Journal of Chemical Technology and Biotechnology *J Chem Technol Biotechnol* **81**:505–510 (2006)
DOI: 10.1002/jctb.1465

Investigations of cell immobilization in alginate: rheological and electrostatic extrusion studies

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Abstract: In this study, the process of electrostatic extrusion as a method for cell immobilization was investigated. We have assessed the effects of concentrations of yeast cells (as a model cell type) and Na alginate on the size of the resulting microbeads and attempted to rationalize the obtained findings by rheological characterization of the cell–alginate suspensions. Under the investigated conditions, microbeads, 50–600 µm in diameter, were produced and the increase in both alginate and cell concentrations resulted in larger microbeads with their sizes having higher standard deviations. 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 (5×10^8 and 1×10^9 cells mL^{-1}) did not significantly affect the rheological properties of the Na alginate solution. Finally, we have investigated the kinetics of alginate gelation with respect to the quantity of Ca^{2+} ions and the presence of cells. The molar ratio of α-L-guluronic acid units to Ca^{2+} ions of 4:1 provided complete crosslinking. The presence of cells decreased the rate of network formation as well as the strength of the obtained Ca alginate hydrogel.
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Keywords: cell immobilization; rheology; alginate; electrostatic extrusion

INTRODUCTION

Hydrogel-immobilized cell systems are attractive for a variety of applications in biotechnology and biomedicine. Natural polysaccharides (e.g. agar, alginates, κ-carrageenan), gel-forming proteins (e.g. gelatin) and synthetic polymers (e.g. polyacrylamide) have gained a leading role as cell carriers in entrapment or encapsulation techniques.[1] Among these, spherical matrices based on calcium alginate gels are the most widely used supports for immobilization of living cells.[2]

Alginates are naturally derived linear copolymers of 1,4-linked β-D-mannuronic acid and α-L-guluronic acid residues.[3–5] The ratio and sequential distribution of β-D-mannuronic acid (M) residues and α-L-guluronic acid (G) residues along the length of the alginate chain vary in alginates of different origins (brown seaweeds, certain bacteria).[3,4,6] 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 gelating ions, generally Ca^{2+}.[7]

In recent years, several techniques have been developed and established for the production of very small hydrogel beads with immobilized cells. Controlled production of uniform microbeads is especially significant for potential bioreactor applications. One of the promising techniques, 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.[8,9] Under optimal operating conditions it was possible to obtain alginate microbeads down to 50 µm in diameter.[9,10] Nevertheless, 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.[11] 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 (i.e. micro hydrodynamics within

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Contract/grant sponsor: Ministry of Science and Environmental Protection of the Republic of Serbia; contract/grant number: 1776
Contract/grant sponsor: Ministry of Science and Environmental Protection of the Republic of Serbia; contract/grant number: 548
(*Received 2 June 2005; revised version received 27 October 2005; accepted 30 October 2005*)
Published online 24 February 2006

the capillary via electrostatic and physical interactions of the cells).

The presence of cells can also influence gelation kinetics and the mechanical properties of the final Ca alginate microbeads. Although most studies of alginate bead production were carried out in an excess of hardening solution (i.e. $CaCl_2$) over prolonged times in order to ensure complete gelation,[3,12] immobilization of highly sensitive mammalian cells (e.g. bone marrow cells) can require minimal exposure to $CaCl_2$ solution. Assessment of gelation kinetics can be therefore essential for optimization of immobilization techniques for these cells.

The objective of this study was to further investigate and rationalize the process of cell immobilization by the electrostatic droplet generation technique. In specific terms, we aimed to assess the effects of cell and alginate concentrations on the size of the resulting microbeads and then to characterize the cell–alginate suspensions in order to get an insight in the phenomenon of electrostatic extrusion. In the present work, we have studied rheological properties of pure Na alginate solutions and cell–alginate suspensions at different temperatures. Finally, we attempted to analyze the gelation kinetics using limited supplies of Ca^{2+} ions with pure polymer solutions as well as the effects of the addition of cells on gelation kinetics.

