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Ion exchange in alginate gels – dynamic behaviour revealed by electron paramagnetic resonance

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The formation of alginate gel from low molecular weight alginate and very low molecular weight alginate in the presence of divalent cations was investigated using Electron Paramagnetic Resonance (EPR) spectroscopy. The transition from sol to gel in the presence of divalent cations was monitored by the changes in the dynamics of spin labelled alginate. The immobilisation of the spin labelled alginate in the gel reflects the strength of interaction between the cation and alginate chain. Diffusion experiments showed that both the cation and alginate polyanion in the gel fibres can exchange with molecules in solution. In particular, we showed that dissolved alginate polyanions can replace alginates in the gel fibres, which can hence diffuse through the bulk of the gel. This illustrates the surprisingly highly dynamic nature of these gels and opens up the possibility of preparing multicomponent alginate gels via polyanion exchange process.

Introduction

Hydrogels based on naturally occurring polysaccharides find many applications in biotechnology,[1] heterogeneous catalysis[2] and other areas. One of the most common gelators of this type is alginate which is isolated from brown marine algae. Thanks to its biocompatibility and low toxicity, alginate is commonly used in biomedical research,[3] as well as in the food and beverage industry as a thickening, gelling or colloidal agent.[4]

Alginic acid and its alkaline metal salts (alginates) are linear polysaccharides composed of (1-4) linked β-D-mannuronate (M) and α-L-guluronate (G) residues (Fig. 1) arranged in homopolymeric blocks.[2,5-7] The exact sequence of M and G blocks depends on the biological source, growth and seasonal conditions and these factors thus influence physicochemical properties of the alginates. A range of alginates can also be produced as biofilms by bacterial biosynthesis (*Azotobacter*, *Pseudomonas*) – this can be particularly problematic in the mucus-rich lungs of cystic fibrosis patients, where *pseudomonas aeruginosa* is associated with increased morbidity and mortality.[3,8,9]

Alginate is water soluble and forms gels in the presence of various divalent cations which act as cross-linkers for polymer chains. Ca^{2+} ions are most commonly used to prepare alginate gels and the rheological properties of these gels have been extensively studied.[1-8,10] The metal ions are coordinated by the G blocks due to the $^1C_4$ conformation of the G units and the ability of the paired G units to generate cavities in the shape of an "egg-box" that are capable of accommodating ions.[6,11,12] The G residue content of alginate therefore influences the rheological properties of the cation cross-linked gels. Thus, a higher G unit content leads to the formation of alginate gels with more ordered structure and higher stiffness.[3,13] Other divalent alkaline-earth ions such as Ba^{2+} and Sr^{2+} also form alginate gels; the Ca^{2+} gels are weaker than those of the other cations.[4,14] The Mg^{2+} cation represents an exception as the Mg-alginate system does not form a gel under normal conditions, which is proposed to be due to weaker polymer-ion interactions.[6,15,16] A recent study reported the formation of a Mg-alginate gel at higher concentration of ions and alginate compared with those generally used for other divalent cations,

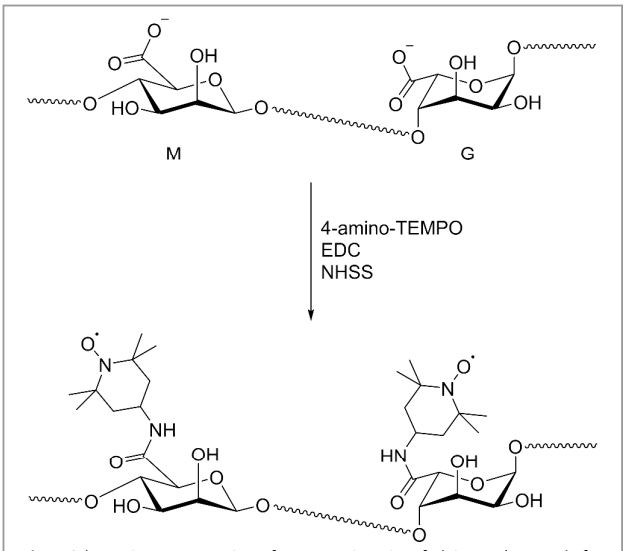

Fig. 1 Schematic representation of monomeric units of alginates (M stands for mannuronate and G stands for guluronate) and of spin labelled units.

