External Versus Internal Source of Calcium During the Gelation of Alginate Beads for DNA Encapsulation

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Abstract: Alginate gels produced by an external or internal gelation technique were studied so as to determine the optimal bead matrix within which DNA can be immobilized for in vivo application. Alginates were characterized for guluronic/mannuronic acid (G/M) content and average molecular weight using ^{1}H-NMR and LALLS analysis, respectively. Nonhomogeneous calcium, alginate, and DNA distributions were found within gels made by the external gelation method because of the external calcium source used. In contrast, the internal gelation method produces more uniform gels. Sodium was determined to exchange for calcium ions at a ratio of 2:1 and the levels of calcium complexation with alginate appears related to bead strength and integrity. The encapsulation yield of double-stranded DNA was over 97% and 80%, respectively, for beads formed using external and internal calcium gelation methods, regardless of the composition of alginate. Homogeneous gels formed by internal gelation absorbed half as much DNAse as compared with heterogeneous gels formed by external gelation. Testing of bead weight changes during formation, storage, and simulated gastrointestinal (GI) conditions (pH 1.2 and 7.0) showed that high alginate concentration, high G content, and homogeneous gels (internal gelation) result in the lowest bead shrinkage and alginate leakage. These characteristics appear best suited for stabilizing DNA during GI transit. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **57**: 438–446, 1998.
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INTRODUCTION

DNA is used as a trap or target to monitor levels of food-related carcinogens in the gastrointestinal (GI) tract (Alexakis et al., 1995; Quong et al., 1996). Previously, microencapsulated polyethyleneimine was used for this purpose as a DNA surrogate (Povey et al., 1986, 1987; Povey and O'Neill, 1990), due to the tendency for carcinogens to form adducts with DNA or related chemical structures. It is possible to consider use of DNA in place of the surrogate, if the DNA can be protected during gastrointestinal transit from hydrolytic, enzymatic, acidic, and microbial attack. The DNA must be retained and protected within a capsular structure (artificial cell), which is accessible to lower molecular weight carcinogens or mutagens. Subsequent recovery and purification of the DNA and DNA adducts is necessary for molecular biological assay to determine the level and nature of adduct formation, and extent of DNA damage. The formation of an adduct is a precursor to possible tumor development within the colorectal cavity (O'Neill, 1993; O'Neill et al., 1993).

DNA has been immobilized in alginate microspheres using the emulsification/internal gelation technique (Poncelet et al., 1992) and dosed to rats through oral administration (Quong et al., 1995). Beads were recovered magnetically from feces after GI transit by coencapsulating iron magnetite within the microspheres. Alginate beads were recovered intact with recoveries approaching 60% (Alexakis et al., 1995) but containing low residual levels of DNA. The high microsphere recovery suggested that alginate beads can tolerate harsh GI conditions and avoid digestion within the tract; however, the low DNA recovery suggested that DNA was subjected to hydrolysis, degradation, and release. To increase DNA yield after GI transit, the DNA must be protected and retained, while maintaining free diffusional access to low molecular weight carcinogens. As a result, the bead matrix requires optimization in terms of its alginate composition and matrix structure.

Alginate is a polysaccharide obtained from brown algae and is composed of guluronic and mannuronic acid residues varying in proportions and molecular weight (Smidsrød, 1974; Smidsrød et al., 1968). In the presence of divalent cations (i.e., calcium) the alginate is crosslinked and forms a gel. The mild conditions during bead production are desirable to insure fully intact DNA following encapsulation. The conventional technique for alginate bead production involves extrusion of droplets into a calcium chloride solution. Calcium from the external source diffuses into the droplet, causing gelation of the alginate. Alternatively, the

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internal gelation method uses fine, insoluble calcium carbonate microcrystals as an internal calcium source for gelation (Poncelet et al., 1992). Calcium is released from calcium complex upon pH reduction from 7.0 to 6.5. Depending on the compositions of alginate and the conditions for producing beads, different intracapsular gel structures would form and may subsequently alter DNA distributions and affect the level of DNA protection and retention within the beads. The purpose of this study was to compare external and internal gelation methodologies with respect to gel matrix structure for DNA immobilization and protection. This will be evaluated in terms of alginate, calcium, and DNA distributions within the bead structure; shrinkage or swelling by weight ratio changes during formation, storage, and simulated GI conditions; alginate loss during formation; and nuclease exclusion.

MATERIALS AND METHODS

Commercial samples of sodium alginate SG80, SG150, SG300, and S550 were kindly donated by Systems Bio-Industries (France). SetaCarb calcium carbonate was donated by Omya (France). Vantocil IB or poly(hexamethylenebiguanidinium chloride) was donated by ICI Americas Inc. Canola oil was donated by Canada Packers (Montreal). Calf thymus DNA (highly polymerized) was purchased from Sigma (St. Louis, MO). DNAse I was purchased from Boehringer (Montreal). All other chemicals were reagent grade.

Alginate Characterization

Low angle laser light scattering (LALLS) measurements at 632.8 nm were performed with a DAWN-F multiangle laser photometer (Wyatt Technology, Santa Barbara, CA) instrument equipped with a He–Ne laser. DawnF and SkorF software were used for data acquisition and analysis, respectively. The angular dependence of scattering was determined at 15° intervals between 20° and 150° at 25°C. Sodium chloride (0.2 *M*) was used as solvent. All samples were dialyzed against 0.2 *M* NaCl, and dialysate was used as diluent. A value of 0.162 cm^3/g was used as the specific refractive index increment at 632.8 nm in this study according to Wedlock et al. (1986). The solutions and the diluent were optically clarified by 0.45- and 0.22-μm filters (Chromatographic Specialties Inc.), respectively. The alginate content was measured by poly(hexamethylenebiguanidinium chloride) ($PHMBH^+Cl^-$) reaction (Kennedy and Bradshaw, 1987).