EXPERIMENTAL

Electrostatic extrusion

The investigated polymer was low viscosity sodium alginate Protanal LF 20/40 (FMC Biopolymer, Drammen, Norway). The content of guluronic acid of the commercial sample was in the range 65–75%. For calculation purposes the guluronic content was assumed to be 70%. 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 1×10^7 to 5×10^8 cells mL^{-1}. 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 grounded hardening solution was applied. Hardening solution was $CaCl_2$ 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 to 7.5 kV. The 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 h^{-1}. Three batches were produced for each set of experimental parameters. A sample of 30 microbeads was taken from each batch and the diameters of the microbeads were measured with an accuracy of 10 µm 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 using 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 s^{-1}. Pure Na alginate solutions in the concentration range 2–4% (w/w) as well as the suspension of yeast cells at the concentration of 5×10^8 cells mL^{-1} were analyzed at room temperature, 30 and 37 °C. Viscosities of alginate solutions at lower concentrations (below 2%) could not be measured by the Rheometrics instrument because of the instrument's lower limitation of 1 Pas. The liquid samples were analyzed at a constant strain of 30% and complex dynamic viscosities (η^*) as well as the storage (G') and loss (G'') moduli were recorded. 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 was analyzed by rheological examinations of mixtures of Na alginate (1–4% w/w) and $CaCl_2$ (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 $CaCl_2$ solutions were varied to obtain ratios of G units to Ca^{2+} ions in the range from 15:1 to 1:1 in order to determine the quantity of 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 $CaCl_2$ was performed under vigorous mixing. From the obtained homogeneous mixtures discs fitting the size of the measuring compartment were stamped out and submitted to rheological measurements. The storage (G') and loss (G'') moduli of the hydrogel were recorded over time at 20 °C and a constant frequency of 6.28 rad s^{-1}.

RESULTS AND DISCUSSION

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 1×10^7 to 4×10^7 cells mL^{-1} of 1.5% (w/w) alginate are

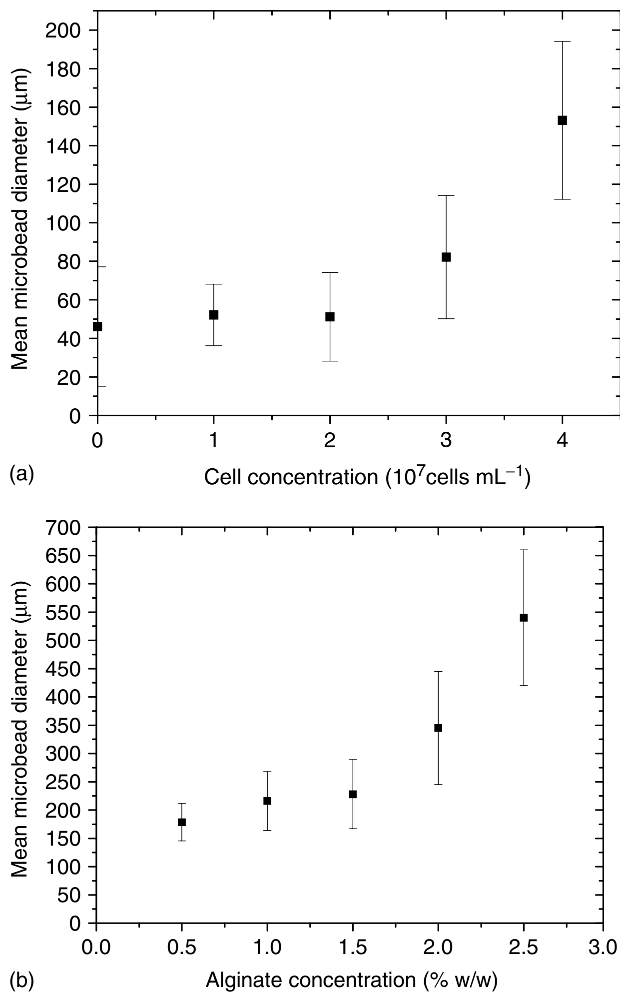

Figure 1. Mean microbead diameter as a function of: (a) cell concentration (1.5% (w/w) Na alginate concentration, 7.5 kV applied potential, 22 G needle, 13.9 mL h^{-1} flow rate, 2.5 cm electrode distance, data represent averages of $n = 3$); (b) Na alginate concentration (5×10^8 cells mL^{-1}, 6.5 kV applied potential, 20 Gs needle, 13.9 mL h^{-1} flow rate, 2.5 cm electrode distance).