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and highlighted that high content and longer chains of guluronic units favor formation of Mg/alginate gel.[17] Other divalent ions like Pb^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+} or Co^{2+} also cross-link alginate to form gels.[4,18] Given the toxicity of the latter cations, this ability to gelate alginate has found applications in water purification.[19] These applications are based on the ability of alginate gels to exchange cations with solution. This cation exchange is a fairly fast process governed by the thermodynamic parameters (e.g., equilibrium constants).[20]

Here, we report an EPR study of alginate gelation and ion exchange in alginate gels. EPR spectroscopy and the spin labelling approach have long been successfully applied to probe the formation of colloidal and supramolecular structures. Thanks to the sensitivity of EPR to rotational diffusion on the nanosecond time scale and interspin distances up to several nanometres, this technique can provide unique information about the environment in nanostructures.[21] This method has also been used to study properties of supramolecular and covalent gels,[22] and in particular has been shown to provide detailed and powerful insights into the dynamics of these materials.[23] Surprisingly, however, spin labelling and EPR spectroscopy have hardly been applied to probe the properties of polysaccharide assemblies. The rare examples include an EPR study combined with rheology to monitor the dynamics of spin-labelled insulin in the chitosan gel and its release from gel.[24] Spin labelled dextran and chitosan incorporated into poly(isobutylcyanacrylate) nanoparticles were used to study mobility of the nanoparticle surface groups.[25] The EPR method has been applied to study paramagnetic metal ion sorption by polysaccharide surfaces, for instance, extracellular polysaccharides produced by cyanobacteria *Anabaena spiroides.*[26] Interactions of various types of starch with transition metal dications[27] and the effect of Cu^{2+} on gelation of gellan solution have also been studied by EPR.[28] We reasoned that EPR and the spin-labelling technique could provide useful new insights into the dynamics of alginate gel formation, which could be of wider significance in understanding the behaviour of these materials – the results of our studies are reported here.

Results and discussion

Preparation of spin-labelled alginate

The mannuronate and guluronate units of alginates were randomly spin labelled by reaction of the carboxyl groups with 4-amino-TEMPO in the presence of EDC and NHSS (Fig. 1). The EPR spectra of both spin-labelled low molecular weight alginate (4-NH_2T-ALG-L) and spin-labelled very low molecular weight alginate (4-NH_2T-ALG-VL) (Fig. 2) showed a fast motion of the paramagnetic moiety covalently attached to the polysaccharide chain. The τ_c values were 4.48×10^{-10} s and 3.80×10^{-10} s for 4NH_2T-ALG-L and 4NH_2T-ALG-VL, respectively. The latter (lower viscosity) sample showed higher mobility as expected.

The EPR spectra of labelled alginates showed that the rotational diffusion of the label is fast but anisotropic. The

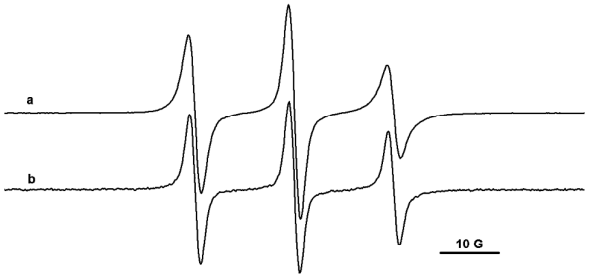

Fig. 2 The EPR spectra of 0.1 % aqueous solutions of spin-labelled low molecular weight alginate (4NH_2T-ALG-L) (a) and very low molecular weight alginate (4NH_2T-ALG-VL) (b).

