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Controlling the size of alginate gel beads by use of a high electrostatic potential

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The effect of several parameters on the size of alginate beads produced by use of an electrostatic potential bead generator was examined. Parameters studied included needle diameter, electrostatic potential, alginate solution flow rate, gelling ion concentration and alginate concentration and viscosity, as well as alginate composition. Bead size was found to decrease with increasing electrostatic potential, but only down to a certain level. Minimum bead size was reached at between 2–4 kV/cm for the needles tested. The smallest alginate beads produced (using a needle with inner diameter 0.18 mm) had a mean diameter of ~300 µm. Bead size was also found to be dependent upon the flow rate of the fed alginate solution. Increasing the gelling ion concentration resulted in a moderate decrease in bead size. The concentration and viscosity of the alginate solution also had an effect on bead size as demonstrated by an increased bead diameter when the concentration or viscosity was increased. This effect was primarily an effect of the viscosity properties of the solution, which led to changes in the rate of droplet formation in the bead generator. Lowering the flow rate of the alginate solution could partly compensate for the increase in bead size with increased viscosity. For a constant droplet size, alginates with a low G block content ($F_{GG} \approx 0.20$) resulted in ~30% smaller beads than alginates with a high G block content ($F_{GG} \approx 0.60$). This is explained as a result of differences in the shrinking properties of the beads.

Keywords: Alginate, alginate beads, bead size, electrostatic bead generator, encapsulation.

Introduction

Occurring as a structural component in marine brown algae (*phaeophyceae*), and as capsular material in some soil bacteria, alginate is a polysaccharide that is quite abundant in nature (Moe *et al.* 1995). Alginate consists of unbranched $1 \rightarrow 4$ glycosidically linked β-D-mannuronic acid (M) and its C-5 epimer α-L-guluronic acid (G). The relative amount of M and G, and their distribution along the polymer chain in a specific alginate, depends upon the organism and the tissue from which the polymer is isolated (Moe *et al.* 1995, Smidsrød and Moe 1995). Because of its ability to retain water and its gelling, viscosifying and stabilizing properties, alginate has been used in several applications (Smidsrød and Skjåk-Bræk 1990, Indergaard and Østgaard 1991, Christenson *et al.* 1993, Onsøyen 1996).

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Immobilization of living cells or other biomaterials in alginate beads is a commonly used technique involved in an increasing number of applications, which include treatment of diseases such as diabetes and brain cancer (Lim and Sun 1980, Soon-Shiong *et al.* 1994, Hoffman *et al.* 1998, Read *et al.* 1999). One parameter of particular importance in many applications is the control of bead size. In particular, for the immobilization of living cells, diffusion limitations require that bead size is small (i.e. well below 1 mm in diameter). This is because diffusion limitations within the beads will limit cellular metabolism (Papas *et al.* 1999). In particular, depletion of nutrients, such as oxygen, to the interior of the beads will lead to cell death as a result of consumption from the surrounding cells (Colton 1995, De Vos *et al.* 1996). Also, smaller beads are likely to be more resistant to shear and compressive forces and may be less immounogenic (Hallé *et al.* 1994).

Various techniques for the production of small spherical beads with a narrow size distribution have been investigated (Hallé *et al.* 1994, Brandenberger and Widmer 1998, Dulieu *et al.* 1999). Among these, the electrostatic bead generator has been demonstrated to be a good alternative (Dorian and Cochrum 1994, Hallé *et al.* 1994, Gåserød 1998). This instrument is commonly used by different workers to immobilize cells in small spherical beads, and the high voltage has been confirmed not to damage the encapsulated cells (Gåserød 1998, King *et al.* 1999). The instrument is based upon using an electrostatic potential to pull droplets from a needle tip into a gelling bath. A voltage is applied between the needle feeding the alginate solution and an electroconductive solution underneath. The voltage forces the droplets to fall off the needle tip before it has grown to the point were it falls off due to its own weight. The electroconductive solution most often contains gelling ions (e.g. Ca^{2+}), and beads are formed when droplets fall into the solution (Morris *et al.* 1978, Stokke *et al.* 1997). The droplet size is easily changed by adjusting the voltage. However, for a predictable outcome, careful control of several parameters is required (Poncelet *et al.* 1994). These, in particular, include the applied electrostatic potential, flow rate of polymer solution, needle diameter, gelling conditions, as well as alginate solution viscosity, alginate composition and molecular weight distribution.