presented in Fig. 1(a). Addition of cells up to the concentration of 2×10^7 cells mL^{-1} had a negligible effect on the microbeads' diameter. However, when the cell concentration was increased to 4×10^7 cells mL^{-1} a three-fold increase in the microbeads' diameter was observed (i.e. from 51 ± 17 μm to 153 ± 51 μm). At the given operating conditions (needle size 22 G), further increase in the cell concentration resulted in the non-uniform outflow of the cell–polymer suspension and a three modal distribution of microbead sizes (data not shown). In order to assess the effects of polymer concentration in cell suspensions with a high cell concentration (5×10^8 cells mL^{-1}) on the microbead size, we have used a larger needle (20 Gs) and varied the Na alginate concentration in the range from 0.5 to 4% (w/w). Under the operating conditions used, uniform spherical microbeads were obtained up to the concentration of 2.5% (w/w) (Fig. 1(b)) and additional increase resulted in non-spherical microbeads.

The increase in alginate concentration up to 1.5% (w/w) had little effect on the average microbead diameter whereas further increase up to 2.5% (w/w) resulted in an approximately two-fold increase in the microbeads' size (Fig. 1(b)). It should be mentioned that an earlier study demonstrated that the mean size of droplets increased by as much as four- to five-fold as the polymer viscosity was increased from 1 to 10 Pas.[13] In the present study, spherical droplets were obtained using a narrow range of alginate concentrations (0.5–2.5% w/w) exhibiting viscosities of the order of magnitude of 1 Pas so that the observed effects were less pronounced.

It should be noted that the increase in alginate concentration resulted in the increase in the standard deviations of the diameters of the microbead (Fig. 1(b)). The observed results are in agreement with mechanisms of droplet formation under action of the electrostatic field.[14,15] 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.[11] 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 non-uniform size distribution. Results obtained in this study implied 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, implying changes in fluid flow and droplet formation mechanism.

Rheological measurements

Rheological characterization of cell–alginate suspensions

In order to get an insight in the process of electrostatic extrusion of cell–polymer suspensions we have performed rheological characterizations of pure Na alginate solutions and suspensions of yeast cells at high concentrations (5×10^8 and 1×10^9 cells mL^{-1}). 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 (e.g. mammalian cells) rheological characterizations were also performed at 30 and 37 °C.

Complex dynamic viscosities (η^*) of Na alginate solutions in the concentration range 2–4% (w/w) at 21 °C as functions of frequency are presented in Fig. 2. The results indicated non-Newtonian, pseudoplastic behavior. For all samples a shear thinning behavior above shear rate 2 rad s^{-1} 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 the molecules,

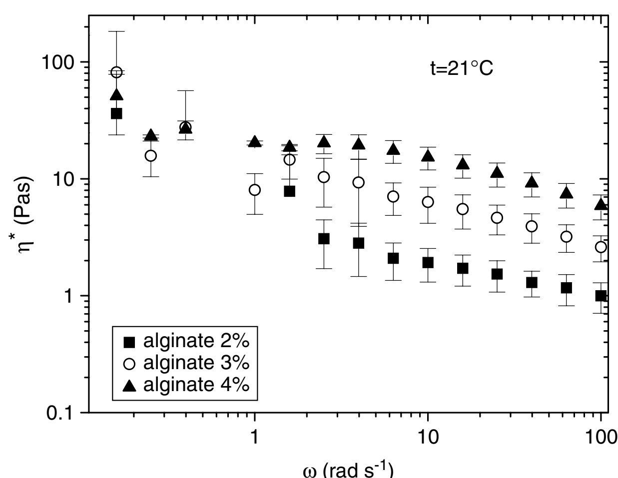

Figure 2. Complex dynamic viscosities (η^*) of Na alginate solutions of different concentrations as functions of frequency at 21 °C (data represent averages of $n = 4$).

i.e. 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 seven-fold at a frequency of 1 Hz (Fig. 2).