lineshape is quite unusual for TEMPO derivatives. In most cases, TEMPO units show fastest rotation along the axis parallel to the N-O bond which results in EPR spectra in which the low field line shows the highest intensity (in the fast motion regime). However, spin-labelled alginates show EPR spectra that are indicative of a more complex rotational motion and/or tilt of the rotational diffusion axes with respect to the molecular axes. This suggests that the environment around the TEMPO units in the spin labelled alginates is conformationally restricted, which is consistent with the recently suggested structure of alginate solutions that include an extended polymer network surrounded by large water pools.[29]

At higher concentration of spin labelled alginates (ca. 1%), the EPR lines showed further broadening typical of hindered rotational motion (Fig. S1, S2). This is probably due to self-assembly and entanglement of alginate polymers in these solutions. In the absence of cationic cross-linkers, however, such self-assembly results simply in increased viscosity rather than formation of a sample-spanning gel network. A similar effect was observed when spin labelled alginate was mixed with unlabelled alginate. Although the spectra remained in the fast motion regime even at 3% concentration of alginate (Fig. S3, S4), restricted tumbling was clearly visible at total alginate concentrations above 1%. For example, in 3% alginate solution the τ_c value for 4NH_2T-ALG-L increases to 9.33×10^{-10} s and for 4NH_2T-ALG-VL to 7.72×10^{-10} s. The latter (lower viscosity alginate) sample is still more mobile as expected. As we were primarily interested in the changes in the EPR spectra brought about by the formation of the gel phase in the presence of metal ions, we used a total alginate concentration of 1% in subsequent experiments. The spin-labelled alginate concentration was kept at 0.1% to make sure that EPR spectra are not affected by spin-spin interactions between adjacent spin labels.

Gel formation in the presence of divalent cations

The alginate gels can either be formed by adding the alginate solution dropwise to the solution of a divalent cation to form gel 'beads' or by adding the solution of a cation to the alginate solution resulting in the formation of a gel monolith (Fig. 3). The gels thus formed incorporate a certain volume of solution which is almost independent of the method of gel preparation.

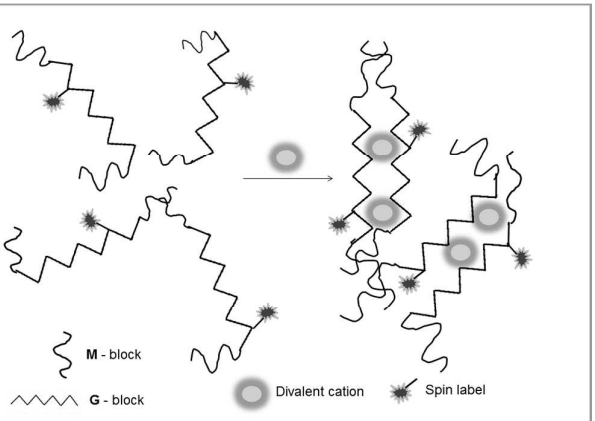

Fig. 3 Formation of the alginate gels in the presence of divalent cations.

The EPR spectra of alginate gels formed in the presence of Ca^{2+} salt reveal two components (Fig. 4a). One component shows very restricted molecular motion, while the rotational tumbling of the other component is similar to that of metal-free alginate (Fig. 2). The slowly moving component can undoubtedly be attributed to the alginate molecules in the gel fibres. Due to highly restricted dynamics of this component, it is likely to belong to spin-labelled units in close proximity to the cross-linking points of the gel, possibly in the G block of the alginate polymer. It cannot correspond to spin labelled sugar units which are directly involved in cross-linking as the carboxylate groups are attached to the spin labels and are therefore not available for the cross-linking interactions with the metal ions.

