

# Immobilization of cells by electrostatic droplet generation: a model system for potential application in medicine

V Manojlovic<sup>1</sup>  
 J Djonlagic<sup>1</sup>  
 B Obradovic<sup>1</sup>  
 V Nedovic<sup>2</sup>  
 B Bugarski<sup>1</sup>

<sup>1</sup>Faculty of Technology and Metallurgy,

<sup>2</sup>Faculty of Agriculture, University  
 of Belgrade, Belgrade, Serbia and  
 Montenegro

**Abstract:** The process of electrostatic extrusion as a method for cell immobilization was investigated that could be used for potential applications in medicine. An attempt was made to assess the effects of cell addition and polymer concentration on the overall entrapment procedure, ie, on each stage of immobilization: polymer–cell suspension rheological characteristics, electrostatic extrusion process, and the process of gelation. The findings should contribute to a better understanding of polymer–cell interactions, which could be crucial in possible medical treatments. Alginate–yeast was used as a model system for carrier-cells. The electrostatic extrusion was considered as a complex two-phase flow system and the effects of cell and alginate concentrations on the resulting microbead size and uniformity were assessed. Under investigated conditions, microbeads 50–600 µm in diameter were produced and the increase in both alginate and cell concentrations resulted in larger microbeads with higher standard deviations in size. We attempted to rationalize the findings by rheological characterization of the cell–alginate suspensions. Rheological characterization revealed non-Newtonian, pseudoplastic behavior of cell–alginate suspensions with higher viscosities at higher alginate concentrations. However, the presence of cells even at high concentrations ( $5 \times 10^8$  and  $1 \times 10^9$  cells/mL) did not significantly affect the rheological properties of Na-alginate solution. Lastly, we investigated the kinetics of alginate gelation with respect to the quantity of  $\text{Ca}^{2+}$  ions and cell presence. The gelation kinetics were examined under conditions of limited supply with  $\text{Ca}^{2+}$  ions, which can be essential for immobilization of highly sensitive mammalian cells that require minimal exposure to  $\text{CaCl}_2$  solution. The molar ratio of G units to  $\text{Ca}^{2+}$  ions of 3.8:1 provided complete crosslinking, while the increase in alginate concentration resulted in prolonged gelation times but higher strength of the resulting gel. The cell presence decreased the rate of network formation as well as the strength of the obtained Ca-alginate hydrogel.

**Keywords:** alginate, electrostatic extrusion, rheology, yeast cells

## Introduction

Electrostatic extrusion is a novel, proven technique for immobilization of various cell types, such as brewing yeast cells (Nedovic et al 2001a, 2001b, 2002), insect cells (Bugarski et al 1993, 1994), and plant cells (Sajc et al 1995). The advantages of electrostatic extrusion make the technique a first choice for immobilization procedures in medicine. Compared with other extrusion techniques, it can give much smaller, microscale particles (it is possible to obtain alginate microbeads down to 100 or even 50 µm in diameter: Bugarski et al 1994; Manojlovic et al 2004). In contrast to emulsion techniques (that give particles of nanoscale dimension), this technology is reproducible, controllable, and gives beads that are uniform in size. Further, the technique is easy to use under sterile conditions. The production process is performed under mild stress conditions without the use of any organic solvents that can inhibit cell activity and cause serious damaging effects. Although a high electric field is involved, it has been

Correspondence: V Manojlovic  
 University of Belgrade,  
 Faculty of Technology and Metallurgy,  
 Department of Chemical Engineering,  
 11120 Belgrade, Karnegijeva 4,  
 Serbia and Montenegro  
 Phone +381 1133 70472  
 Fax +381 1133 70387  
 Email manojlovicverica@yahoo.com

proven that the applied potential does not damage cells. No loss in cell activity or viability occurs after immobilization. The productivity of the technique is high enough to produce microcapsules in the scale needed for clinical needs. In addition, the electrostatic extrusion technique has been shown to have high entrapment efficiency with almost no loss in cells during processing.

Electrostatic droplet generation is based on the use of electrostatic forces to disrupt a liquid filament at the capillary–needle tip and form a charged stream of small droplets (Keshavartz et al 1992; Bugarski et al 1994). The process of electrostatic droplet formation is a complex function of a number of parameters such as applied electrostatic potential, needle diameter, electrode distance and geometry, polymer solution flow rate, as well as properties including surface tension, density, and viscosity (Poncelet et al 1999). The situation becomes even more complicated when a cell suspension is introduced within the polymer, which can affect both polymer properties and the extrusion process (ie, micro-hydrodynamics within the capillary via electrostatic and physical interactions of the cells). We have previously investigated effects of several operating and design parameters on electrostatic extrusion of polymer–cells suspension and the resulting microbead size, such as the applied potential, needle size, electrode spacing, and suspension flow rate (Bugarski et al 1994). However, effects of cells on hydrogel properties affecting flow dynamics have not been examined.

The objective of this study was to further investigate and rationalize the effect of cell presence on each stage of the immobilization procedure: polymer rheological behavior, extrusion process, and finally, gelling kinetics, since bead quality is reflected in variations in all these domains. The mutual impact carrier-cell is still under scrutiny and a better understanding of these interactions should contribute to understanding of encapsulated cell behavior, which is very important for possible medical applications. Alginate was used as a test carrier and yeast as a model cell type.