NMR spectra of alginates were determined and analyzed according to Grasdalen et al. (1979) and Skjåk-Bræk et al. (1986). Briefly, alginate (10 mg) was dissolved in D_2O (0.4 mL) at neutral pD with the addition of 3 mg of 3-ethylenetetraminehexacetic acid (TTHA) for the prevention of trace divalent cations from interacting with the monomer units. The ^{1}H-NMR spectra were run at 400 MHz and recorded at 92° with a Bruker WM-400 spectrophotometer. The chemical shifts were expressed in parts per million (ppm) downfield from the signal for sodium 3-(trimethylsilyl)propanesulfonate. The area under each of the partly overlapping peaks in the low-field regions was found by excision and weight.

Alginate was quantified by mixing duplicate samples containing 0.5 mL of alginate solution (1 to 5 mg/ml) with 1.0 mL of 0.3% (w/v) $PHMBH^+Cl^-$ in 1.0% (w/v) sodium acetate (Kennedy and Bradshaw, 1987). The mixture was vortexed, allowed to sit at room temperature for 1 h, then centrifugated at 3000*g* for 5 min. The supernatant was then diluted 100 times prior to measurement at 235 nm against distilled water using a Cary 1 spectrophotometer (Varian Canada, Montreal).

For alginate concentrations less than 0.4 mg/mL, a 200 μL of sample was analyzed with a DC-80 Rosemount Dohrman Total Organic Carbon (TOC) analyzer (Montreal) via UV-enhanced persulfate oxidation reaction.

DNA Quantification Using Hoechst 33258

DNA concentrations less than 500 μg/mL were quantified using the dye Hoechst 33258 and a DyNAQuant 200 fluorometer (Pharmacia Biotech, Montreal). Briefly, 2 μL of DNA sample were mixed with 2 mL of TNE buffer (10 m*M* Tris, 1 m*M* EDTA, 0.2 *M* NaCl, pH 7.4) and the fluorescence quantified at a wavelength of 460 nm.

Alginate Bead Formation

A solution containing 1% to 3% (w/v) sodium alginate was extruded through a syringe tip needle (0.001 in. internal diameter) apparatus into 50 m*M* calcium chloride solution. Because the calcium source was external to the forming bead, the technique is referred to as external gelation. To form internally gelled beads, a solution containing 1% to 3% (w/v) sodium alginate and 0.5% (w/v) suspended sonicated calcium carbonate powder was mixed and extruded dropwise through a syringe needle into canola oil containing 0.2% (v/v) glacial acetic acid. The beads were reacted for at least 1 h prior to washing with 50 m*M* calcium chloride solution containing 1% (v/v) Tween-80 to remove excess oil. Bead size distributions were measured using a video camera mounted on a stereomicroscope. The average bead diameters were determined with image analysis software.

To measure the encapsulation yield of DNA, a 2% (w/v) solution of alginate SG300 containing 0.2% (w/v) calf thymus DNA was gelled as described, then liquified in 500 m*M* EDTA solution and quantified for DNA using Hoechst 33258 dye fluorescence.

Calcium Uptake and Sodium Release

A total of 20 beads were dropped into each reaction vessel containing 5 mL of a 50 m*M* calcium chloride solution for a specific reaction time (i.e., 2, 13, 20, 30 min), according to the external gelation protocol. The bulk solution was

sampled and analyzed for calcium, sodium, and alginate. Calcium and sodium were determined by diluting liquid samples in deionized water and analyzing using a Smith–Hieftje II Atomic Absorption Spectrophotometer (Thermo Jarrell Ash, Canada) at 422.7 and 389.0 nm for calcium and sodium, respectively.

Alginate Bead Shrink/Swell in Simulated GI Conditions

The bead shrinkage or volume reduction was quantified by measuring the weight difference between 20 alginate droplets before gelification with the weight of the same 20 beads incubated in 50 m*M* calcium chloride solution for 1 hour followed by storage in distilled water for 24 h. To simulate GI transit in vitro, the same 20 beads were exposed to simulated gastric solution (pH 1.2) for 24 h and then 72 h in distilled water at pH 6.5 to 7. Alginates varying in molecular weight, guluronic content, and alginate concentration were tested and measured for weight changes in the GI simulation time course.

DNAse Diffusion Within External and Internal Gelled Alginate Beads

The absorption of DNAse into beads formed using the external and internal gelation techniques was determined by placing 20 beads and 3 mL of 7 μg/mL DNAse in a quartz glass cuvette. A nylon mesh restricted beads to the bottom of the cuvette and away from the light path of the spectrophotometer. Cuvette contents were mixed (Spectrocell Inc.) and the absorbance at 280 nm continuously measured with a Cary 1 spectrophotometer (Varian Canada, Montreal).