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, the complex dynamic viscosities of 2 and 4% (w/w) alginate solutions decreased from 3 and 21 Pas to values of 2 and 12 Pas, respectively.

Complex dynamic viscosities (η^*) of cell suspensions with a cell concentration of 5×10^8 cells mL^{-1}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 Fig. 3. In addition, similar results were obtained for suspensions with the higher cell concentration (1.0×10^9 cells mL^{-1}), indicating that the presence of cells at the investigated concentrations did not have any significant influence on the rheological parameters in the experimental range of frequencies, probably due to cell elasticity and orientation in the shear stress field.

However, the effects of cell concentration on the mechanism of electrostatic droplet formation and the microbead size (Fig. 1(a)) still need to be explained, possibly by modifications of fluid flow in the capillary or electrostatic interactions.

Rheological determination of gelation kinetics

In order to optimize $CaCl_2$ concentration and duration of alginate exposure to $CaCl_2$, we have investigated gelation kinetics of Na alginate at different molar ratios of G units to Ca^{2+} ions, as well as in the presence of cells.

In the first experimental series we used rheological measurements in order to estimate the maximal molar ratio of G units to Ca^{2+} ions, which provides gelation. Figure 4 shows storage (G') and loss (G'') moduli as functions of time during the gelation process of the 2% alginate gels at two different molar ratios of G units to 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 4:1 allowed physical crosslinking and gelation after 40 min. The gelation kinetics is characterized by a significant increase in G' while G'' stayed approximately constant. However, the smaller quantity of Ca^{2+} ions (G units to Ca^{2+} ions 8.5:1) did not provide crosslinking and hydrogel formation over a period of an hour, since the viscous component stayed dominant compared with the elastic modulus of the mixture (Fig. 4).

In the third experimental series, the effects of cell addition (5×10^8 cells mL^{-1}) on the gelation kinetics of 2% (w/w) alginate at the ratio of G units to Ca^{2+}

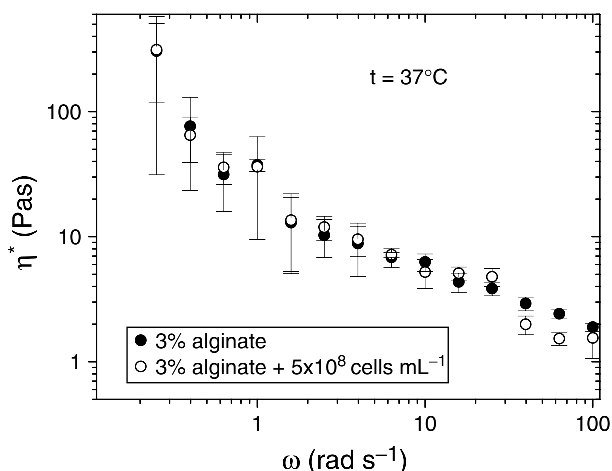

Figure 3. Effects of cells on rheological properties: complex dynamic viscosities (η^*) of pure 3% (w/w) Na alginate solutions and with added cells at a concentration of 5×10^8 cells mL^{-1}as functions of frequency at 37 °C (data represent averages of $n = 5$).

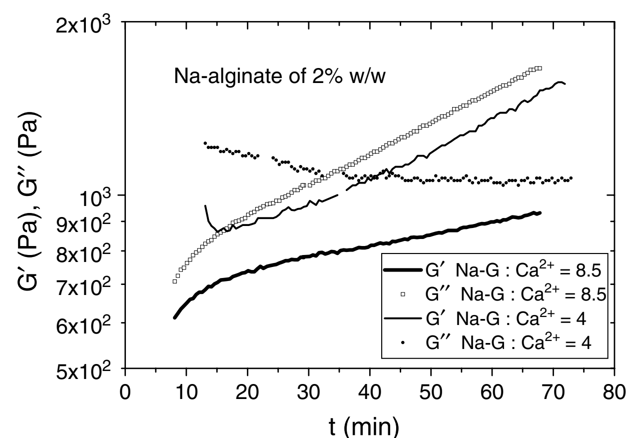

Figure 4. Gelation kinetics of 2% (w/w) Na alginate: storage (G') and loss (G'') moduli at 6.28 rad s^{-1} and at 20 °C vs time, after adding $CaCl_2$ in molar ratios of G units to Ca^{2+} ions of 8.5:1 and 4:1 (data represent averages of $n = 3$; STD = ±6%).

