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Gel beads from novel ionic polysaccharides

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Abstract

Stable gelling systems were obtained by mixing polyanion solutions with solutions containing suitable polycations. Carboxymethyl cellulose was chosen as the polyanion, whilst a number of polycations, with different molecular weights and charge densities, were tested. In particular, both low molecular weight polyamines and new synthetically aminated polysaccharides, derived from pullulan and scleroglucan, were used. Stable gels, in the absence of phase separation, were obtained only with flexible polycationic species. The morphological characterisation of the gel beads obtained were studied by scanning electron microscopy and NMR microscopy.

Keywords: Gel beads; Mixed gels; NMR microscopy; Scanning electron microscopy; Aminopropyl pullulan; Aminopropyl scleroglucan; Carboxymethyl cellulose

1. Introduction

Gel forming systems are interesting subjects because of their possible use in sophisticated applications in different scientific and technological fields. In addition, advanced experimental techniques may be used for the investigation of their morphology. Although different polymeric and non-polymeric species can be used to obtain gels, polysaccharides play an important role mainly in the formulation of the so-called weak gels.

One of the aims of this work was to develop new systems for the production of weak gels by mixing solutions of differently charged polymeric species. In particular, conformationally semi-rigid polyanion (carboxymethyl cellulose) and polycations exhibiting different flexibility were tested. Following this idea, new polycationic derivatives were prepared from two fungal polysaccharides, pullulan and scleroglucan.

Pullulan is a flexible water soluble neutral glucan produced by different species of fungi, such as *Cryphonectria parasitica*, *Phaeoacremonium aleophylum*, *Phaeomoniella chlamydospora* and *Aureobasidium pullulans*. Commercial pullulan is mainly obtained from *A. pullulans*. In this polymer, maltotriose repeating units are linked by means of alpha 1 → 6 glycosidic bonds (Catley, 1970, 1971; Ueda, Fujita, Komatsu, & Nakashima, 1963; Wallenfels, Keilich, Bechtler, & Freudenberger, 1965) forming a linear chain. The flexibility of pullulan is mainly due to the presence of 1 → 6 linkages (Kato, Okamoto, Tokuya, & Takahashi, 1982). Although the introduction of positive charges onto the pullulan chain might decrease the intrinsic flexibility, the presence of 1 → 6 glycosidic bonds should keep a certain degree of flexibility.

In contrast to pullulan, scleroglucan, produced by *Sclerotium* spp., is considered as a rigid polysaccharide. Besides the contribution of the cellulosic polymer backbone, the rigidity also depends on the triple-helix structure assumed by scleroglucan in water (Bluhm, Deslandes, Marchessault, Perez, & Rinaudo, 1982). Previous studies on some scleroglucan derivatives showed that derivatisation did not alter very much the chain aggregation ability of the polymer (Gianni et al., 2002).

In addition to aminoalkyl pullulan and scleroglucan derivatives, commercial low molecular weight 'polyamines' were also used for interaction tests with carboxymethyl cellulose.

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The gel beads structure was investigated by means of NMR microscopy and scanning electron microscopy (SEM).

2. Experimental

2.1. Materials

Two samples of pullulan were purchased from Hayashibara Biochemical Laboratories Inc. (Okayama, Japan, lot no. 902101 and no. PI20) exhibiting average M_n equal to 73,000 and 108,000 g mol^{-1}, respectively. The polysaccharide was used without further purification.

The aminoalkyl derivatives were obtained using an SN_2 reaction, both in water and in water-*iso*-propanol mixture. Either 2-chloroethyl amine or 3-chloropropyl amine (Aldrich, sold as 98% chlorhydrate) were used as the nucleophylic reactant to obtain aminoethyl pullulan and aminopropyl pullulan, respectively.