Alginates are widely used as immobilizing materials for cells or tissue in the development of artificial organs and have the potential to be used in the treatment of a variety of diseases (Melvik and Dornish 2004). Alginates are naturally derived linear copolymers of 1,4-linked  $\beta$ -D-mannuronic acid and  $\alpha$ -L-guluronic acid residues (Martinsen et al 1989; Moe et al 1995; Gombotz and Wee 1998). The ratio and sequential distribution of  $\beta$ -D-mannuronic acid (M) residues and  $\alpha$ -L-guluronic acid (G) residues along the length of the alginate chain vary in alginates of different origins (brown

seaweeds, certain bacteria) (Martinsen et al 1989; Smidsrod and Skjak-Braek 1990; Gombotz and Wee 1998). There is no regular repeat unit in alginate polymers, and the chains can be described as a varying sequence of regions termed M blocks, G blocks, and MG blocks. Water solutions of polysaccharides form hydrogels in the presence of divalent ions via ionic interactions between acid groups on G blocks and the chelating ions, generally  $\text{Ca}^{2+}$  (Eiselt et al 2000). As a result, calcium alginate gels are physically cross-linked systems with mechanical properties dependent on the proportion and length of the G blocks in a given alginate chain (Martinsen et al 1989; Gombotz and Wee 1998). There is a hypothesis that calcium ions bind only between G blocks of more than 20 units, forming a polymer network, and at high calcium concentrations, multiple cross-linking among alginate chains is possible (Grant et al 1973; Stokke et al 1991, 2000; Kong et al 2003; Simpson et al 2004). The gels are viscoelastic solids, with a network structure described by the “egg-box” model (Grant et al 1973; Stokke et al 2000; Simpson et al 2004).

Coupled with other naturally biodegradable or synthetic polymers, alginates create matrixes, which provide scaffolds for cells, proteins, and genes for a variety of applications. Complexing poly-L-lysine (PLL) with polycation-linked alginates provides a membrane around the alginate beads resulting in either alginate–PLL or alginate–PLL–alginate membranes that have numerous encapsulation applications. As an alternative to PLL, other polymers, such as poly(methylene-co-guanidine), can be used to obtain higher mechanical stability and retain metabolic activity of the encapsulated cells. Implanting recombinant cells encapsulated in alginate microcapsules to express therapeutic proteins has proven effective in treating several mouse models of human diseases (neurological disorders, dwarfism, hemophilia, lysosomal storage disease, and cancer). Novel capsule formulation and encapsulation technologies allow independent adjustments of capsule size, wall thickness, mechanical strength, and permeability, which may offer distinct advantages for immunisolating cells. Different techniques for immunoisolation and immobilization of viable cells within microcapsules have been developed. Here we used a sophisticated droplet generator system based on the use of electrostatic field, which has proven to be applicable for the immobilization of higher-organism cells, such as hybridomas (Goosen et al 1997; Bugarski et al 1999), islets of Langerhans (Bugarski et al 1994, 1997), and parathyroid cells (Rosinski et al 2002). Besides the geometry of a designed electric field, rheological behavior (behavior of cells and polymer

in an extrusion process) should be well understood, since it influences the formation of uniform capsules. Therefore, an attempt was made to phenomenologically consider the influence of cell–polymer interaction in close contact, in order to optimize the parameters for obtaining microcapsules. For medical applications it is very important to obtain uniform beads of a desired diameter. We used yeast cells as a model system, since they are easy to use compared with other cell types (such as mammalian cells), and their behavior during the short process of immobilization is quite similar to that of any other cells. Knowing that the rheological behavior of cells and polymer is a two-phase flow entity could contribute to a universal approach to the encapsulation method.

Cell presence can also influence gelation kinetics and mechanical properties of final Ca-alginate microbeads. Although most studies of alginate bead production have been carried out in an excess of hardening solution (ie,  $\text{CaCl}_2$ ) over prolonged times in order to ensure complete gelation (Martinsen et al 1989; Gilson et al 1990), immobilization of highly sensitive mammalian cells (eg, bone marrow cells) can require minimal exposure to  $\text{CaCl}_2$  solution. Assessment of gelation kinetics can be therefore essential for optimization of immobilization techniques for these cells. In this study we attempted to analyze gelation kinetics with limited supplies of  $\text{Ca}^{2+}$  ions with pure polymer solutions, as well as the effects of cell addition on gelation kinetics.

## Experimental

### $^1\text{H-NMR}$ spectroscopy

The polymer investigated was low viscosity sodium alginate Protanal LF 20/40 (FMC Biopolymer). The molar ratio of mannuronate (M) to guluronate (G) residues (M/G) and the mole fraction of GG, MM, and GM diad sequences  $F_{\text{GG}}$ ,  $F_{\text{MM}}$ , and  $F_{\text{GM}}$  were determined by  $^1\text{H-NMR}$  according to Grasdalen (Grasdalen et al 1979). The alginate sample was partly degraded by very mild hydrolysis with an acid in order to diminish solution viscosity. The hydrolyzed alginate sample was dissolved in  $\text{D}_2\text{O}$  at neutral pD.  $^1\text{H-NMR}$  spectra were run at 400 MHz on a Bruker AC 250 E NMR spectrometer.