The identity of the mobile component is more difficult to establish. We found that if the remaining solution is separated from the gel, it has no EPR signal. Therefore, the mobile component cannot simply belong to alginate molecules dissolved in excess solvent. It could correspond to spin-labelled saccharide residues far away from the cross-linking points, *e.g.,* near the end groups of the polymer, in the M blocks of the polymer, or alternatively to spin labels in free alginate molecules that are not attached to the gel fibres but are entrapped in the gel network.

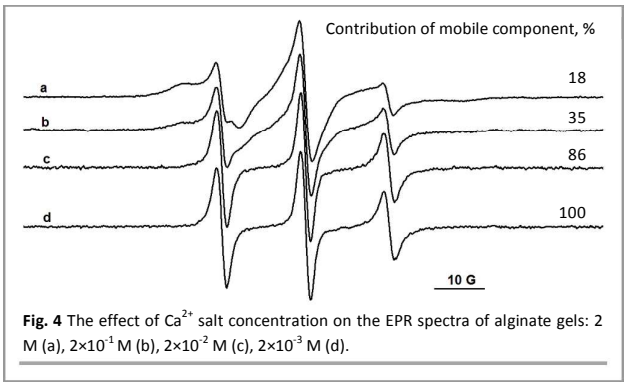

Fig. 4 The effect of Ca^{2+} salt concentration on the EPR spectra of alginate gels: 2 M (a), 2×10^{-1} M (b), 2×10^{-2} M (c), 2×10^{-3} M (d).

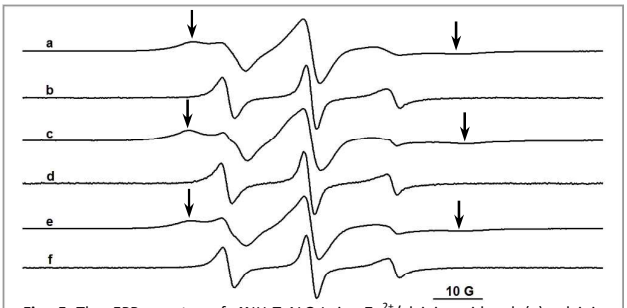

Fig. 5 The EPR spectra of $4NH_2$T-ALG-L in: Zn^{2+}/alginic acid gel (a), alginic acid/Zn^{2+}/EDTA mixture (b), Ba^{2+}/alginic acid gel (c), alginic acid/Ba^{2+}/EDTA mixture (d), Ca^{2+}/alginic acid gel (e), alginic acid/Ca^{2+}/EDTA mixture (f). The outer peaks of the immobile component are shown with arrows.

The ratio of the two components in the EPR spectra depends on the relative concentrations of the alginate and the metal ion. On reducing the Ca^{2+} concentration to 0.2 M (Fig. 4b), the contribution of the immobile component to the EPR spectrum decreases. The gel is still formed under these conditions but its strength is lower.[30] A subsequent further reduction of Ca^{2+} concentration to 20 mM or 2 mM leads to a turbid solution; the gel is not formed (Fig. 4c,d). The EPR spectra of this solution are similar to those in the absence of the dication.

The analysis of the immobile component can shed light on the rigidity of the gel fibres, which in these systems is related to the strength of complexation of dication with alginate chains. EPR spectra Fig. 5a,c,e correspond to the alginate gels formed in the presence of Zn^{2+}, Ba^{2+} and Ca^{2+} (at 2 M concentration). Although all spectra show two components, the distance between the outer lines of the slow component (effective $2A_{zz}$ which is related to the rate of molecular tumbling: the smaller the effective $2A_{zz}$, the faster the tumbling) is different for different metals. In all experiments, the concentrations of salts and alginate were maintained at the same value to ensure the same cation/alginate ratio. The values of effective $2A_{zz}$ for spectra corresponding to the gels formed in the presence of Zn^{2+}, Ca^{2+} and Ba^{2+} are 55.0, 56.0 and 57.2 G, respectively. This order correlates with the strength of the gels, *e.g.,* Ba^{2+} forms a stronger alginate gel than Ca^{2+},[31] and as such, less molecular tumbling is observed.