Gel Heterogeneity and Homogeneity: Distribution of Calcium, Alginate, and DNA

A study of calcium and alginate distribution within gels made by the external and internal gelation techniques was conducted. Gel cylinders were made by sealing 1.7 g of alginate solution in an Eppendorf tube with a dialysis membrane (SpectraPor 1; Fisher Scientific, Montreal) of molecular weight cutoff 6000 to 80,000 Da. To simulate internal gelation, 0.5% (w/v) suspended sonicated calcium carbonate was added. The membrane-end was immersed in a constantly stirred (200 rpm) reservoir at room temperature (21°C) containing either 50 m*M* calcium chloride solution for the external gelation simulation or a solution containing 1% (v/v) Span 80 (Atkemix, Montreal) and 0.2% (v/v) glacial acetic acid dissolved in canola oil for the internal gelation simulation. After 24 h, the gel cylinders were soaked in distilled water for an additional 24 h to remove excess calcium from the gel prior to sectioning the cylinders into 1-mm-thick disks. Each slice was then dissolved in 1 mL of 1% (w/v) sodium citrate solution and analyzed for both calcium and alginate. For the DNA distribution study, a final 2% (w/v) medium G alginate with 0.01% (w/v) DNA was mixed and gelled according to the dialysis techniques described. The DNA concentration within each section was analyzed with a Hoechst 33258 DNA fluorometer (DyNA Quant 200; Pharmacia Biotech, Montreal).

RESULTS

Various alginates were analyzed for guluronic/mannuronic composition and average molecular weight by ^{1}H-NMR and LALLS, respectively. A typical ^{1}H-NMR spectrum of medium G alginate is shown in Figure 1 where the guluronic and mannuronic acid residue peaks are indicated in terms of its monad, dyad, and triad frequencies. Based on the monad frequencies shown in Table I, SG-type alginates had a higher proportion of guluronic than mannuronic residues when compared with an S-type alginate. The average molecular weights ranged from 237 to 1000 kg/mol with SG80 and SG150 being similar in size. Alginate SG80 was not used in the following studies due to its similarity with SG150. For simplicity, alginate S550, SG300, and SG150 are referred to as low, medium, and high G alginate.

Gelation kinetics determined by calcium uptake and sodium release profiles for 3% medium G alginate beads are illustrated in Figure 2 and summarized in Table II. A minimum 30-min reaction time is required to saturate alginate beads with calcium. A reaction time of 60 min was used for subsequent calcium-complexation experiments. The Ca:Na molar exchange ratios at initial and equilibrium time were 0.5 and 0.6, respectively, as shown in Table II.

The yield of DNA encapsulation was measured for both high and low G alginate and for beads formed using the external and the internal gelation methods. Beads formed by external gelation had encapsulation yields of 97%, whereas beads formed using the internal gelation technique had DNA yields of 80%. The composition of alginates (i.e., high, low G) did not affect the yield of encapsulation.

Alginate beads formed by external gelation, but varying

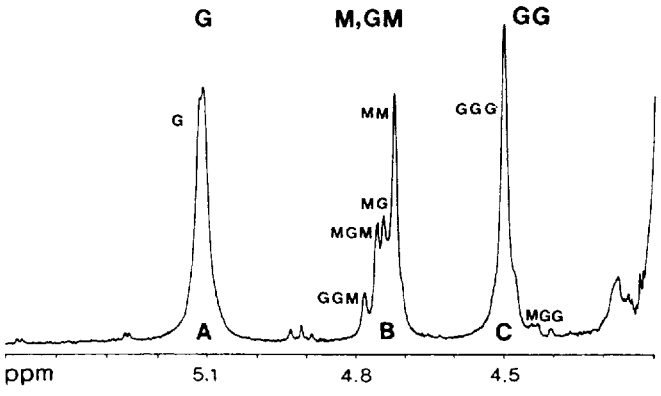

Figure 1. The anomeric region of a typical ^{1}H-NMR spectrum of alginate (F_G = 0.60). The spectrum was recorded at 92° at 400 MHz on a Bruker WM spectrometer. The relative areas of peaks A (G-1), B (M-1 and GM-5), and C (GG-5) contain information on the G/M ratio and the fractions of doublets of nearest neighbors along the intact copolymer chain. For peak assignments see Grasdalen (1983).

Table I. Composition of alignates. Guluronic molarity based on a 3% solution.

Alignate	F_G	F_{GG}	F_{MM}	$F_{GM,MG}$	F_{GGG}	F_{GGM}	F_{MGM}	$N_G > 1$	Molecular weight (kg/mol)	Guluronic molarity (μM)
SG 80	0.71	0.56	0.14	0.15	0.51	0.05	0.10	12.2	237	90
SG 150	0.75	0.60	0.10	0.15	0.56	0.04	0.11	16.0	278	81
SG 300	0.60	0.47	0.27	0.13	0.42	0.05	0.08	10.4	694	26
S 550	0.41	0.26	0.44	0.15	0.22	0.04	0.11	7.7	1000	12

in alginate composition and concentration, were tested over a typical retention time in formulation, storage, and simulated GI conditions to determine bead stability. As illustrated in Figure 3A, decreasing alginate concentration results in a higher degree of shrinkage. A 2% alginate droplet was reduced to 65% of its original weight during gelation but showed no change during storage in distilled water. At pH 1.2, the same beads shrank to 38% of the original weight with a slight recovery in mass at pH 6.5 to 7. The overall shrinkage for fully gelled 2% beads was approximately 33% during simulated GI transit. Figure 3B illustrates the stability of alginate beads with varying G content in formulation and simulated GI conditions. Medium and high G alginates behave similarly during gelation, storage, and at pH 1.2; however, high G polymer shows the greatest shrinkage overall. The final reswelling of high G alginate is much less than with the other two alginates which is indicative of its shrink/swell stability. Low G alginate results in the highest initial shrinkage during gelation.

