10% for the 16% β-GP formulation.

Our results are supported by experiments from Kfufi et al., who described the release of bioactive Insulin from chitosan glycine gel beads [31]. However, their systems showed faster release rates of few hours (e.g., 6 h with 50% release in 60 min). They did also observe incomplete Insulin release. Incomplete release of a FITC albumin from chitosan matrices has also been described by Canadian scientists, who also demonstrated that the addition of lysozyme can speed up the release rate by degrading the chitosan matrix [16].

4. Conclusion

Mixtures of chitosan and β-glycerolphosphate have been investigated with respect to their ability to temperature induced gel formation. Systems composed of 2.5% chitosan and 8/16% β-glycerolphosphate combined low viscosity at room temperature with thermogelling properties. Oscillating rheology was used to characterize the macroviscosities of the sol and gel systems. Gel formation is associated with a dramatic increase of the storage modulus G′.

EPR was applied to shed more light into thermogelling chitosan/β-GP drug delivery systems. An amount of 6% β-GP is necessary to induce gel formation. A pH sensitive low molecular weight nitroxide and spin-labelled Insulin were incorporated into the gelling solutions. It was found that both substances are highly mobile despite the formation of a strong gel, indicating the existence of a low viscous microenvironment. Furthermore, EPR-based pH measurements indicate, the internal pH is slightly below 7 within a narrow range, which fits nicely the physiological pH. Neither the gelation process nor the chitosan/β-GP ratios affect the pH to a significant amount. The EPR studies with spin-labelled Insulin show that Insulin is not immobilized locally within the gel, however, a controlled release over several days was achieved. The incorporation into the gel has no impact on Insulin stability. In conclusion, the chitosan β-GP thermogelling system is an attractive delivery system for peptides and proteins. EPR measurements can help to understand the system's performance by monitoring microviscosity and pH inside the gel.

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