### Electrostatic extrusion

Polymer solutions of different concentrations in the range 1%–4% were prepared by dissolving Na-alginate powder in distilled water. Polymer–cell suspensions were formed by mixing the prepared Na-alginate solutions with a suspension of brewing yeast cells (*Saccharomyces cerevisiae*) at various

volume ratios to obtain final cell concentrations in the range from  $1 \times 10^7$  to  $5 \times 10^8$  cells/mL. Spherical droplets were formed by extrusion of the polymer–cell suspension through a blunt stainless steel needle using a syringe pump (Razel Scientific Instruments, Stamford, CT, USA) and a 10-mL plastic syringe. Electrode geometry with the positively charged needle and a ground hardening solution was applied. The hardening solution was  $\text{CaCl}_2$  at a concentration of 1.5%. The potential difference was controlled by a high-voltage dc unit (Model 30R, Bertan Associates, Inc., New York, USA) and was varied in the range 6.5–7.5 kV. Distance between the needle tip (20 gauge small or 22 gauge) and the hardening solution was 2.5 cm while the flow rate of polymer solution was 13.9 mL/hour. The experimental set-up is presented in Figure 1. Three batches were produced for each set of experimental parameters. A sample of 30 microbeads was taken from each experiment and diameters of microbeads were measured with an accuracy of 10  $\mu\text{m}$  using a microscope (Carlzeiss Jena). The average microbead diameter and standard deviations were then calculated from the measured data, and the average of all three batches was taken as representative. Cell concentrations were determined by cell counting under the microscope using a Thoma chamber.

### Dynamic rheological measurements

Dynamic rheological measurements of alginate samples before and during gelation were performed by a Rheometrics mechanical spectrometer RMS-605 operating in the dynamic shear mode between parallel plates. The plate diameter was 25 mm, and the gap between the plates could be set between 1 and 3 mm. The frequency was varied from 0.1 to 100 rad/second. Pure Na-alginate solutions in the concentration

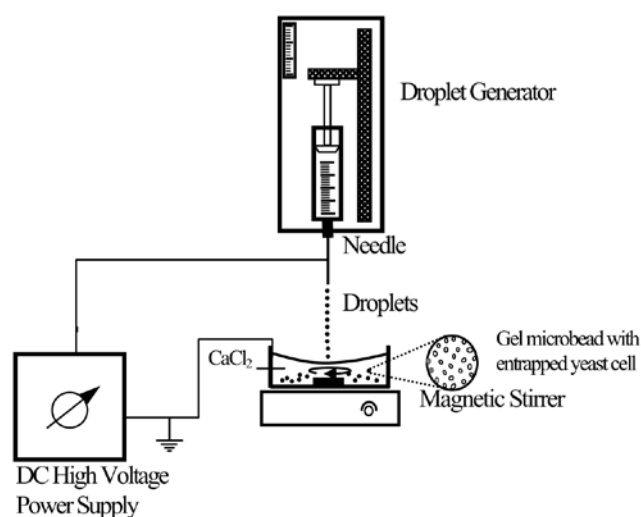


Figure 1 Schematic diagram of the experimental set-up.

range 2%–4% w/w as well as the suspension of yeast cells at the concentration of  $5 \times 10^8$  cells/mL were analyzed at room temperature, 30°C, and 37°C. Viscosities of alginate solutions at lower concentrations (below 2%) could not be measured with the Rheometrics, because of the instrument's lower limitation of 1 PaAs. The liquid samples were analyzed at a constant strain of 30% and complex dynamic viscosities ( $\eta^*$ ) as well as the storage ( $G'$ ) and loss ( $G''$ ) moduli were recorded. The complex dynamic viscosity determined as a function of frequency ( $\omega$ ) was considered as being equal to the steady shear viscosity ( $\eta$ ), a function of shear rate ( $\dot{\gamma}$ ), ie,  $\eta^*(\omega) = \eta^*(\dot{\gamma})$ . For each sample and temperature, up to 16 values could be recorded. Some values at lower concentrations and higher temperatures could not be obtained accurately because of inadequate sensitivities of the measurements.

Gelation kinetics were analyzed by rheological examinations of mixtures of Na-alginate (1%–4% w/w) and  $\text{CaCl}_2$  (1.5%) solutions at a strain of 5% in order to ensure a good contact between the hydrogel and rheometric plates. Volume ratios of Na-alginate to  $\text{CaCl}_2$  solutions were varied to obtain ratios of G units to  $\text{Ca}^{2+}$  ions in the range from 15:1 to 1:1 in order to determine the quantity of  $\text{Ca}^{2+}$  ions necessary for complete gelation. Considering that the reaction of ion exchange is almost instantaneous and the gelation process is diffusion controlled, the addition of  $\text{CaCl}_2$  was performed under vigorous mixing. From the obtained homogenous mixtures, discs fitting the size of the measuring compartments were stamped out and submitted to rheological measurements.  $G'$  and  $G''$  moduli of the hydrogel were recorded over time at 20°C and a constant frequency of 6.28 rad/s.