When the reaction was carried out in water, pullulan was inserted in a jacketed glass vessel. Water was added dropwise to promote a slow swelling. After complete swelling, the system was made homogeneous by magnetic stirring, then the proper amount of water was added to the viscous system. The reactants were added under continuous stirring, in the following order. (1) NaOH 50% w/v solution to a final OH^- equivalents to glucose residues ratio equal 5. This produced a marked decrease in the viscosity of the solution and yellowing of the mass. (2) $NaBH_4$ (Aldrich, purity 99%) up to a molar borohydride to glucose residues ratio of 0.08. (3) Chloroalkyl amine chlorhydrate to a final ratio of amine groups to glucose residues in the range 2–3, depending on the desired degree of substitution. After that, the temperature of the heating water bath was increased up to 68–70 °C and the stirring was continued for 8–24 h to reach different values of the degree of substitution. Finally, the yellowish reaction mixture was cooled down to room temperature and neutralised with 1 M HCl. The derivative was then separated by dropping the solution into abundant cold (4 °C) ethanol. The flock-like precipitate was separated by centrifugation and dissolved in water. The solution was exhaustively dialysed against distilled water, filtered with a Millipore type SM apparatus (pore size 5 μm), frozen in an acetone-CO_2 bath and liophylised in a Modulyo Edwards apparatus.

When using 50% v/v aqueous *iso*-propanol (C. Erba, analytical grade) as the reaction medium, pullulan (2 g) was pestled in a mortar and added with *iso*-propanol (5 ml) so to wet all the solid. Two millilitres of 30% w/v NaOH (C. Erba RP) were then added under grinding. The system became yellowish and hard. One millilitre of water was added to increase the mobility of the system and, possibly, the accessibility of polymer chains to the reactants. Solid $NaBH_4$ and subsequent small amounts of chloroalkyl amine were then added. After each addition of chloroalkyl amine, a careful and prolonged grinding was performed. After that, the reaction mixture was introduced into the jacketed reactor with the aid of 1.5 ml of *iso*-propanol and 1 ml of water added to give a homogeneous, although highly viscous, system. The reactor was heated up to 55 °C and the system was let to react for 4 h. Separation and purification of the derivative were performed as already described.

When using scleroglucan, only the synthesis of the aminopropyl derivative was performed. The synthesis was achieved by swelling 0.75 g of scleroglucan (Applied Biosystems, Actigum CS11™) in 3 M NaOH (15 ml) for 3 h at 60 °C under vigorous stirring. The opalescent and viscous solution of the polysaccharide was then added of 3-chloropropyl amine (0.5 g solubilised in 1 ml of 3 M NaOH) and left under stirring at 60 °C. A similar addition of the amine was repeated twice at 2.5 h intervals. The reaction mixture was stirred for 12 h at 60 °C, then diluted with 30 ml of water, neutralised with 2 M HCl, exhaustively dialysed against distilled water and then freeze dried.

Carboxymethyl cellulose (CMC) was a kind gift from Lamberti S.p.A., Albizzate (Varese, Italy). It was supplied and used in the Na^+ salt form. Its DS, as declared by the producer and verified by potentiometric titration, was 0.8. The crude derivative was purified first by dissolution in water under vigorous mechanical stirring (Silverson Machines Ltd, England), then by dialysis against pure water and filtration with a G3 glass filter. Finally, the clear solution obtained was freeze-dried.

The following polyamines were also used in the interaction tests with CMC: cadaverine dichlorhydrate (Sigma, lot 1275-0447), spermine tetrachlorhydrate (Sigma, lot 54H2637) and spermidine thrichlorhydrate (Sigma, lot 85H0972).

2.2. Instruments and methods

The pH measurements were performed with a PHM240 Radiometer pHmeter (Copenhagen, Denmark), equipped with a GK2401B Radiometer combined electrode.

The elemental analyses were performed using a model 1106 C. Erba Elemental Analyzer.

Conductivity measurements were carried out at 25 °C with a HI 8633 Hanna Instruments conductivity meter and were used to test the effect of the dialysis for the purification of the derivatised polysaccharides.

NMR spectra were recorded on a Bruker AC200 spectrometer. The concentration of the solutions was 25 mg ml^{-1} and the temperature was 300 K. The ^{1}H NMR spectra were recorded at 200.13 MHz, with a spectral width of 2700 Hz, an acquisition time of 1.5 s and 64 scans with pre-saturation of the solvent. The value of 4.75 ppm was assigned to the residual water signal referring to the Na 3-(trimethylsylil)-propionate-2,2,3,3-d_4 (TSP) resonance used as the reference. ^{13}C NMR spectra, decoupled from protons, were recorded at 50.32 MHz, with a spectral width of

16,000 MHz, an acquisition time of 0.5 s and 110,000 scans. Chemical shift values were referred to TSP, attributing the value of 39.6 ppm to the resonance of DMSO, used as the external reference.

