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TRIVALENT CATION STABILIZATION OF ALGINATE GEL FOR CELL IMMOBILIZATION

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Calcium alginate gel can be stabilized with simple treatment with trivalent cation. Gel stength can be increased by a factor of two after washing with 0.1M aluminum nitrate without significant loss of ability for cell immobilization.

Introduction

The immobilization of living whole cells in bioreactors is an attractive method to achieve high cell density and continuous operation. The entrapment of cells in calcium alginate gel beads is a popular procedure because of the simplicity of the method. However, calcium alginate gels are mechanically fragile and chemically unstable in high concentrations of phosphate anion. Many attempts have been made to improve the stability of calcium alginate gels but most procedures are time consuming and/or costly. The objective of this short note is to illustrate that treatment with a trivalent cation could provide a simple solution.

A common approach to improve the stability of calcium alginate is to further crosslink the gel with glutaraldehyde. Other methods generally employs impregnation of the gel with another polymer or polycation. Birnbaum et al. (1981) attempted several methods which included treatment of alginate gel with polyethyleneimine followed by glutaraldehyde, and with carbodimmide and N-hydroxysuccinimide followed by polyethyleneimine. Veliky and Williams (1981) used poly- cations such as polyethyleneimine or polypropyleneimine. Kuu and Polack (1983) impregnated alginate with polyacrylamide. These procedures all led to improved gel strength, but it is apparent that these methods were not particularly attractive, as none were pursued any further.

Better understanding of the gel formation kinetics is crucial in improving the alginate gel strength. Alginic acid contains polymannuronic and polyguluronic acid, the composition of which is dependent on the source of brown algae. The gelling is a result of the formation of an "egg-box" structure consisting of chains of either guluronic acid or mannuronic acid units in parallel being held together (crosslinked) by divalent cations such as calcium (Grant et al., 1973). The gelling process is dependent on many factors, one of which is the apparent calcium ion concentration. If the calcium ion concentration

is high, the gelling reaction is much faster than the diffusion of the cation. The result is preferential gel formation near the surface. However, if the calcium concentration is too low there is either not enough calcium cation to provide a firm gel structure or the curing time is excessively long. One can regulate the final texture of the gel by controlled release of calcium ion or the addition of a polycation (Kelco, 1979; Flink and Johansen, 1985). However, the addition of a cationic polymer such as polyethyleneimine involves the diffusion and simultaneous chemical reaction of a polymer into another polymer. The resulting system is complicated and not amenable to simple analysis and scale-up. It is our feeling that a simple solution to the problem would be to add a trivalent cation (e.g. aluminum) to the already formed gel (made by the addition of 0.1M $CaCl_2$ to a 1.0% sodium alginate solution). Not only is the diffusion of aluminum less complicated than a polycation, but there is also absolutely no question of toxicity in a dilute aqueous solution. The procedure is described in the following sections and results are presented for some representative gels used in cell immobilization work.

Experimental Methods

Aqueous 1% sodium alginate solutions were used throughout. For the rheological tests, all gels were made *in situ* using a Rheometrics Fluid Rheometer in the parallel plate mode with 400 grit sandpaper glued to the plates to prevent sample slippage. The alginate solution was poured onto
the bottom plate (cup) to make a uniform layer 2 mm thick and 7 cm in diameter. The cup was then flooded with 10 ml of $CaCl_2$ for 35 min to allow for gel formation, and the excess $CaCl_2$ solution was removed before beginning a dynamic experiment over the frequency range 0.1-10 ω/s at 5% strain. The gap between the two parallel plates was kept in the 1.4-1.6 mm range, a thickness typical of gel bead dimensions in cell immobilization. The sample was loaded with a small residual axial force to help prevent slippage. Precautions also were taken to avoid very uneven surfaces or trapping of air pockets due to shrinkage. Both the storage (G') and the loss (G") moduli were obtained as a function of frequency. To test the effect of the trivalent cation, the $CaCl_2$ solution was allowed to sit for 30 min, drained, and aqueous 0.1M $Al(NO_3)_3$ was added for a 5 min treatment before removal for dynamic testing. Each test was repeated several times to check for reproducibility.

To test whether the aluminum treated gels are suitable for cell immobilization, *Saccharomyces cerevisiae* (ATCC 24858) was used. The yeast cells are entrapped in 0.6 mm diameter gel beads extruded from a two-phase atomizer. Untreated calcium alginate beads were used as control. Gelling times for calcium or aluminum treatment

were similar to those used in the dynamic testing experiments. Both treated and untreated beads were cultured in 1% glucose growth medium in a shake flask. Spent medium was replaced by fresh medium every day for 9 days. Samples of gel beads were taken daily and preserved in 4% formaldehyde. All samples were later embedded in paraffin, sectioned into 8 μm slices, and stained with hematoxylin.