## Results and discussion

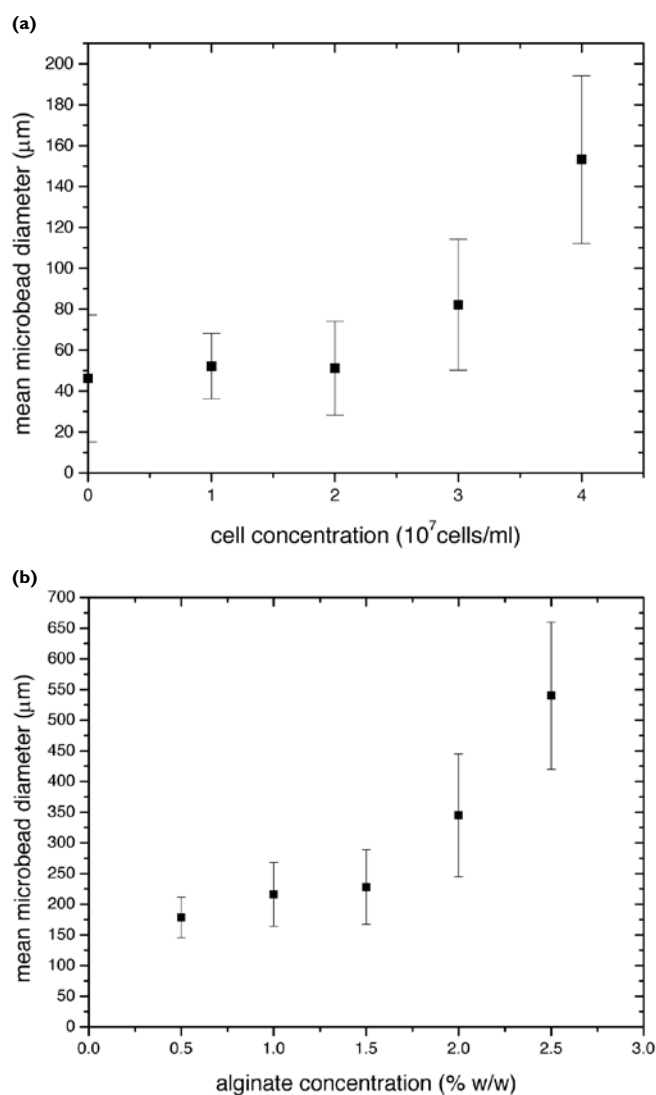
### $^1\text{H-NMR}$ spectroscopy

Chemical characterization results of Protanal LF 20/40 obtained by  $^1\text{H-NMR}$  spectroscopy revealed that alginate used in this study had a high content (67%) of guluronic residues and was rich in GG diad blocks ( $F_{\text{GG}} = 55\%$ ). Mole fractions of MM and GM diad sequences  $F_{\text{MM}}$  and  $F_{\text{GM}}$  were 21% and 12%, respectively. The H-NMR spectra showed no significant peaks originating from proteins or other impurities.

### Electrostatic extrusion

The first aim of this study was to assess effects of cell and alginate concentrations on the mean microbead size obtained by the electrostatic extrusion technique. The effects of cell

concentration in the range  $1 \times 10^7$  to  $4 \times 10^7$  cells/ml of 1.5% w/w alginate are presented in Figure 2a. Addition of cells up to the concentration of  $2 \times 10^7$  cells/mL had a negligible effect on the microbead diameter. However, when the cell concentration was increased to  $4 \times 10^7$  cells/mL a 3-fold increase in the microbead diameter was observed (ie, from  $51 \pm 17 \mu\text{m}$  to  $153 \pm 51 \mu\text{m}$ ). At the given operating conditions (needle size 22 G), a further increase in the cell concentration resulted in the nonuniform outflow of the cell–polymer suspension and a three-modal distribution of microbead sizes (data not shown). In order to assess the effects on microbead size of polymer concentration in cell suspensions with a high cell concentration ( $5 \times 10^8$  cells/mL), we used a larger needle (20 Gs) and varied the Na-alginate concentration in the range



**Figure 2** Mean microbead diameter as a function of (a) cell concentration (1.5% w/w Na-alginate concentration, 7.5 kV applied potential, 22 G needle, 13.9 mL/hour flow rate, 2.5 cm electrode distance); (b) Na-alginate concentration ( $5 \times 10^8$  cells/mL, 6.5 kV applied potential, 20 Gs needle, 13.9 mL/hour flow rate, 2.5 cm electrode distance).

0.5%–4% w/w. Under the operating conditions used, uniform spherical microbeads were obtained up to the concentration of 2.5% w/w (Figure 2b); higher concentrations resulted in nonspherical microbeads.

The increase in alginate concentration up to 1.5% w/w had little effect on the average microbead diameter, whereas a further increase up to 2.5% w/w resulted in an approximately 2-fold increase in microbead size (Figure 2b). It should be mentioned that an earlier study demonstrated that the mean size of droplets increased by as much as 4–5-fold as the polymer viscosity was increased from 1 to 10 PaAs (Jayasinghe and Edirisinge 2004).