FT-IR spectra of CMC were recorded in the range 400–4000 cm^{-1} by a double beam System 2000 Perkin–Elmer spectrophotometer. Samples were dispersed in KBr (concentration, 1–2%) and disks were prepared using a hand oil press (10 t) connected to a vacuum system.

Molecular weights and molecular weight distributions of polysaccharides were measured using a PU-880 Jasco HPLC apparatus with Rheodyne 9125 injection, equipped with a TSK Pwxl G6000 + G5000 + G3000 300×7.8 mm^2 Toso Haas columns. Experimental conditions were: temperature, 40 °C; mobile phase, 0.15 M NaCl; flow, 0.8 ml min^{-1}. Chromatix CMX-100 LALLS and Waters Differential Refractive Index 410 were used as the detectors (temperature, 32 °C).

SEM measurements were carried out by a Leica model 430i Stereoscan apparatus, cooled by liquid nitrogen. The sample was frozen by contact with a glass surface, cooled by solid CO_2. The solid sphere (2–3 mm in diameter) was cut by a cold blade and put onto the cold sample holder with the plain surface up. The temperature in the vacuum sample room was increased from − 170 up to − 85 °C to have better images. The quality of the images was sufficiently good to avoid the need for gold sputtering.

NMR Microscopy measurements were performed at 24 °C using a Bruker AM300 spectrometer equipped with a standard microimaging unit and coupled to a Spectrospin vertical wide bore superconducting magnet operating a 7.05 T (300 MHz for ^1H). The gel spheres were placed at the bottom of a 5 mm NMR tube in the absence of the mother liquor, to avoid interference. Two-dimensional images were obtained with a modified spin-echo pulse sequence (Hsu et al., 1995), which reduced the effect of diffusion. The matrix size was 128×128 and the field of view was 6 mm, giving an in-plane resolution of 47 μm. Average NMR parameters were calculated from slice projections. Longitudinal (T_1) and transverse (T_2) relaxation times were obtained using the above mentioned spin-echo sequence. T_2 values were calculated from nine projections acquired with an echo time (TE) ranging between 10.5 and 60.5 ms. T_1 values were calculated from a series of 11 projections obtained at 11 different repetition times, TR (TR = 0.2–15 s). Diffusion coefficients were obtained using the pulse gradient spin echo (PGSE) sequence (Stejskal & Tanner, 1965). Seven projections with the diffusion sensitising gradient g up to 0.45 T m^{-1} applied along z axis were measured. The duration of the PGSE pulses δ was 3 ms and their separation, Δ, was set to 11 ms, thus giving the b attenuation factor values up to 1.29×10^9 m^2 s^{-1}. The diffusion coefficient D was calculated using a linear fit of $\ln I$ against b according to the equation

$$\ln I = \ln I_0 - \gamma^2 g^2 \delta^2 (\Delta - \delta/3) D = \ln I_0 - bD$$

where I and I_0 are the signal intensities in the presence and absence of the diffusion sensitising gradient, respectively. M_s/M_o ratio, where M_s and M_o are the magnetisation in the presence and absence of saturation, respectively, was computed following the procedure suggested by Wolff and Balaban (1989). A 1×10^{-5} T saturation pulse of 10 or 3 kHz offset was applied for 2 s before each scan to obtain the M_s values. T_1 and T_2 relaxation maps were also calculated from a series of 2D images obtained with the spin-echo sequence and the TE and TR values described above.

2.3. Gel formation tests

Gel beads formation tests were carried out by pouring the polycation solution (e.g. aminopropyl pullulan solution) into a beaker gently stirred with the aid of a magnetic stirrer. A single drop of the polyanion solution (e.g. CMC solution) was added with a syringe. Experiments were also performed by dropping the polycation solution into the polyanion one. Drops of aminopropyl pullulan solution into CMC concentrated solution, however, did not produce any gel beads. In this case, an increase of both the scattering and adherence of the solution was only observed.

In the event of gel formation, stirring was stopped and the gellish drop was allowed to age some hours or days before investigation by SEM and/or NMR microscopy.