Results in Figure 3C indicate that there is a higher degree of shrinkage with beads formed using the external vs. the internal gelation techniques, ranging from 32% to 25% reduction after gelation, respectively. In simulated gastric conditions, both external and internal gelled beads behaved similarly, shrinking to 35% and 36% of their original weight in acidic conditions but swelling to 45% and 52% at pH 7, respectively.

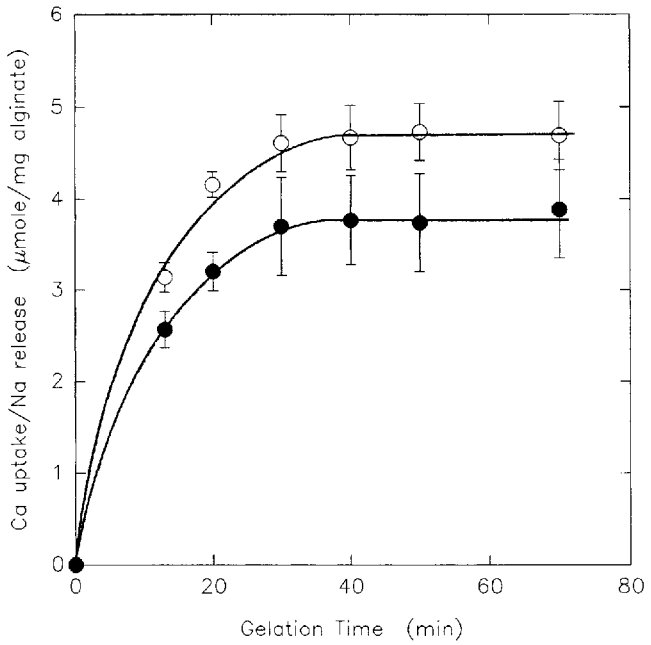

Figure 2. Calcium uptake (●) and sodium release (○) kinetic profiles for 3% (w/v) medium guluronic alginate beads formed using the external gelation technique in 50 m*M* calcium chloride. Error bars represent standard deviations of three replicates about the mean.

Beads varying in alginate concentration and G content were analyzed for total alginate release after 1 h of gelation (Table III). The amount of alginate lost is low at less than 4%. Increasing polymer concentration results in a decrease in polymer loss. Higher polymer viscosity may decrease the tendency of alginate to partition into the calcium solution. Low G alginate lost four times as much alginate as medium and high G alginate because there were fewer G sites available for calcium complexation.

Calcium and alginate profiles obtained through gels formed using simulated external and internal gelation techniques were compared in relation to initial polymer concentration and alginate G content. Figure 4 summarizes the results for medium G alginate varying in polymer concentration from 1% to 3%. In the external gelation simulation, increasing calcium levels were observed with higher gel concentrations (Fig. 4A). The initial calcium concentration in the bulk solution was the same for each case (50 m*M*), yet the calcium loading observed near the gel surface was more than double that concentration for 2% and 3% gels. A concentration gradient may be seen with steeper gradients observed at the higher alginate concentrations. In the internal gelation simulation, initial calcium concentrations in the gels prior to gelation were similar, resulting in similar profiles, irrespective of polymer concentration (Fig. 4C). A calcium gradient was observed near the gel surface for 2% and 3% alginates, but the calcium concentration was fairly uniform throughout the gel. The alginate profiles for the

Table II. Calcium and sodium initial rates and total uptake and release at 60 minutes for 3.5 mm diameter alignate beads of medium G molarity formed in 50 mM calcium chloride.

Time (min)	Initial Ca uptake rate (μmole/mg min)	Initial Na release rate (μmole/mg min)	Total Ca uptake (μmole/mg)	Total Na release (μmole/mg)	Ca:Na molar ratio
0–10	0.34	0.71	–	–	0.5
60	–	–	3.1	4.7	0.6