An objective of the present work was, therefore, to extend the current knowledge about the influence of these parameters with respect to the final size of alginate beads produced by an electrostatic bead generator. To characterize the influence of each parameter, alginate beads were generated under similar conditions, but with each parameter varied separately. The size of the resulting beads was thereafter determined.

Materials and methods

Alginates

Samples of sodium alginate isolated from *Laminaria hyperborea* stipe (PRONOVA^{TM} LVG and MVG, designated high G alginates) and *Ascophyllum nodosum* (PRONOVA^{TM} LVM and MVM, designated low G alginates) were obtained from Pronova Biomedical as (Oslo, Norway). Alginate composition and sequential structure was determined by NMR spectroscopy, and viscosity

Table 1. Viscosity, molecular weight and chemical composition and sequential structure of the alginates used.

Name	Batch number	Viscosity$_{1\%}$ [mPa s]	Molecular weight [g/mole]	F_G	F_M	F_{GG}	F_{MM}	F_{GM}	$N_{G<1}$
PRONOVA^{TM} UP LVG	612-256-01	175	NA	0.73	0.27	0.61	0.16	0.12	16
PRONOVA^{TM} LVG	611-256-07	158	189 000	0.73	0.27	0.56	0.11	0.17	16
PRONOVA^{TM} LVG	804-256-04	99	NA[1]	0.69	0.31	0.55	0.17	0.14	12
PRONOVA^{TM} UP MVG	701-256-08	316	231 000	0.73	0.27	0.60	0.13	0.14	25
PRONOVA^{TM} LVM	210-241-02	148	209 000	0.38	0.62	0.18	0.43	0.19	4
PRONOVA^{TM} MVM	210-242-01	200	226 000	0.36	0.64	0.20	0.49	0.16	5

[1] NA = Not available.

by rotational viscometry. For alginates where the molecular weight was determined, size-exclusion chromatography combined with low angle laser light scattering (SEC-LALLS) was used. The viscosity, molecular weight and compositional characteristics (Moe *et al.* 1995) of the alginates used are shown in table 1.

Preparation of beads

The beads were made using an electrostatic bead generator designed at the Norwegian University of Science and Technology (Professor Gudmund Skjåk-Bræk, NTNU) and produced by SINTEF Unimed (Trondheim, Norway). The generator mainly consists of a variable power supply delivering 0–10 kV, a switch for fine-tuning of the voltage, an autoclavable needle holder and a safety cover with an electrical safety switch. A magnetic stirrer was used for stirring the gelling bath containing a 50 mM $CaCl_2$ solution (except from when the gelling ion concentration was varied). The needles used in the instrument are made of steel and are commercially produced for computer plotters (Staedler Mars GmbH & Co., Nürnberg, Germany). In the present work, two needles with an inner diameter of 0.18 and 0.40 mm were used. Inhomogeneous beads were made as the alginate solution was dropped into the gelling bath containing gelling ions (Ca^{2+}) only. The alginate solution was fed into the bead generator by a syringe pump (Cole-Parmer 74900-05, Cole Parmer Instrument company). The size distribution of beads produced with the electrostatic bead generator used in these experiments may be as low as 3–6% (Gåserød 1998).

When a specific parameter was not varied, it was held constant at: voltage 5 kV, flow rate 10 ml/h, distance between needle tip and gelling bath 1.2 cm, gelling ion (Ca^{2+}) concentration 50 mM and alginate solution concentration 1.5% (w/v).

Measurement of bead size

The beads were stored in the $CaCl_2$-solution for at least 30 min before measurement of bead size to allow the gel network to stabilize. Bead diameter was determined by laser diffraction using a laser diffraction instrument (Mastersizer-S, Malvern Instruments). To present the data, the volume mean diameter was used. To collect and calculate the data, the software Mastersizer-Sv2.18 was used.