3. Results and discussion

Data on the chemical and macromolecular characterisation of the aminopropyl pullulans synthesised are reported in Table 1. As it can be seen, the molecular weights and the polydispersity indexes of samples obtained in different experiments were very similar. In addition, the indexes of polydispersity were low (1.60 ± 0.01). Aminoethyl

Table 1
Conditions for aminopropyl pullulan synthesis

Label	P15d	P17d	P19d	P20d	P21d
Amine/pullulan[a]	2/1	2/1	3/1	3/1	3/1
NaOH/pullulan[a]	5/1	5/1	5/1	5/1	5/1
$NaBH_4$/pullulan[a]	0.08/1	0.08/1	0.08/1	0.08/1	0.08/1
Final NaOH conc. (N)	1.54	1.38	1.4	1.4	1.4
T (°C)	70	68	70	68	68
t (h)	24	24	8	16	14
DS_{EA}[b]	0.092	0.098	0.102	0.23	0.16
DS_{NMR}[c]	0.086	0.1	–	–	0.143
$\langle M_n \rangle$	20300	23300	–	–	30400
$\langle M_w \rangle$	32600	37300	–	–	48200
Polydispersity index	1.61	1.60	–	–	1.59

[a] Moles of reactant per moles of repeat units of the polysaccharide.
[b] Degree of substitution determined by elemental analysis.
[c] Degree of substitution determined by ^1H NMR.

Table 2
Sample experiments for the formation of gel beads

n[a]	Solution A	Solution B	Effect of the interaction
1	Spermidine 0.5N, pH = 12	CMC1 1.5% w/v, pH = 3	Diffusion of the drops in the solution without appreciable change in viscosity
2	CMC1 1.5% w/v, pH = 7	Cadaverine 1N, pH = 8	Transparent streaks, that disappear after stirring. The surface tension appears changed where the solution B was dropped
4	Aminopropyl pullulan (DS = 0.54) 0.123N, pH = 8	CMC 6% w/v, pH = 7–8	The drops are opalescent and compact. Upon stirring, precipitate is formed
5	CMC 6% w/v, pH = 7–8	Spermine 1.16N, pH = 7	Milky wakes are formed, without increase in the apparent viscosity
26	Aminopropyl pullulan (DS = 0.12), 4.87×10^{-2}N, pH = 7–8	CMC 6% w/v, pH = 7–8	The falling drops maintain their shape, with the presence of a microprecipitate. The drops are resistant against touch with a tool. Opalescence and apparent viscosity increase upon further additions
30	Aminopropyl pullulan (DS = 0.2), 6.9×10^{-2}N, pH = 8	CMC 1.2% w/v (0.05N), pH = 7–8	The drops become clear spheres, that depositate on the bottom. The drops are resistant against touch with a tool. A microgel is visible in the spheres. A few days after, the drops are white and less rigid
44	Aminopropyl pullulan (DS = 0.098) 3.88×10^{-2}N in 1:1 v/v H_2O/EtOH, pH = 7–8	CMC 1.2% w/v (0.05N); pH = 7–8	Spherical drops settle on the bottom. They are resistant against touch with a tool. They become opalescent with time
45	Aminopropyl pullulan (DS = 0.23), 7×10^{-2}N, pH = 7–8	CMC 1.2% w/v (0.05N), pH = 7–8	The drops are clover-shaped. A few minutes later, they become white
59	Aminopropyl scleroglucan (DS = 0.18), 5.8×10^{-4}N	CMC 1.2% w/v (0.05N), pH = 7–8	The drops maintain their structure; a few minutes later, white precipitate is formed

[a] Number of the experiment.

pullulans, obtained under the same conditions, exhibited a much lower DS (0.02).

The aminopropyl scleroglucan samples used for the gel formation tests had a DS of 0.18.

The index of polydispersity of CMC obtained is surprisingly high. M_w is about 10^6 and the M_n is about 1.7×10^5 g mol^{-1}. It is probable that aggregation is responsible for the unusually high M_w value found compared with industrially derivatised celluloses.