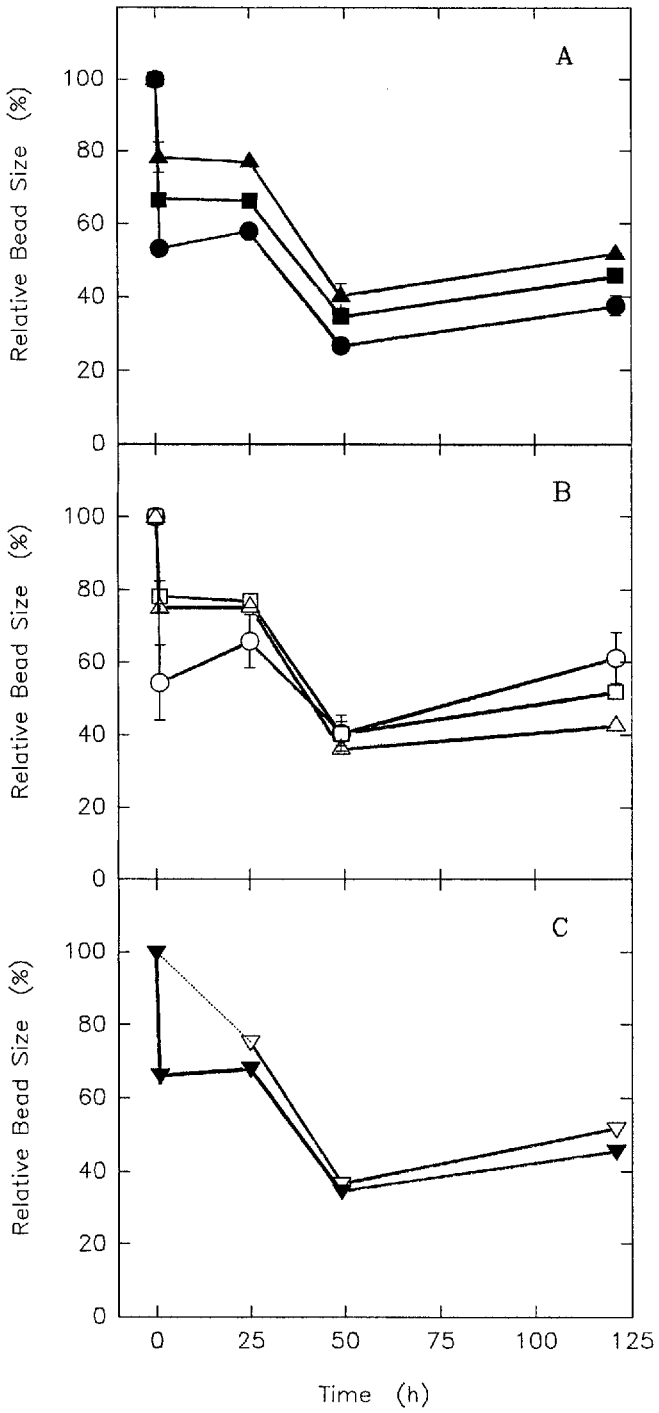

Figure 3. Bead mass reduction during gelification for 1 h, followed by storage in water for 24 h, simulated GI incubation for 24 h at pH 1.2, followed by 72 h of incubation at pH 7.0. (A) Percent relative bead size for 1 (●), 2 (■), and 3% (▲) medium guluronic alginate. (B) Percent relative bead size for low (○), medium (□), and high (△) guluronic alginate at 3% concentration. (C) Percent relative bead size for 2% (w/v) medium G alginate beads formed using the external (▼) and internal (▽) gelation techniques. Error bars represent standard deviations of three replicates about the mean.

Table III. Alginate released into 50 mM calium chloride solution after 1 hour of gelation.

Guluronic molarity (μM)	Alginate (%)	Bead Diameter (mm)	% Total Alginate Released
12	3.0	3.5	3.7 ± 0.1
	1.0	3.1	1.3 ± 0.2
26	2.0	3.4	1.1 ± 0.1
	3.0	3.5	0.7 ± 0.2
81	3.0	3.5	0.7 ± 0.2

external gelation technique had a sharper gradient through the core, decreasing with decreasing alginate concentration (Fig. 4B). For the internal gelation technique, there was a slight alginate gradient similar to its calcium gradient profile at the gel surface followed by a uniform alginate distribution within the core (Fig. 4D). Low polymer (1%) concentration resulted in an approximate final alginate concentration of about 1.5% and 1.8% for external and internal gels, respectively.

Figure 5 summarizes the calcium and alginate gel profiles for 3% alginate varying in guluronic molarity from 12 to 81 μ*M*, formed using both the external and internal gelation techniques. Results for gels formed via external gelation showed high calcium gradients from the surface to minimal concentrations at the inner core. The amounts of calcium complexed was similar for different alginate types. The calcium profiles measured for internal gels were similar and independent of alginate G content. In contrast to external gels with steep gradients, a calcium and alginate gradient existed near the surface of the internal gels but exhibited uniformity through the core of the gel.

DNA distributions within 2% medium G alginate cylinders were studied using both the external and internal gelation methods (Fig. 6). External calcium gelation of alginate produced DNA gradient profiles at the gel periphery and gradually decreased within the core matrix. In contrast, internal gelation produced homogeneous DNA distributions within the gels.

Alginate beads (2%) of medium G content, formed using external and internal gelation methods, were examined for DNAse absorption kinetics. Figure 7 indicates that beads formed by external gelation absorbed 30% of the DNAse, whereas internal beads formed by internal gelation absorbed only 15% after 1.5 h of incubation.

DISCUSSION

Alginates provided by Systems Bio-Industries were characterized in terms of G/M content and average molecular weight. SG-type alginates had a higher G residue content than an S-type alginate. SG-type alginates are purified from stipes of brown seaweeds, *Laminaria hyperborea,* whereas S-types are leafy portions from either *Laminaria digitata* or *Ascophyllum nodosum.* SG80 and SG150 had similar proportions of G and M residues, however, SG150 had the highest mean block length (N_G = 16.0). For the purpose of