Results and Discussion

The treatment of calcium alginate gels with trivalent aluminum nitrate generally resulted in gels which were firmer. The untreated gel beads could easily be crushed between two fingers while the aluminum treated beads showed greater integrity in this *finger test*. To quantify this behavior, the dynamic moduli of the gels under the different treatment conditions were examined. The storage G'(ω) and loss G"(ω) moduli are plotted in Figure 1 for a representative sample and the average values of the moduli over the frequency range studied are reported in Table 1.

The dynamic storage modulus G' is a measure of the materials ability to store elastic energy (Ferry, 1972). For a *nearly perfectly elastic* material, G' should be independent of frequency over the range covered in the present experiments . The loss modulus G" is a measure of the viscous energy dissipated by the material. Typically for a gel, G'>> G" and both moduli are only a weak function of frequency. The *gel strength* is reflected in the magnitude of G' and the *gel resilience* can be inferred from the ratio G'/G". The larger this ratio, the greater the amount of elastic energy stored as compared to loss due to viscous dissipation, hence, the more *resilient* the gel. Simply put, the best indication of gel structure is the frequency independence of G' and the best measure of the effectiveness of the gel formation process is a large increase in both G' and the ratio G'/G".

One important point which should be emphasized is that gel formation is a kinetic process. The dynamic data are reported for an experimental time which we consider to be the minimum required for most cell immobilization procedures. Thus the results are comparative in nature and the absolute values of the moduli are dependent on the experimental conditions chosen.

From Table 1 it is apparent that, in agreement with the visual observations, the storage moduli increased with the addition of trivalent crosslinking agent ($Al(N0_3)_3$) in all cases. On average, the increase was by a factor of two, which is a substantial improvement in gel strength. Within the limits of discrimination in these type of experiments, it appears that gel strength, G', the increase in gel strength, G'_{Ca}/G'_{Al}, and the gel resilience, G'/G", are all approximately the same for the 0.05M and 0.1M

Table 1 Keltone (1.0 wt%) Gel Properties With and Without aluminum Cation Treatment. The G' and G" are averaged values over the frequency range 0.1-10 rad/s at a strain of 5%. The subscripts Ca and Al stand for gels without and with aluminum treatment.

$CaCl_2$ M	$Al(NO_3)_3$ M	G' 10^{-3} dyne/cm^2	G" 10^{-3} dyne/cm^2	G'/G"	G'_{Ca}/G'_{Al}
0.05	--	9.3	1.4	6.8	
0.05	0.1	18.5	0.82	22.5	2.0
0.1	--	7.4	1.0	7.1	
0.1	0.1	19.0	1.1	17.4	2.6
0.2	--	9.3	1.1	8.2	
0.2	0.1	17.6	1.8	9.5	1.9