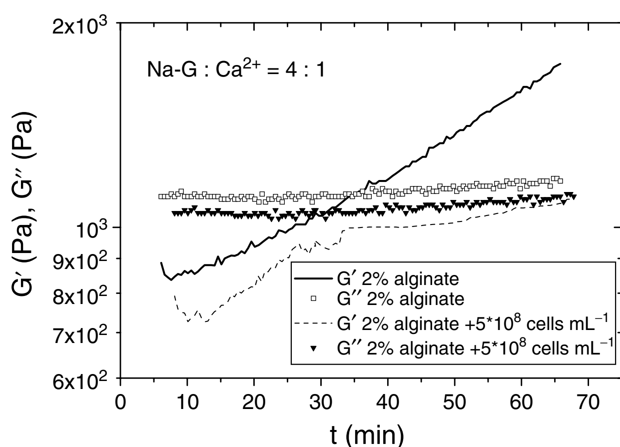

Figure 5. Effects of cell addition (5×10^8 cells mL^{-1}) on the gelation kinetics of 2% (w/w) Na alginate: storage (G') and loss (G'') moduli at 6.28 rad s^{-1} vs time, after adding $CaCl_2$ in the molar ratio of G units to Ca^{2+} ions of 4:1 (data represent average of $n = 4$; STD = ±14%).

ions of 4:1 were investigated (Fig. 5). The presence of cells caused gelation to occur over a prolonged time as compared with the pure alginate solution due to reduction of space available for 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 $t = 70$ min.

Previous investigations have also shown that the presence of yeast cells slowed down the gelation process.[12] The presence of 10% yeast (dry weight basis) caused an increase in gelation time of 31% compared with the similar yeast-free beads.[12]

In addition, the presence of yeast cells in our studies caused reductions in G' (between 8% and 34%) and G'' (∼7%) as compared with the values obtained for alginate systems without cells. Other investigations also indicated reduction of gel strength in the presence of microorganisms.[12,16] The obtained results imply that immobilized microorganisms could cause irregularities in the network structure and reduce the gel elasticity. Furthermore, these effects could be even more pronounced with the cell growth in the culture. This phenomenon should be considered before exposing immobilized cell systems to severe environmental stresses in industrial applications.

CONCLUSIONS

This study was focused on investigation of the electrostatic extrusion process as a technique for cell immobilization in alginate microbeads. We aimed to assess the effects of concentrations of yeast cells (as a model cell type) and Na alginate on the size of the resulting microbeads and to rationalize the obtained results by rheological characterization of the cell–alginate suspensions. Finally, we have investigated the kinetics of alginate gelation with respect to the quantity of Ca^{2+} ions and the presence of cells in order to optimize the concentration of $CaCl_2$ solution and duration of microbead exposure to $CaCl_2$, important for cells sensitive to high ion concentrations (e.g. 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 larger standard deviations in size, implying changes in fluid flow and the mechanism of electrostatic droplet formation.

Rheological characterization of pure Na alginate solutions revealed that the observed effects of alginate concentration on the microbeads' size could be related to the 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 (5×10^8 and 1×10^9 cells mL^{-1}) did not significantly influence rheological properties of Na alginate, indicating that the observed effects of cells on the microbeads' size should be further explored and elucidated.

The kinetics of gelation was analyzed by monitoring rheological parameters over time at the constant frequency of 1 Hz and under conditions of limited addition of Ca^{2+} ions, at different Na alginate concentrations as well as in the presence of cells. The results have shown that the molar ratio of G units to Ca^{2+} ions of 4:1 provided crosslinking, which was not the case for the higher molar ratio (8.5:1). Finally, 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 get an insight into the process of electrostatic extrusion by determining and relating the effects of cell and alginate concentrations as two operating parameters 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 fully describe and optimize the electrostatic droplet generation technique.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Science and Environmental Protection of the Republic of Serbia (Grants 1776 and 548).

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