Ion exchange experiments in alginate gels

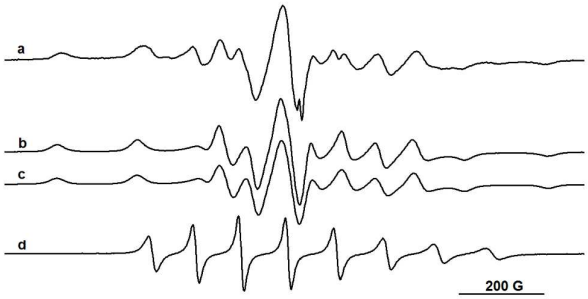

Fig. 6 EPR spectra of vanadyl cation in alginate gel at 293 K (a), 170 K (b), simulation at 170 K (c) and after diffusion of $ZnSO_4$ into the gel.

The sensitivity of EPR to the molecular environment makes it possible to use this method for monitoring ion exchange processes. For instance, addition of a strong chelating agent such as EDTA to the alginate-metal ion gel should result in the extraction of metal ions from the gel until its dissolution. The faster tumbling of spin-labelled alginate molecules released from the gel can be monitored by EPR, thus reporting on the progress of cation exchange. These experiments were carried out as follows. A 2 M solution of a dication salt (0.2 mL) was added to a 1% solution of alginate (0.6 ml) to form a gel. The excess solvent was removed and replaced with the same volume of a 2 M EDTA solution. The EPR spectra in Fig. 5b,d,f show only one rapidly tumbling component consistent with the breakdown of the gel fibres. These spectra do, however, show somewhat slower tumbling rates than those observed for free alginate in water (Fig. S5a), in 2 M EDTA (Fig. S5b) or 4 M NaCl (Fig. S5c). This suggests that the difference in the tumbling rates cannot be attributed to increased ionic strength. We conclude that some degree of self-assembly persists and that extraction of metal ions from the alginate complexes using EDTA treatment in this manner is incomplete.

The gels can also be modified by ion exchange, i.e., replacement of one metal ion with another. The EPR of spin-labelled alginates cannot be used to monitor this process as the mobility of the gel fibres will not change significantly. However, ion exchange can be monitored by using a paramagnetic metal ion. In this work, we used VO^{2+} to replace Zn^{2+} ions from the alginate gels.[32]

Just like other dications, vanadyl ions are capable of gelating alginate. Fig. 6a,b shows EPR spectra of vanadyl-alginate gels at room temperature and in a frozen matrix. The magnetic parameters of vanadyl-alginate gels calculated from the frozen matrix spectra ($g_{||}$ = 1.935, $g_{\perp}$ = 1.974, $A_{||}$ = 530 MHz, $A_{\perp}$ = 195 MHz, Fig. 6c) are typical of vanadyl-dicarboxylate complexes[33] which confirms that the vanadyl ions cross-link two alginate units through formation of dicarboxylates. Comparison of the spectra at room temperature (Fig. 6a) and in the frozen matrix (Fig. 6b) shows that at room temperature, the vanadyl units at the cross-linking points possess very little, if any, mobility on the EPR timescale. Small changes to the spectral lineshape at room temperature (Fig. 6a) can be explained by the presence of small amount of a more mobile component (with a spectrum similar to that in Fig. 6d).[34]

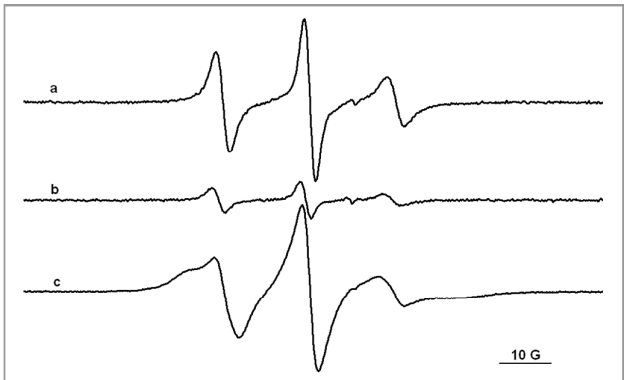

Fig. 7 The EPR spectra of a spin-labelled alginate solution (a) and after mixing with unlabelled Zn^{2+}/alginate gel, mother liquor (b) and gel phase (c).