In the present study, spherical droplets were obtained using a narrow range of alginate concentrations (0.5%–2.5% w/w) exhibiting viscosities of about 1 PaAs, so that the observed effects were less pronounced.

It should be noted that the increase in alginate concentration increased standard deviations of microbead diameters (Figure 2b). The observed results are in agreement with the mechanisms of droplet formation under an electrostatic field (Bugarski et al 2004, 2005). As the electrostatic field is applied, the almost spherical shape of the liquid meniscus at the tip of the needle is deformed into a conical shape. Consequently, the alginate solution flows through this weak area at an increasing rate, causing formation of a neck. In the experiments with pure Na-alginate solutions, the neck formation was more pronounced as the alginate concentration was increased from 0.8% to 1.5% w/w, so that at the latter concentration the neck elongated up to 1 mm before detachment (Bugarski et al 2005). Furthermore, detachment of the drop was, in that case, accompanied by detachment of the linking filament, which then broke up into a large number of smaller droplets resulting in nonuniform size distribution. Results obtained in this study suggested a similar mechanism of droplet formation at higher alginate concentrations in the cell suspension.

Electrostatic extrusion experiments revealed an increase in microbead size with the increase of both cell and alginate concentrations, suggesting changes in fluid flow and droplet formation mechanism.

## Rheological measurements

### Rheological characterization of cell–alginate suspensions

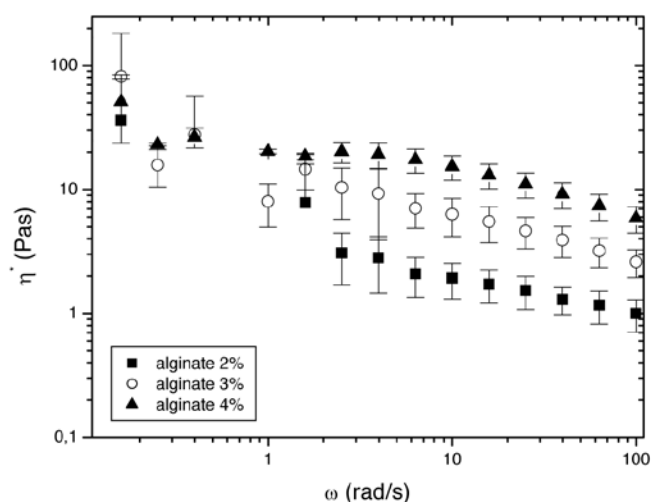
To get an insight into the process of electrostatic extrusion of cell–polymer suspensions, we performed rheological characterizations of pure Na-alginate solutions and suspensions of yeast cells at high concentrations ( $5 \times 10^8$

and  $1 \times 10^9$  cells/mL). The experiments were performed at room temperature, representing the usual conditions for electrostatic extrusion. However, in order to simulate conditions for immobilization of temperature-sensitive cells (eg, mammalian cells) rheological characterizations were also performed at 30°C and 37°C.

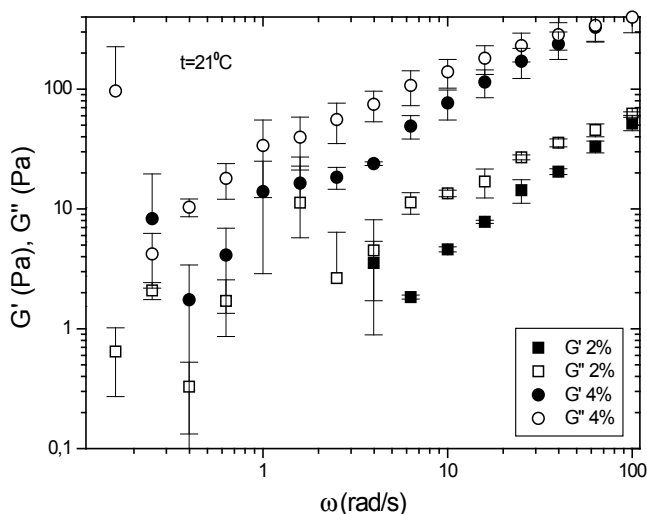
$\eta^*$  of Na-alginate solutions in the concentration range 2%–4% w/w at 21°C as functions of frequency are presented in Figure 3. The results indicated non-Newtonian, pseudoplastic behavior. For all samples a shear thinning behavior above a shear rate 2 rad/second was found. The complex dynamic viscosities decreased with increasing shear rate as a consequence of polymer chain orientation. The viscosity of an alginate solution depends on the alginate concentration and the length of molecules, ie, the number of repeating units in the chains. As the alginate concentration was increased from 2% to 4% w/w, the complex dynamic viscosity also increased as much as 7-fold at a frequency of 1 Hz (Figure 3).