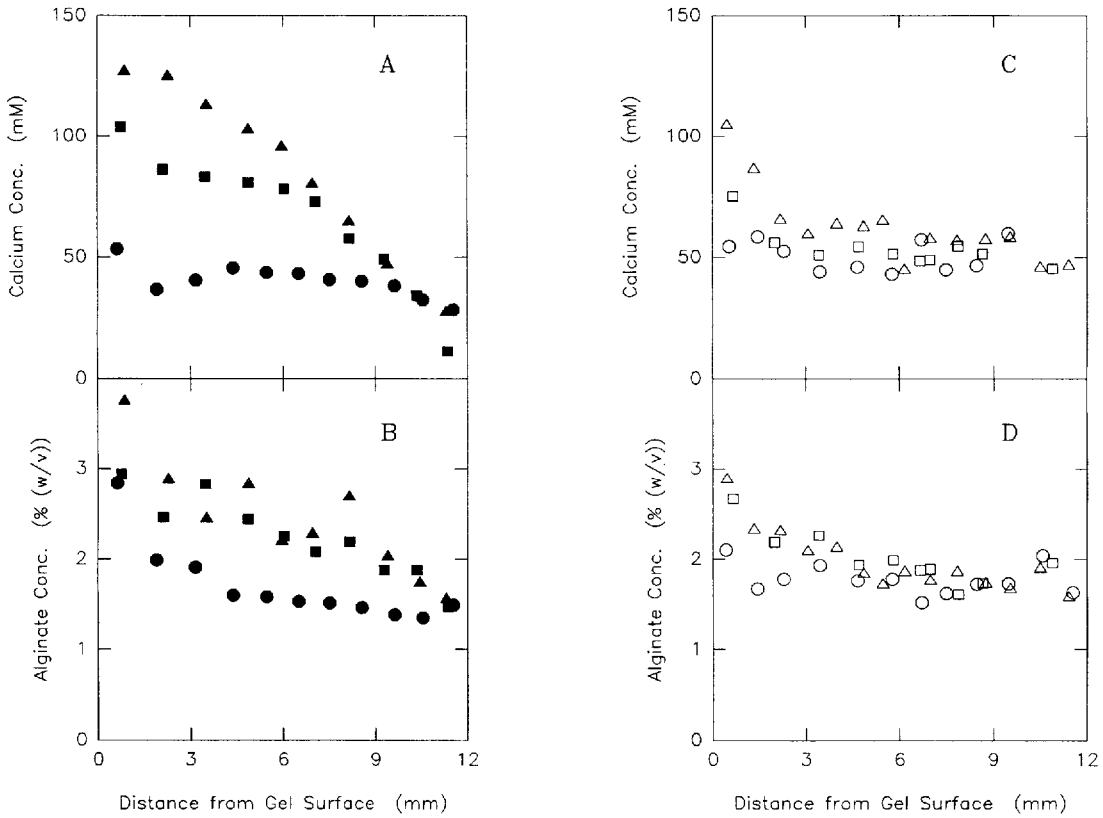

Figure 4. Profiles of gels formed using simulated external (A, B) and internal (C, D) gelation techniques. Gels were formed using 50 m*M* calcium chloride solution (external) or 0.5% calcium carbonate suspension (internal) to which 0.2% glacial acetic acid was added to initiate gelation. Medium G alginate was used at 1 (●, ○), 2 (■, □), and 3% (▲, △) alginate concentrations.

this study, alginates with low (0.41), medium (0.60), and high (0.75) G content were selected for further study.

There are two techniques for forming alginate beads, based on the source of calcium. In conventional bead formation, alginate droplets are gelled in a calcium chloride solution. The calcium source is external to the droplet and gelation is initiated from the bead surface and works toward the inner core. This is referred to as external gelation. For the internal gelation method, the alginate solution is preloaded with insoluble calcium carbonate, liberating calcium with a pH adjustment from 7 to 6.5 (Poncelet et al., 1995). The calcium carbonate microcrystals, distributed within the aqueous droplet, gel the alginate using an internal calcium source.

During external gelation, exchange of sodium for calcium ions occurs at a ratio of 2:1 limiting the gelation rate to the rate of calcium diffusion. A 30-min time interval is required for calcium saturation in contrast to a few seconds with calcium carbonate and 20 s when calcium citrate is used as the calcium source in internal gelation (Poncelet et al., 1995).

The stability of alginate beads during formation and GI transit is critical for in vivo protection of DNA (Alexakis et al., 1995; Quong et al., 1996). The beads must retain DNA with minimal swelling to avoid leakage of low molecular weight DNA fragments. During gelation, alginate beads decreased in weight to less than 80% of initial weight due to syneresis. Low alginate concentration, low G alginate, and external gelation gave rise to the highest shrinkage during bead formation. Subsequent storage in water showed stability of beads due to their constant weights; however, low G alginates, being weaker in strength, were also more flexible. These low G alginates reswelled during storage, resulting in a more permeable gel. In simulated GI conditions, the beads shrank to less than 40% of the initial weight at pH 1.2 and swelled slightly at pH 7.0. High G alginate offered the greatest bead stability during simulated GI transit. External and internal gelled beads appeared to behave similarly under simulated GI conditions with a 66% weight reduction. Medium G alginate (2%), formed by internal gelation, showed a 65% bead size reduction during GI transit in rats (Quong et al., 1996).