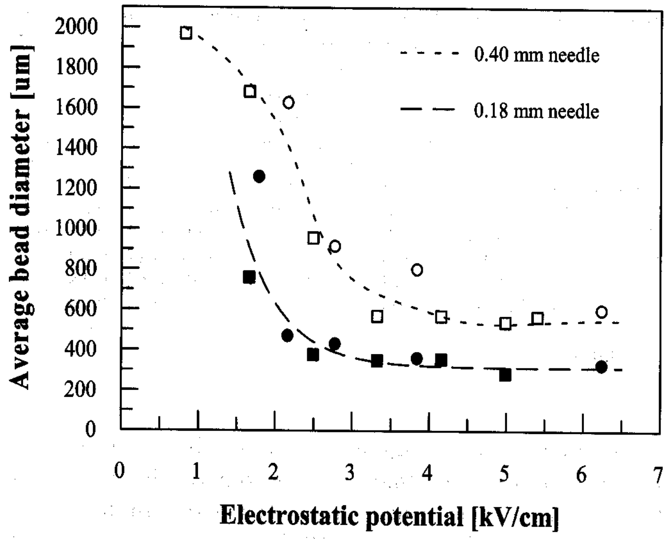

Figure 1. Average bead diameter as a function of electrostatic potential. ● Distance adjusted (0.18 mm needle); ○ Distance adjusted (0.40 mm needle); ■ Voltage adjusted (0.18 mm needle); □ Voltage adjusted (0.40 mm needle). The experiment was performed with 0.18 and 0.40 mm needles (inner diameter) and with a 1.5% (w/v) alginate solution (PRONOVA$^{\text{TM}}$ UP LVG, batch 612-256-01). The alginate solution flow rate was 10 ml/h. Each point is the average of two measurements. The dotted lines were drawn by generating a spline curve between the data for the 0.40 mm and the 0.18 mm needle, respectively.

Results and discussion

In the present work, the electrostatic field was kept constant; however, electrostatic pulses may also be used for droplet generation (Hallé *et al.* 1994). In the first experiment, alginate beads were made from a 1.5% alginate solution. Beads with different sizes were produced by adjusting the voltage of the bead generator in one experiment, and the distance between the needle tip and gelling bath in another. The experiments were performed with 0.18 and 0.40 mm needles (inner diameter). In figure 1, the results from these two experiments are combined and shown as the average bead diameter as a function of the electrostatic potential (voltage/distance). The results show that the two ways of changing the electrostatic potential have similar effects on bead size. Bead diameter was found to decrease rapidly until the electrostatic potential reached ~2.5 kV/cm for the 0.18 mm needle and ~3.5 kV/cm for the 0.40 mm needle. Above this value, there seems to be no further (or a very small) decrease in bead diameter. The smallest bead diameter achieved was ~0.60 mm for the 0.40 mm needle and ~0.40 mm for the 0.18 mm needle. This is similar to the outer diameter of the needles. The alginate solution may be moisturising the edges of the needle tip, and the droplet size may, therefore, be influenced by the outer diameter of the needle (Gåserød 1998). This effect will also be dependent upon the actual shape and material of the needle.

The data are supported by earlier work (Gåserød 1998) and implies that increasing the distance between needle tip and gelling bath may be compensated

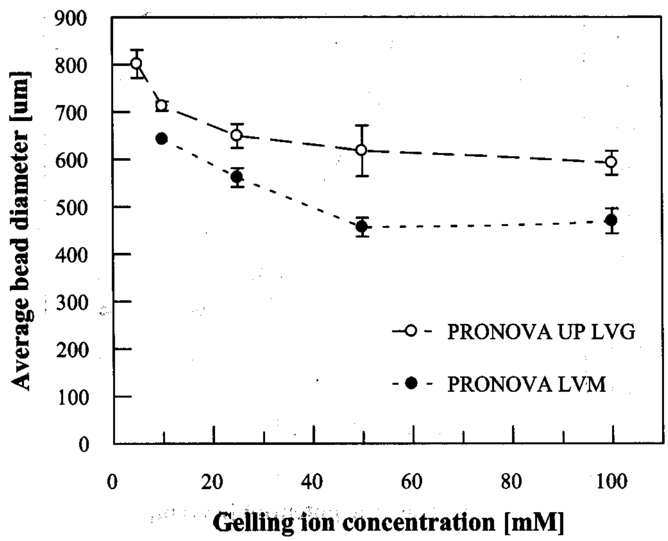

Figure 2. Average bead diameter as a function of gelling ion (Ca^{2+}) concentration. The beads were made from 1.5% (w/v) solutions of PRONOVA(UP LVG (batch 612-256-01) and PRONOVA™ LVM (batch 210-241-02). The electrostatic voltage was 5 kV, the flow rate 10 ml/h, and the distance between the needle tip and the gelling bath was 1.2 cm. The experiment was performed with a 0.40 mm needle (inner diameter). Each point (with SE) is the average of two independent experiments.