Similar trends were also found at the other two investigated temperatures (data not shown). Since temperature defines the energy state of any molecule, it thus influences the response of alginates to shear forces. As a general rule, a temperature increase of 1°C leads to a viscosity drop of approximately 2.5%. Results obtained in this study confirmed this rule: when the temperature was raised from 21 to 37°C,  $\eta^*$  2% and 4% w/w alginate solutions decreased from 3 and 21 PaAs to values of 2 and 12 PaAs, respectively.

$G'$  and  $G''$  moduli of Na-alginate solutions of 2% and 4% w/w as functions of frequency, presented in Figure 4, were characteristic of viscoelastic liquids, with  $G''$  being much higher than  $G'$ . Furthermore, in the experimental range



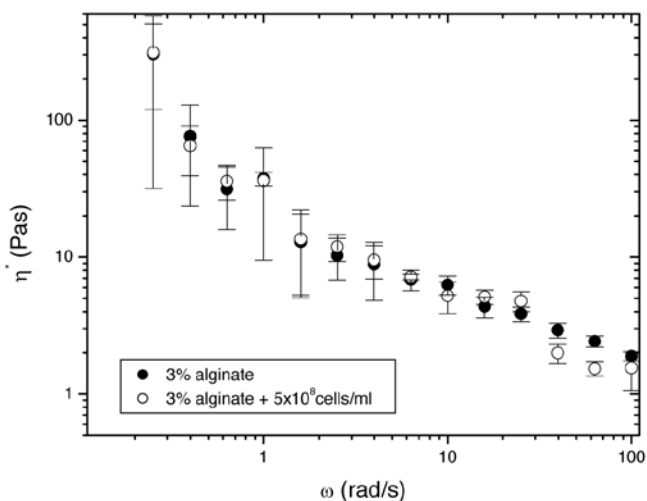
**Figure 3** Complex dynamic viscosities ( $\eta^*$ ) of Na-alginate solutions of different concentrations as functions of frequency at 21°C.



**Figure 4** Storage ( $G'$ ) and loss ( $G''$ ) moduli of Na-alginate solutions (2% and 4% w/w) as functions of frequency at 21°C.

of low frequencies  $\omega$ , both rheological parameters showed behavior characteristics for viscoelastic liquids, ie, with  $\log(G')$  vs  $\log(\omega)$  and  $\log(G'')$  vs  $\log(\omega)$  having slopes of  $\sim 2$  and  $\sim 1$ , respectively (Figure 4).

$\eta^*$  of cell suspensions with a cell concentration of  $5 \times 10^8$  cells/mL and Na-alginate at concentrations in the range 2%–4% w/w at all investigated temperatures were not significantly different from those measured in pure Na-alginate solutions. A representative set of results for 3% w/w Na-alginate at 37°C is presented in Figure 5. In addition, similar results were obtained for suspensions with the higher cell concentrations ( $1.0 \times 10^9$  cells/mL), indicating that the cell presence at the investigated concentrations did not have any significant influence on the rheological parameters in the experimental range of frequencies, probably because of cell elasticity and orientation in the shear stress field.



**Figure 5** Effects of cells on rheological properties: complex dynamic viscosities ( $\eta^*$ ) of pure 3% w/w Na-alginate solutions and with cells added at a concentration of  $5 \times 10^8$  cells/mL as functions of frequency at 37°C.

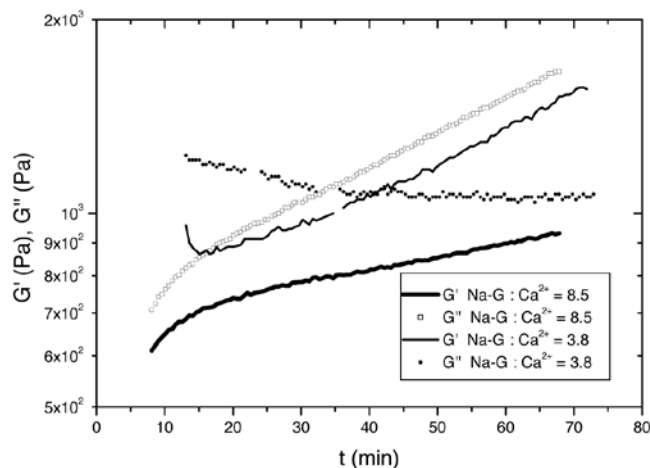
However, the effects of cell concentration on the mechanism of electrostatic droplet formation and the microbead size (Figure 2a) still need to be explained, possibly by modifications of fluid flow in the capillary or by electrostatic interactions.

### Rheological determination of gelation kinetics

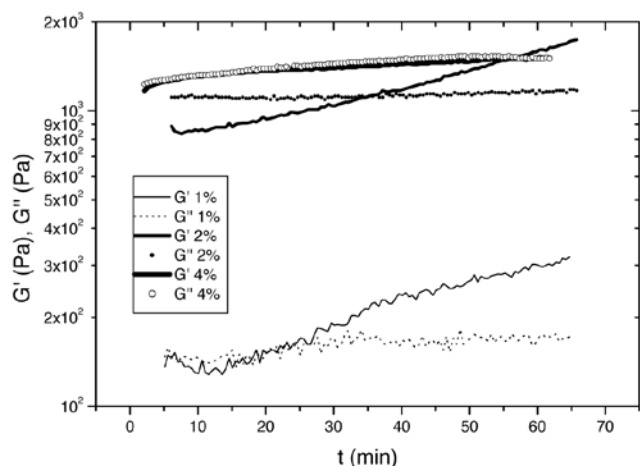
In order to optimize  $\text{CaCl}_2$  concentration and duration of alginate exposure to  $\text{CaCl}_2$ , we have investigated gelation kinetics of Na-alginate at different molar ratios of G units to  $\text{Ca}^{2+}$  ions, at different Na-alginate concentrations as well as in the presence of cells.