As a consequence of the reaction conditions chosen, both pullulan and scleroglucan derivatives exhibited a rather low degree of substitution (DS, i.e. the ratio of moles of amino groups to glucose residues) rather low. Different DS values were obtained by varying the molar ratio of amine to polysaccharide but keeping the other reaction parameters unchanged (Table 1).

A number of variables were taken into account in the polycation/polyanion interaction tests: the nature of the (poly)amines, the concentration of polyelectrolytes in the interacting solutions, pH, ionic strength and nature of solvent.

As a first attempt, we tested monomeric polyamines as network promoters in CMC solutions. Many tests were performed, both dropping polyamines solution into CMC solution and vice versa, and all were unsuccessful. Then we moved to aminopropyl polysaccharide derivatives that in general appeared more effective in linking the polyanion. However, on dropping a polyelectrolyte solution into the other one only a few of the almost 70 tests performed resulted in gel bead formation. The experimental conditions used in some of the tests performed are reported in Table 2.

It is worth pointing out that the most stable gel beads were formed by using aminopropyl pullulan in the following conditions: degree of substitution ranging from 0.1 to 0.2; high concentration of the aminopropyl pullulan solutions (i.e. concentrations ranging from 7 to 14% w/v); CMC concentration, 1.2% (w/v); pH ranging from 7 to 8; operating procedure: CMC solution dropped into the polycation solution. Noticeably, the beads were particularly stable when the aminopropyl pullulan solutions were prepared in 1:1 (v/v) water–ethanol. SEM and NMR microscopy studies were performed on these beads after ageing for 24 h.

In contrast on mixing monomeric polyamines with CMC almost no effect was observed. In these cases, a small change in the viscosity of the solution, as empirically observed by the motion of small bubbles, and/or the presence of stripes on the surface or inside the solution as a consequence of mixing by a needle, were the only detectable effects. Apparently, the molecular size of these amines is too low to promote the formation of a polymeric network (Table 2).

Aminopropyl scleroglucan exhibited very different behaviour. In fact, upon mixing aminopropyl scleroglucan and CMC solutions, phase separation occurred that revealed a strong interaction between the two charged polysaccharides. However, it was impossible to find the conditions to obtain a weak gel (Table 2) probably because

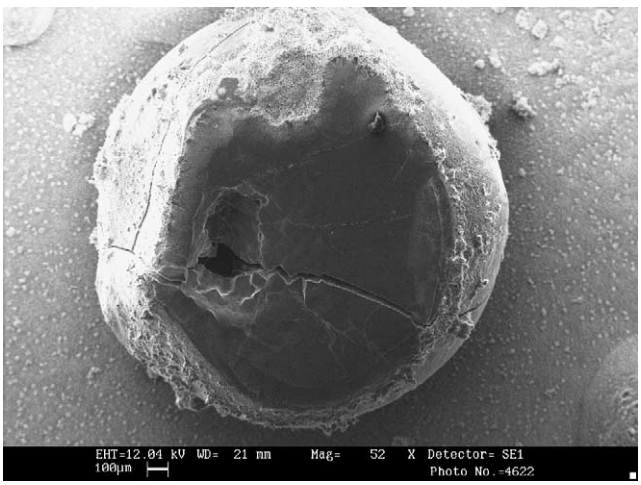

Fig. 1. SEM microimage of a gel bead obtained by dropping carboxymethyl cellulose aqueous solution into 1:1 water–ethanol aminopropyl pullulan solution. $T = -90$ °C.

the rigid structure of scleroglucan prevented the formation of suitable flexible regions needed to set up the gel network.

SEM analysis showed that an external shell and a core were clearly recognisable in intact spheres (Figs. 1 and 2). The structure of the core appeared to be morphologically homogeneous. Very likely, most aminopropyl pullulan molecules could not reach the inner part of the bead and remained confined in the external shell because of strong interactions with CMC.