for by a simultaneous rise in electrostatic voltage. It has been proposed that the reduction in bead size with increasing potential is primarily a result of a fall in the surface tension of the alginate droplets generated at the needle tip (Dulieu *et al.* 1999). The electrostatic potential forces ions in the alginate solution towards the surface of the alginate droplet and thereby counteracts the surface tension of the droplet. At one point, the surface tension reaches a minimum, and no further decrease in bead diameter is achieved.

The effect of varying the gelling ion concentration was studied for 1.5% solutions of a high G alginate and a low G alginate. The low G alginate resulted in smaller beads than the high G alginate for all Ca^{2+} concentrations (figure 2). Furthermore, the data indicate a decrease in bead size for calcium concentrations below ~50 mM. This is in accordance with earlier work (Martinsen *et al.* 1987) and the effect is likely explained by tightening of the gel network with increasing Ca^{2+} concentration, resulting in smaller beads. When there are sufficient Ca^{2+} ions to fill up the cavities made up by the G-residues in the polymer chain to a certain level (the 'egg-box' model, Stokke *et al.* 1997), no further decrease in bead size is achieved. For the low G alginate, it was not possible to make beads at a Ca^{2+} concentration below ~10 mM, while the high G alginate resulted in beads also at a concentration of 5 mM. This may be explained by the higher content of available G blocks (F_{GG}) in high G alginates, giving a stronger gel structure even at low Ca^{2+} concentrations.

The effect of alginate solution concentration and viscosity on final bead size was examined (figure 3). The beads were made from high G alginate with three different concentrations. The data show that an increase in concentration, and thereby also in viscosity, is followed by an increase in bead diameter at

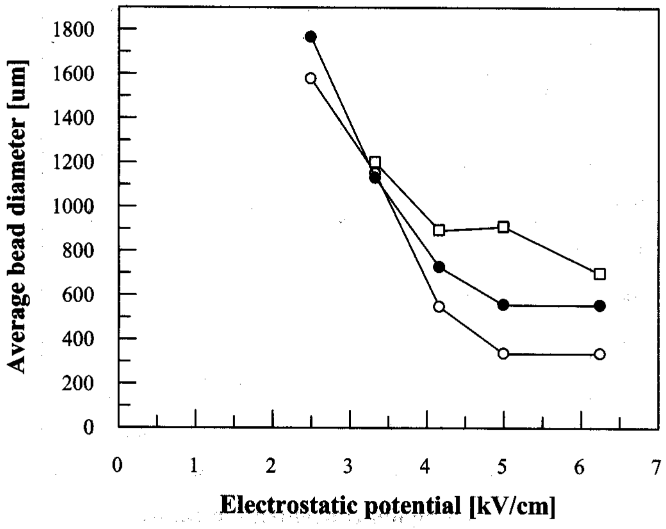

Figure 3. Average bead diameter as a function of electrostatic voltage for three PRONOVA™ UP LVG (batch 612-256-01) alginate solutions of different concentrations: ○ 0.5% (w/v) (viscosity 36.1 mPa s); ● 1.5% (w/v) (viscosity 473 mPa s); □ 2.5% (w/v) (viscosity 2358 mPa s). The alginate solution flow rate was 10 ml/h, and the distance between the needle tip and the gelling bath was 1.2 cm. The experiment was performed with a 0.40 mm needle (inner diameter). Each point is the average of two measurements.

identical voltages above ~3–4 kV/cm. It seemed impossible to compensate for the higher solution viscosity by increasing the electrostatic voltage further. Another experiment was performed in order to examine if reducing the flow rate of the alginate solution could compensate for the effect of higher solution viscosity (figure 4). The results show that smaller beads may be made at lower solution flow rates. This effect was more pronounced for beads made of higher viscosity solutions. With the 2.5% solution (viscosity 2865 mPa s) bead diameter was reduced from above 600 µm down to ~450 µm in the experiment. The relationship between diameter and flow rate seems to be linear within the tested range (figure 4), and the dependency was also similar for different needle diameters (data not shown). The data are supported by work done by Hallé *et al.* (1994) and Gåserød (1998). With increasing flow rate, more alginate solution is passing through the needle per time, but because of the viscosity of the solution the rate at which the droplets fall off the needle will not rise correspondingly. The alginate droplets will then grow to a larger size before they are pulled off the needle tip.