Addition of Zn^{2+} ions (2 M) to the vanadyl-alginate gels resulted in immediate release of the vanadyl into solution as evident from the resultant fast motion EPR spectra, Fig. 6d. The reverse process (e.g., replacement of Zn^{2+} from the alginate gel with VO^{2+} using a saturated solution of $VOSO_4$) is also possible. This was confirmed by the EPR spectrum corresponding to VO^{2+} immobilised in the gel structure (Fig. S6). This suggests that cation exchange can readily occur in such materials.

While the ability of metal-alginate gels to exchange metal ions is known,[20] a related polymer ion exchange process (i.e., exchange of gel-immobilised alginates with other anionic alginates in solution) has not been explored. We studied this self-exchange by adding a 1% solution of spin-labelled alginate to unlabelled Ca^{2+}/alginate gel or Zn^{2+}/alginate gel. After equilibration for a few hours, the spectra of both gel and liquid phases were recorded. The intensity of EPR spectra in the liquid phase decreased (Fig. 7a,b), while the gel phase spectrum showed two components (Fig. 7c). The latter spectrum is similar to those of spin-labelled alginate gels, however the proportion of the faster tumbling component is higher, and the tumbling rate of the slower moving component is faster. This suggests that the spin-labelled alginate has replaced some of the polyanion in the 'solid-like' gel network. Similarly, addition of unlabelled alginate to a gel formed from spin-labelled alginate, led to the release of the latter into solution as confirmed by EPR (Fig. S7).

In order to test whether this polymer anion exchange process occurs just at the gel surface, or if polymer chains can diffuse long distances through the gel, we carried out the following experiment. A 2 cm high column of alginate gel was prepared by addition of alginate solution to Zn^{2+} salt. This column was then placed on top of a solution of spin-labelled alginate (0.1 %) (Fig. 8a). After three hours, the signal of spin-labelled alginate in solution decreased (Fig. 8b). The column was then cut in two parts and briefly washed with water. The EPR spectra of the two pieces (Fig. 8c,d) demonstrated that spin-labelled alginate has diffused through the first piece of the gel into the second, although the EPR intensity of the top piece is clearly weaker. The EPR spectra of both gel pieces also show immobilised spin label, consistent with the incorporation of the polymer chains in the gel fibres.

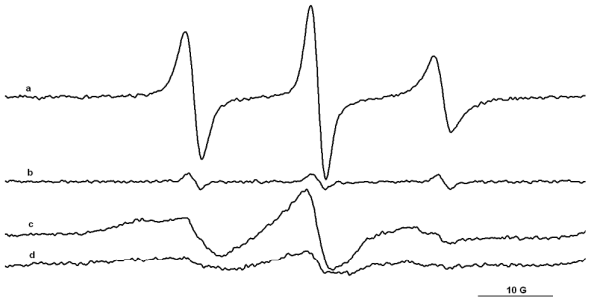

Fig. 8 EPR spectra of spin-labelled alginate in the initial solution (a) and after mixing with the Zn^{2+}/alginate gel: mother liquor (b), the bottom piece of gel (c), the top piece gel (d).

This ability of alginate polyanion to diffuse through alginate gels and exchange with the polymers in the fibres in solution is rather surprising, taking into account the large number of carboxylate groups in each polymer molecule which are involved in gel cross-linking. This polymeric anion exchange confirms that these cross-links have a significant dynamic nature, and also suggests that other polycarboxylates, or indeed other polyanions, may be incorporated into the existing alginate gels through a simple exchange process – providing these materials with significant potential further scope for applications than may have been suspected.