Similarly, alginate leakage may also signify bead instability. The degree of leakage is dependent on polymer concentration and guluronic content for beads formed using the external gelation method. Increasing alginate content decreases the leakage of polymer. Alginate loss from beads formed by internal gelation was not anticipated because the beads were dispersed in oil. Martinsen et al. (1989) showed that the diffusion coefficients of solutes for high molecular weight polymer beads are low in comparison to that of low molecular weight polymer beads. In this study, high man-

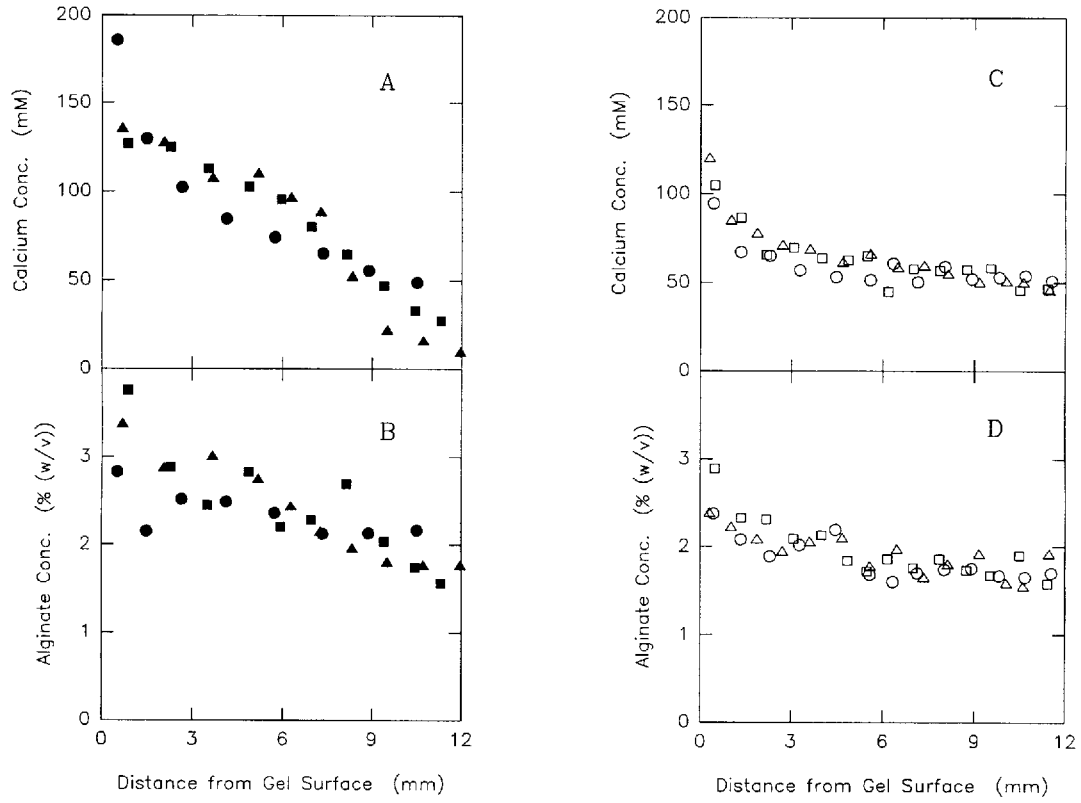

Figure 5. Profiles of gels formed using simulated external (A, B) and internal (C, D) gelation techniques. Gels were formed using 50 m*M* calcium chloride solution (external) or 0.5% calcium carbonate suspension (internal) to which 0.2% glacial acetic acid was added to initiate gelation. Three percent (w/v) alginate was used for low (●, ○), medium (■, □), and high (▲, △) G alginate.

nuronic alginate lost more polymer because mannuronic blocks have not been crosslinked. In all cases, alginate loss was less than 4% of the total alginate. For in vivo application, a total alginate loss of this magnitude would seem insignificant because alginate is biocompatible and nontoxic. Ultimately, high G content and high alginate concentration would minimize sol fraction (short alginate chains with minimal G blocks which do not contribute to the gel network) or solute leakage (Stokke et al., 1991, 1993).

The calcium and alginate distributions within gels obtained from external and internal gelation simulation were different. When using the external gelation method, a high calcium and polymer gradient exists near the gel surface, decreasing slightly as it approaches the core. Sectioned alginate beads with hollow cores have been observed experimentally, suggesting an alginate gradient. When gel forming ions such as calcium diffuse into an alginate solution, the rapid site binding of the crosslinking ion and formation of the gel network produce an inward moving gelling zone. Alginate will diffuse from the center of the gel toward the gelling zone, leading to a depletion of alginate in the core. Mikkelsen and Elgsaeter (1995) suggested that the polymer gradient is essentially governed by the relative diffusion rate between the soluble alginate molecules and the calcium ions. In general, high polymer concentration, low molecular weight alginate, and low concentration of gelling ions offer the highest inhomogeneity, whereas a high molecular weight alginate, high concentration of gelling ions, and a high concentration of nongelling ions give rise to increased homogeneity (Skjåk-Bræk et al., 1989). In contrast, very homogeneous alginate gels can be achieved in systems where the initial concentration distribution of Ca^{2+} and free alginate is homogeneous. Such homogeneous initial conditions can be achieved by mixing alginate and a fine dispersion of an insoluble calcium salt together with a chemical system that converts insoluble calcium to calcium ions. Homogeneous gels have been made by internal release of calcium from Ca:EDTA or Ca:citrate, or from $Ca(CO_3)_2$ in the presence of a slow acidifier like D-glucono-δ-lactone (GDL) (Draget et al., 1991). In the internal gelation simulation, the gradients are more uniform through the core; however, a slight gradient also exists near the gel surface. This surface gradient of calcium and polymer within internally gelled alginate may be a result of the gelation conditions between the acidified organic external phase and the aqueous alginate droplet. These gels are termed homogeneous, relating to the more uniform calcium and alginate distributions within the core. The method for internal gelation in the present study diverges from the GDL method because it involves diffusion of gel-inducing ions (protons) from an external reservoir. Although the calcium ions are homogeneously distributed in the alginate solution initially, the diffusion of protons into the beads will induce gelation initially at the surface, giving rise to polymer gradients. Protons,