In the first experimental series we used rheological measurements to estimate the maximal molar ratio of G units to  $\text{Ca}^{2+}$  ions, which provides gelation. Figure 6 shows  $G'$  and  $G''$  moduli as functions of time during the gelation process of the 2% alginate gels at two different molar ratios of G units to  $\text{Ca}^{2+}$  ions. As a crossover of  $G' - t$  and  $G'' - t$  curves corresponds to the gel point, it can be deduced that the molar ratio of 3.8:1 allowed physical crosslinking and gelation after 40 minutes. The gelation kinetics are characterized by a significant increase in  $G'$  while  $G''$  stayed approximately constant. However, the smaller quantity of  $\text{Ca}^{2+}$  ions (G units to  $\text{Ca}^{2+}$  ions 8.5:1) did not provide crosslinking and hydrogel formation over an hour, since the viscous components stayed dominant compared with the elastic modulus of the mixture (Figure 6).

In the second experimental series we investigated gelation kinetics of Na-alginate of different concentrations at the ratio of G units to  $\text{Ca}^{2+}$  ions of 3.8:1 (Figure 7). With the increase in alginate concentration from 1% to 4% w/w the rate of gelation process is decreased owing to higher mass transfer



**Figure 6** Gelation kinetics of 2% w/w Na-alginate: storage ( $G'$ ) and loss ( $G''$ ) moduli at 6.28 rad/second and at 20°C vs time, after adding  $\text{CaCl}_2$  in molar ratios of G units to  $\text{Ca}^{2+}$  ions of 8.5:1 and 3.8:1 (data presented are averages of  $n=3$ ;  $\text{STD} = \pm 6\%$ ).



**Figure 7** Gelation kinetics of Na-alginate of different concentrations: storage ( $G'$ ) and loss ( $G''$ ) moduli at 6.28 rad/second and at 20°C vs time, after adding  $\text{CaCl}_2$  in the molar ratio of G units to  $\text{Ca}^{2+}$  ions of 3.8:1 (data presented are averages of  $n=3-5$ ;  $\text{STD}=\pm 9\%$ ).

resistances for the diffusion of  $\text{Ca}^{2+}$  ions. In the investigated geometry of the system, about 20 minutes were needed to form a gel using 1% w/w Na-alginate solution. The time needed for gelation of 2% w/w alginate was approximately twice as long. Finally, the 4% w/w alginate exhibited elastic behavior as much as viscous behavior during the first hour of gelation, and after about 60 minutes values of the storage modulus ( $G'$ ) became slightly above values of the loss modulus ( $G''$ ) (Figure 7).

Results obtained in the present study imply much longer gelation times (20–70 minutes) compared with earlier reports (Gilson et al 1990; Webber and Shull 2004). However, it should be emphasized that the time to achieve complete gelation strongly depends on the system geometry used. For beads with diameters of 2–6 mm in excess of  $\text{CaCl}_2$ , times to achieve complete gelation varied from 3 to 25 minutes (molar ratios of G units to calcium ions were up to 1:1) (Gilson et al 1990). In studies of alginate bead production, duration of bead exposure to the hardening solution varied between 30 minutes and 12 hours to ensure complete gelation (Williams and Munnecke 1981). In the present experiments, gelation kinetics were investigated under conditions of limited  $\text{Ca}^{2+}$  ion supply in the parallel plate geometry in which the diffusion rate of  $\text{Ca}^{2+}$  ions influenced the rate of gelation and the time needed to achieve the gel point.

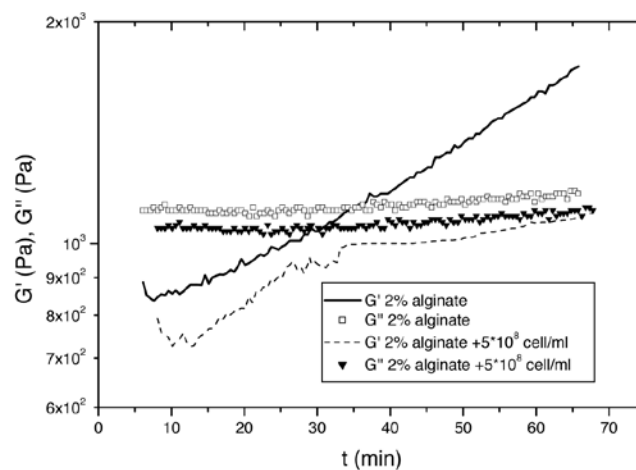
The obtained values of  $G'$  and  $G''$  for 4% w/w alginate gel were about 1000 Pa and approximately 5-fold higher compared with the values measured for 1% w/w alginate gel (Figure 7). These results are consistent with earlier studies (Martinsen et al 1989), which indicated an increase in gel strength as alginate concentration was increased. However, as higher alginate concentrations result in higher

fluid viscosities (Figure 3), leading to changes in droplet formation mechanism and nonuniformity of the produced microbeads (Figure 2b), a compromise has to be determined for each application.