To study the effect of alginate composition on bead size further, six different PRONOVA™ alginates (table 1) of various compositions and at different concentrations were used for bead production. In figure 5(*a*), it can be seen that an increase in the alginate concentration for all samples resulted in an increase in bead diameter. In general, the beads were also found to be larger for the high G alginates. This difference is more clearly shown for the same experiment in figure 5(*b*), where the alginate concentration has been replaced by solution viscosity. Thus, the data demonstrate a difference between high G alginates and low G alginates at identical viscosities. Alginates with a relative low G-block

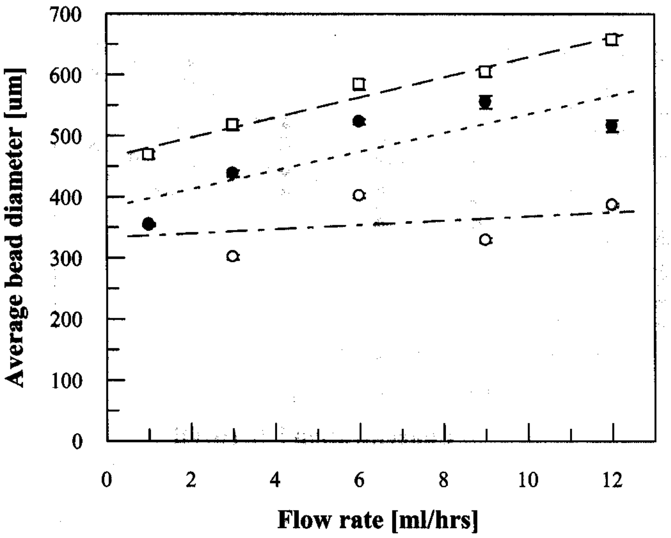

Figure 4. Average bead diameter as a function of flow rate for three alginate solutions, PRONOVA^{TM} UP LVG (batch 612-256-01), of various concentration: ○ 0.5% (w/v) (viscosity 37.1 mPa s); ● 1.5% (w/v) (viscosity 538 mPa s); □ 2.5% (w/v) (viscosity 2865 mPa s). The electrostatic voltage was 5 kV, and the distance between the needle tip and the gelling bath was 1.2 cm. The experiment was performed with a 0.40 mm needle (inner diameter). Each point (with SE) is the average of three measurements. The dotted lines were fitted by the method of least squares (r^2 was 0.142, 0.969 and 0.694 for the data from the 0.5% (w/v), 1.5% (w/v) and 2.5% (w/v) solutions, respectively).

content (PRONOVA^{TM} LVM and PRONOVA^{TM} MVM), $F_{GG} \approx 0.20$, resulted in ~30% smaller beads than alginates with a high G-block content (PRONOVA^{TM} (UP) LVG and PRONOVA^{TM} UP MVG), $F_{GG} \approx 0.60$. This effect is also illustrated in figure 2, where the low G alginate results in smaller beads than the high G alginate.

The data (figure 3) shows that an increase in concentration (and thereby also in viscosity) of the polymer solution causes an increase in bead diameter that may not be compensated for by increasing the electrostatic voltage. Figure 4, on the other hand, illustrates that smaller beads can be made from the high viscosity solution if the flow rate is sufficiently low. Furthermore, the data in figure 5 suggests that bead size is more strongly correlated to solution viscosity than to the actual alginate concentration. From the data in figure 3, it may be seen that all the beads in figure 5 are produced at an electrostatic potential giving minimum droplet size during bead production. Thus, the variation in bead size seen in figure 5 for one type of alginate are likely not influenced by different conditions during bead formation, but is rather an effect of the solution viscosity alone. An alginate gel bead will also normally shrink somewhat during gelling (Smidsrød and Haug 1972, Martinsen *et al.* 1989), and this likely accounts for some of the observed concentration-dependent differences in bead size.