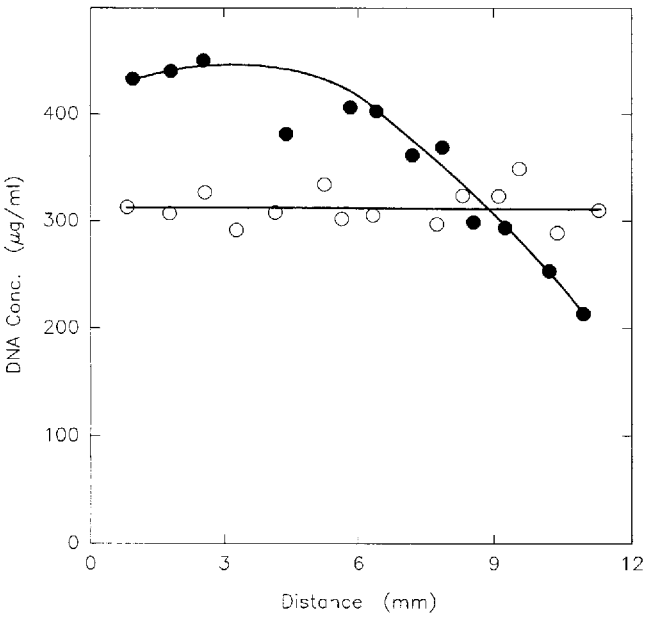

Figure 6. DNA gradients found within 2% alginate gels formed using the external calcium (50 m*M* $CaCl_2$) gelation method (●) and the internal calcium (0.5%) gelation method (○). Dialyzed gels were sectioned, weighed, liquified, and analyzed for DNA.

being smaller in size than calcium ions, diffuse faster, resulting in a shorter gelation time. The diffusion coefficients of protons, calculated using the Wilke–Chang equation (Wilke and Chang, 1955), and calcium (Lin, 1991) in water were found to be 7.7×10^{-5} and 4.0×10^{-6} cm^2/s at 25°C, respectively.

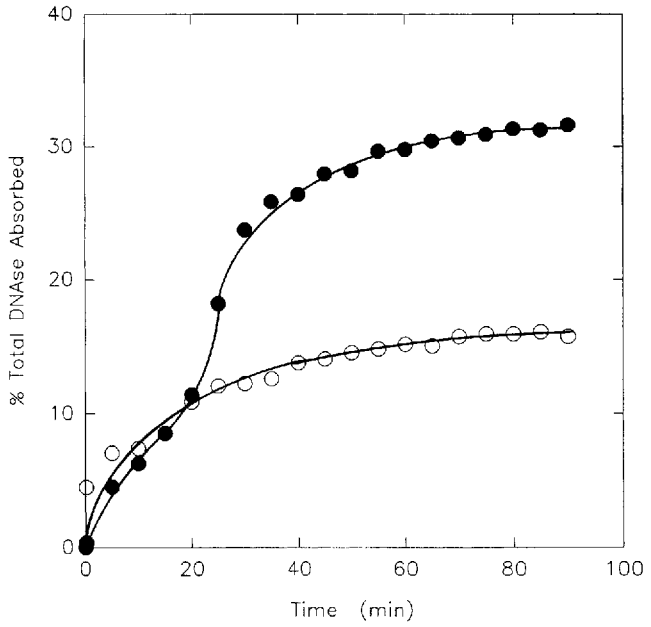

Figure 7. Percent total DNAse absorption into 2% (w/v) alginate beads of medium guluronic alginate formed using external (●) and internal (○) gelation techniques. The percent total values were normalized with respect to bead volume in cubic millimeters.

The DNA distributions within gels created by both external and internal gelation methods were clearly different. The external gelation method resulted in a DNA concentration gradient, whereas a homogeneous DNA distribution was formed using the internal gelation method. The calcium–polymer distribution within a gel contributes to the distribution of the immobilized DNA. In the external gelation method, the high calcium availability at the gel surface creates the driving force for the movement of polymer to the gel-forming front near the bead surface at the expense of polymer within the gel core. During this gelation time, the DNA migrates along with the polymer and becomes immobilized within the crosslinked matrix. For the internal gelation method, the slight polymer gradient formed at the gel surface (oil/bead interface) may prevent the leakage of DNA during bead formation. Internal gelation appears fast and does involve the diffusion of protons from the continuous oil phase to the gel inner core. This fast diffusion allows the gelation within homogeneous gels to occur quicker than in nonhomogeneous gels such that the DNA is immobilized more rapidly with minimal migration and minimal gradient formation.

The steep alginate gradients near the bead surface, within beads formed by the external calcium gelation method, increase the level of DNA retained. Internally gelled beads with more homogeneous distributions are more porous than externally gelled beads as deduced by the loss of DNA fractions during bead formation. The compactness of the surface of externally formed gels offers a higher resistance to diffusion in contrast to the more homogeneous gel formed by internal gelation.

In terms of DNAse diffusion, beads formed using the internal gelation technique minimized DNAse adsorption by half when compared with beads formed using the external gelation technique. DNAse, found in the GI tract, increases the likelihood of DNA hydrolysis once in contact with immobilized DNA. Neither external nor internal gelation methods alone can fully exclude DNAse; however, a combination of alginate beads with a membrane coat may improve DNAse exclusion.

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