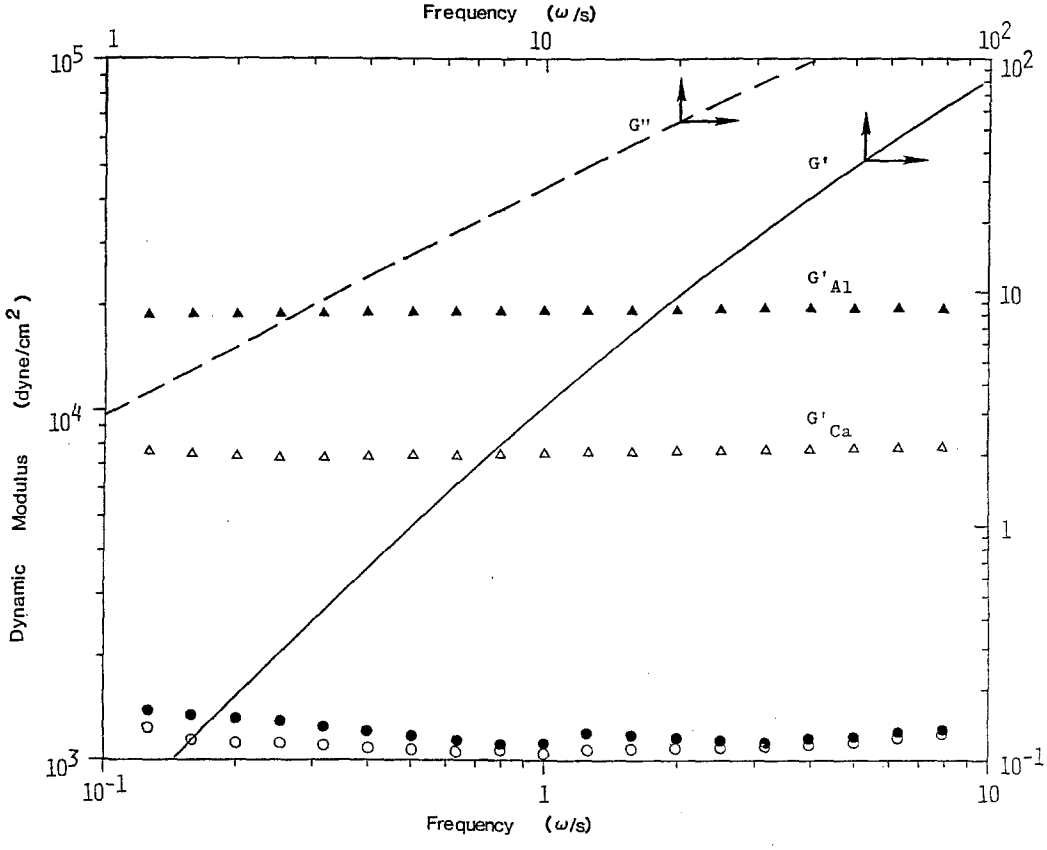

Figure 1 The storage (G') and loss (G") dynamic moduli as a function of frequency. Gel with 0.1M $CaCl_2$: G'(△), G"(○). Gel with 0.1M $CaCl_2$ and 0.1M $Al(NO_3)_3$: G'(▲), G"(●). 1% sodium alginate solution: G'(solid line) and G"(dotted line).

$CaCl_2$ gels. The only major difference between these two $CaCl_2$ levels was the rate of gel formation, which was observed but not measured. The use of 0.05M $CaCl_2$ leads to a much slower initial gelling rate.

In comparing the 0.2M $CaCl_2$ gel to the other two, it is seen that the gel strength and increase in gel strength are comparable in all cases, but the gel resilience (G'/G") is quite different. This can be explained by examining the technique for making the gels. When a relatively high concentration of divalent crosslinking agent is placed in contact with the alginate solution, the rate of gel formation is very fast so that most of the surface sites are occupied quickly and large diffusional resistances develop. Thus, one is left with a material which has a high surface crosslink density but poor distribution of crosslinked sites. With the 0.05M and 0.1M $CaCl_2$ gels, the gelling rates are slower so the crosslinking agent is able to penetrate farther from the surface and distribute crosslinking sites more evenly. With the addition of the trivalent crosslinking agent, which initiates a very fast gelling process, the diffusional resistances set up by the first crosslinking step are magnified. The result is that the effectiveness of the trivalent crosslinking of the 0.2M $CaCl_2$ gel is greatly reduced. One is left with a material with a stiff outer shell and a soft interior, which leads to a higher loss modulus G' and less resilient (low G'/G") gel. This effect can also be seen to a lesser degree in comparing the G'/G" data for the 0.05M and 0.1M $CaCl_2$ gels after addition of the trivalent aluminum ion. The 0.05M $CaCl_2$ gel, which is believed to have the lowest degree of diffusional resistance after the first gelling step, appears to be the most resilient, probably indicating that the crosslink sites are distributed more evenly throughout the gel matrix. Once again, it should be noted that the differences between the 0.05M and the 0.1M $CaCl_2$ gel properties are minor, so that for many applications the *rate* of gel formation may be the governing parameter. For cell immobilization applications, we recommend initial gelling with 0.1M $CaCl_2$ followed by a 0.1M $Al(NO_3)_3$ washing. While gelling with a 0.05M $CaCl_2$ solution has similar final results, a comparatively longer time is required for complete structure development. Since the gelling process in cell immobilization is typically carried out in a stirred vessel, the gel particle which is slower to form is more susceptible to shear deformation and break-up.

As was mentioned earlier, the clearest indication of gel structure in a solution is the frequency independence of the storage modulus G'. The dynamic moduli as a function of frequency for the 0.1M $CaCl_2$ solution are plotted in Figure 1. It can be seen that G' is only a *weak* function of frequency over the entire frequency range studied. The corresponding data for the 1% sodium alginate solution are shown as solid lines in Figure 1. In this case both moduli have a strong frequency dependence and in addition,

the loss modulus G" is considerably greater than the storage modulus G' at all frequencies. This highlights the differences between concentrated solution and lightly crosslinked gel behavior and demonstrates the sensitivity of the dynamic experiment to the presence of gel structure.

The results of the cell growth tests confirm that yeast cells can grow very well in the aluminum treated gel beads. From microphotograph of sections of 6 mm aluminum treated gel beads, the entrapped yeast population within the gel matrixis fairly uniform with slightly higher density near the peripheral region. (The microphotographs are available from the authors.) The cell mass distribution and cell growth progression photographs of both treated and untreated beads are also similar. With a larger gel bead one would expect to observe even more significant diffusional effects and preferrential cell growth near the bead surface (Ryu et al., 1984).

We can conclude that the properties of a gel prepared from sodium alginate with a calcium chloride divalent crosslinking agent can be substantially improved by a simple washing with a trivalent cation, $Al(NO_3)_3$, after the intial gelling process. The procedure is straightforward, nontoxic and the presence of aluminum ion also stabilizes the calcium alginate in high phosphate content media.

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