Experimental

Materials

Low-viscosity sodium alginate and very low-viscosity sodium alginate, 4-amino-TEMPO, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), *N*-hydroxysulfo succinimide (NHSS) and pH 7 phosphate buffer solution (1 M), were obtained from Sigma-Aldrich. Salts of divalent cations were obtained from Fluka. The following salts of divalent cations were used: $Zn(NO_3)_2$, $Ca(NO_3)_2$, $Ba(NO_3)_2$. In experiments with VO^{2+}-containing gels, $ZnSO_4$ and $VOSO_4$ were used.

Procedure for spin-labelling alginates. Sodium alginate (150 mg) was dissolved in a pH 7 phosphate buffer (1 M, 15 mL) to give a 1% solution. EDC (80 mg) and NHSS (50 mg) were added to this solution in order to activate the carboxylic acid groups of the alginate. After 30 min, a solution of 4-NH_2TEMPO (30 mg in 1 ml of pH 7 buffer) was added to the alginate solution and allowed to react overnight. The spin labelled alginate was precipitated by adding excess acetone and washed with acetone to remove unreacted amino-TEMPO. The precipitate was dried under reduced pressure. Two types of sodium alginate were used: with low and with very low viscosity. Isolated yields of spin-labelled alginate were approximately 70%. The degree of spin labelling (e.g., proportion of spin labelled repeat units) was ca. 0.5 % as estimated from EPR peak intensity.

The unlabelled and labelled samples of sodium alginate with low viscosity are referred to as ALG-L and 4-NH_2T-ALG-L, respectively. The unlabelled and labelled samples of sodium alginate with very low viscosity are referred to as ALG-VL and 4-NH_2T-ALG-VL, respectively.

Alginate gel formation. The alginate gels were prepared by either adding a solution of divalent cation to the alginate solution in one portion, or by dropwise addition of the alginate solution to the salt solution. In the former case, the gel separated from solution as a monolith, while in the latter case gel capsules were formed. Obviously, the size of the gel capsules depends on the size of the alginate droplets. For most experiments presented here, the concentration of divalent cations was 2 M. Formation of Ca^{2+} alginate gel was also analysed as a function of Ca^{2+} cation concentration in the range 2×10^{-3} – 2 M.

Instruments

The EPR spectra were recorded on a Bruker EMX-Micro spectrometer equipped with a temperature control unit. The general EPR parameters were: modulation amplitude 1 G; power 1 mW; sweep width 100 G for measurements nitroxides, and 1500 G for vanadyl derivatives.

For EPR spectra in the fast motion regime, the rotational correlation times τ_c (in seconds) were determined using the following equation:

$$\tau_C = 6.51\times10^{-10}\,\Delta H_0\left[\left(\frac{h_0}{h_{-1}}\right)^{1/2}+\left(\frac{h_0}{h_{+1}}\right)^{1/2}-2\right]$$

where ΔH_0 is the peak-to-peak width (in Gauss) of the central line, h_{-1}, h_0 and h_{+1} are the heights of the low, central, and the high field lines, respectively.[35]

Conclusions

EPR spectroscopy is a powerful tool for studying molecular organisation and exchange processes in gels. The sensitivity of EPR to the rotational motion of the spin labels on a nanosecond time scale makes this method ideal for monitoring the formation and collapse of gels, and exploring the molecular environment within them. Our results show the presence of two distinct environments in alginate gels cross-linked with metal ions; these environments probably correspond to the sugar units in different positions with respect to the cross-linking points of the gel. Ionotropic gelation of alginate leads to dynamic gels which can exchange not only metal cation, but also alginate polyanions with the external solution. We believe that these observations are of considerable significance in suggesting new approaches for the future preparation of multifunctional alginate gels through polymer exchange or potential intervention in biofilm-forming processes in bacterial infections.

Acknowledgements

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Cation and polyanion exchange in alginate gels were monitored by spin labelling and EPR spectroscopy