Contrarily to this, a clear supramolecular structure was evident in the shell, where two different regions may be recognised. In both regions, the basic structure of the system was represented by microspheres of very similar size, exhibiting a diameter of about 1 μm. Enlargement of the image of the inner part of the shell showed that this region

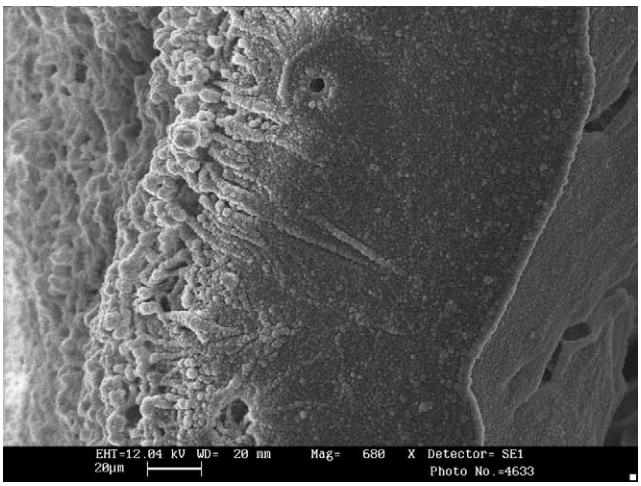

Fig. 2. SEM microimage of the shell of a gel bead obtained by dropping carboxymethyl cellulose aqueous solution into 1:1 water–ethanol aminopropyl pullulan solution. Right side, external skin of the shell. Left side, inner part of the shell. $T = -150$ °C.

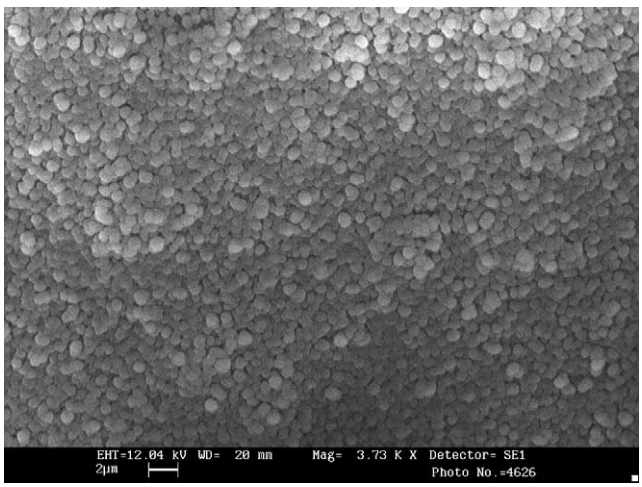

Fig. 3. SEM microimage of the inner part of the shell of the gel bead shown in Fig. 2. $T = -150$ °C.

was composed of microspheres, randomly distributed and very close to each other (Fig. 3). In contrast, the outer layer the microspheres were arranged in a regular network (Fig. 4). This structure is strongly reminiscent of the ‘string of beads’, that is believed to be the basic structure when colloids or gel are formed as a consequence of thermal denaturation of proteins (Glazer, McKenzie, & Wake, 1963; Hatta, Kitabatabe, & Doi, 1986).

For proteins, hydrophobic interactions are invoked as the driving force for the formation of the strings. In our systems, it can be hypothesised that hydrogen bonds and/or ionic interactions between the globules are responsible for the building up of the strings.

Although in Fig. 2 the transition from the unstructured heap of microspheres to the network seems to be continuous, the micrograph in Fig. 4 shows that, at least in some regions, a clear-cut surface divides the two kinds of structures. It may be speculated that the more external layer was formed as soon

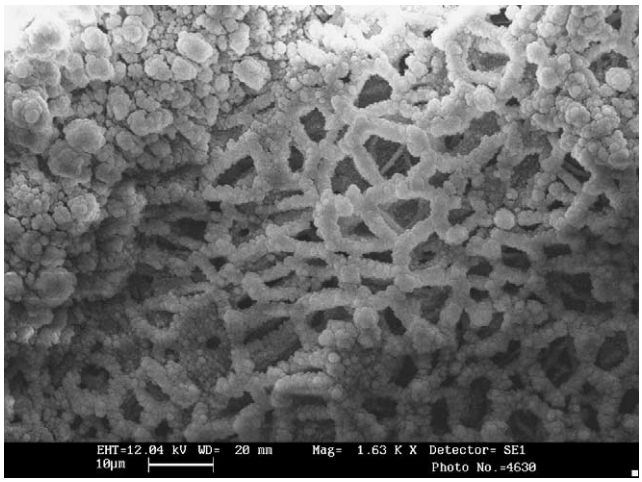

Fig. 4. SEM microimage of the outer part of the shell of the gel bead shown in Fig. 2. $T = -150$ °C.