In the third experimental series, effects of cell addition ( $5 \times 10^8$  cells/mL) on gelation kinetics of 2% w/w alginate at the ratio of G units to  $\text{Ca}^{2+}$  ions of 3.8:1 were investigated (Figure 8). The presence of cells caused gelation to occur at a prolonged time compared with the pure alginate solution owing to the reduction of space available for  $\text{Ca}^{2+}$  ion diffusion. As a consequence, the value of  $G'$  had not reached the value of  $G''$  even after an hour of gelation. Considering the tendency of increase in  $G'$ , it can be expected that the crossover of  $G' - t$  and  $G'' - t$  is reached at about  $t=70$  minutes.

Previous investigations have also shown that the presence of yeast cells slowed down the gelation process (Gilson et al 1990). The presence of 10% yeast (dry weight basis) increased gelation time by 31% compared with the similar yeast-free beads (Gilson et al 1990).

In addition, the presence of yeast cells in our studies reduced  $G'$  (8%–34%) and  $G''$  (~7%) compared with the values obtained for alginate systems without cells. Other investigations also indicated that the presence of microorganisms reduced gel strength (Gilson et al 1990; Camelin et al 1993), implying that immobilized microorganisms could cause irregularities in the network structure and reduce gel elasticity. Furthermore, these effects could be even more pronounced with cell growth. This phenomenon should be considered before exposing immobilized cell systems to severe environmental stresses in industrial applications.



**Figure 8** Effects of cell addition ( $5 \times 10^8$  cells/mL) on the gelation kinetics of 2% w/w Na-alginate: storage ( $G'$ ) and loss ( $G''$ ) moduli at 6.28 rad/second vs time, after adding  $\text{CaCl}_2$  in the molar ratio of G units to  $\text{Ca}^{2+}$  ions of 3.8:1 (data presented are averages of  $n=4$ ;  $\text{STD}=\pm 14\%$ ).

## Summary

This study investigated electrostatic extrusion as a technique for cell immobilization in alginate microbeads for possible medical applications. We aimed to assess the effects of concentrations of yeast cells (as a model cell type) and Na-alginate on the resulting microbead size and to rationalize the obtained results by rheological characterization of the cell–alginate suspensions. Finally, we investigated the kinetics of alginate gelation with respect to the quantity of  $\text{Ca}^{2+}$  ions and cell presence to optimize the concentration of  $\text{CaCl}_2$  solution and duration of microbead exposure to  $\text{CaCl}_2$ , important for cells sensitive to high ion concentrations (eg, mammalian cells).

Both cell and alginate concentrations affected the size and distribution of microbeads produced by the electrostatic extrusion method. The increase in both parameters resulted in larger microbeads with higher standard deviations in size, suggesting changes in fluid flow and the mechanism of electrostatic droplet formation.

The investigated polymer was a low viscosity type of alginate with a high content (67%) of guluronic residues and rich in GG diad blocks ( $F_{\text{GG}} = 55\%$ ). Rheological characterization of pure Na-alginate solutions revealed that the observed effects of alginate concentration on the microbead size could be related to viscosity, which increased as the concentration was increased. In addition, the investigated solutions exhibited non-Newtonian, pseudoplastic behavior. However, the presence of cells even at high concentrations ( $5 \times 10^8$  and  $1 \times 10^9$  cells/mL) did not significantly influence rheological properties of Na-alginate, indicating that the observed effects of cells on the microbead size should be further explored and elucidated.

The kinetics of gelation were analyzed by monitoring rheological parameters over time at the constant frequency of 1 Hz and under conditions of the limited addition of  $\text{Ca}^{2+}$  ions, at different Na-alginate concentrations as well as in the presence of cells. The gel time was determined as the time needed for the loss modulus to acquire the value of the storage modulus. The results showed that the molar ratio of G units to  $\text{Ca}^{2+}$  ions of 3.8:1 provided crosslinking, which was not the case for the higher molar ratio (8.5:1). The increase in alginate concentration resulted in prolonged gelation times but higher strength of the resulting gel. Lastly, the presence of yeast cells in alginate solutions influenced the kinetics of network formation as well as the final properties of Ca-alginate. The network formation of Ca-alginate was slower, with lower crosslinking density causing lower hydrogel strength compared with the hydrogel without cells.

In this study an attempt was made to gain an insight into the process of electrostatic extrusion by determining the effects of cell and alginate concentrations as two operating parameters and relating these to the rheological characterization of the cell–alginate suspensions. Further studies of two-phase flow in the capillary, electrostatic, and physical interactions of cells, as well as development of mathematical models relating all the phenomena in the electrostatic extrusion process to the operating parameters and properties of the resulting microbeads, are needed to describe fully and optimize the technique for generating electrostatic droplets.

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