Figures 3, 4 and 5 also show that for lower alginate concentrations, beads may be formed even with a diameter below the outer diameter of the needle (0.40 and 0.60 mm). At sufficiently low alginate concentrations (viscosities), accordingly, the bead diameter may perhaps be less limited by the outer needle diameter as

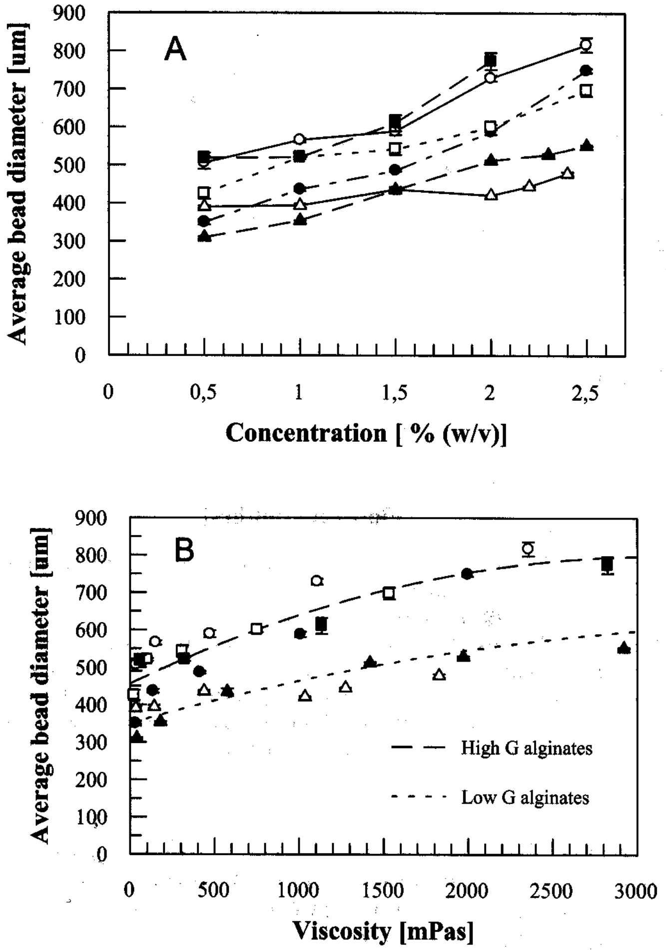

Figure 5. Average bead diameter as a function of concentration (*a*) and viscosity (*b*) for six different alginate batches: ○ UP LVG (Batch nr. 612-256-01); ● LVG (Batch nr. 611-256-07); □ LVG (Batch nr. 804-256-04); ■ UP MVG (Batch nr. 701-256-08); △ LVM (Batch nr. 210-241-02); ▲ MVM (Batch nr. 210-242-01). The electrostatic voltage was 5 kV, the flow rate 10 ml/h, and the distance between the needle tip and the gelling bath was 1.2 cm. The experiment was performed with a 0.40 mm needle (inner diameter). Each point (with SE) is the average of three measurements. The dotted lines in (*b*) were drawn by generating a spline curve between the data for high G and low G alginates, respectively.

long as the surface tension of the droplets is sufficiently low. The lower viscosity of the solutions, causing the droplet to fall off the needle tip even somewhat before the size of the outer needle diameter is reached, explains this. However, shrinkage of the beads during gelling also needs to be accounted for in order to confirm this.

The present data clearly confirms that bead size may change in the gelling solution due to alterations in the polymer network during gelling. In figure 5(*b*), coincidental curves are suggested for the four high G alginates and for the two low G alginates, and clearly the low G alginates resulted in smaller beads than the high G alginates. This effect is also seen in figure 2. The differences are explained by differences in the gel forming and shrinking properties of alginates of various compositions. The droplet size generated by the bead generator for a certain viscosity is very likely the same for all six alginates. The diameter of the beads made from the low G alginates was, nevertheless, found to be ~30% smaller than for the beads made from high G alginates at identical viscosities. This is explained by differences in shrinking properties of the beads due to different arrangement of the polymer network in the gels. In work done by Martinsen *et al.* (1989), a volume reduction of ~50% was seen for beads made from low G alginate after 24 h of storage in 50 mM $CaCl_2$. For high G alginates, the shrinkage was 30%. This confirms the smaller size of the beads made from low G alginate observed here. The different alginate batches studied here were also different with respect to molecular weight (table 1). The present data implies, within the experimental variations, that this factor has no or very little influences on the size of the final beads. This is demonstrated by comparison of the data for the high viscosity and low viscosity batches (figure 5).

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