Table 3
Magnetic parameters detected with beads (experiment no. 44; see Table 2)

Parameter	Average value
T_1 (s)	1.17
T_2 (ms)	27.4
D ($\times 10^{-9}$ m^2 s^{-1})	0.76
M_s/M_o at 10 kHz	0.99
M_s/M_o at 3 kHz	0.94

as the CMC solution drop reached the aminopropyl pullulan solution bath. In contrast, the inner shell was formed by diffusion of aminopropylpullulan molecules from the external layer. Under this hypothesis, the polymers concentration play a major role in gel morphology formation.

NMR microscopy allowed us to obtain information on some physical properties of the gel beads. As revealed by Degrassi, Toffanin, Paoletti, and Hall (1998), who investigated NMR imaging parameters in alginate gels, the transverse relaxation time T_2 is closely connected with the gel strength. In our investigation, T_1, T_2, diffusion and magnetisation transfer measurements were carried out on the spheres previously examined by SEM. The average values obtained for these parameters are reported in Table 3, whilst a selected spin-echo microimage is shown in Fig. 5.

The low T_2 values obtained indicated that the gel matrix of our systems was characterised by low dynamics. As a reference, a gel of 4% w/v calcium alginate has an average T_2 of 48 ms (Degrassi et al., 1998). This is in agreement with the relatively high concentration of the interacting polyelectrolytes and the whitening of the beads upon ageing, that reveals a further ordering of the mixed polymers as a function of time.

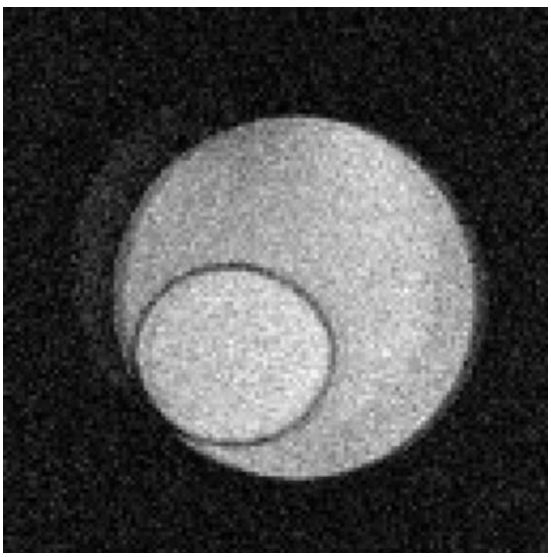

Fig. 5. Proton density-weighted spin-echo NMR microimage of a gel bead obtained by dropping carboxymethyl cellulose aqueous solution into 1:1 water–ethanol aminopropyl pullulan solution.

The low water diffusion coefficient (see Table 3) found for the gel beads confirmed the presence of a tightly structured system.

The M_s/M_o ratio may be used to evaluate, although indirectly, the percentage of water bound by the polymeric network. This parameter is 1 when all the water present is free. The value of 0.94 found for a 3 kHz offset (see Table 3) revealed that the fraction of water bound to polymers in the spheres was very low. On the other hand, the average T_2 value for the whole spheres revealed a high rigidity of our system.

4. Conclusions

Structured systems, in the form of beads, were obtained upon mixing CMC (degree of substitution, 0.8) and aminopropyl pullulans with low degree of substitution (namely, in the 0.1–0.2 range). Moreover, they were obtained in a narrow range of concentrations and pH. Stable beads were obtained in water/ethanol. No gel or phase separation were obtained when using either low molecular weight polyamines or aminoalkyl scleroglucan, indicating that the selection of the proper polymeric backbone is a critical parameter to obtain stable structures necessary to gel formation. As to the interaction between the CMC and the aminopropyl pullulans, it is reasonable to imagine that the derivatized pullulan chains act as network promoters towards the cellulosic chains because of the large difference between the two polysaccharides in terms of both molecular weight and flexibility. The aggregates formed promoted supramolecular structures responsible for the formation of the shells observed in the bead outer domains. The core of the beads here studied might consist in a very weak gel, principally constituted by CMC dispersed in water, where the water molecules still maintain high mobility.

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