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Electrophoretic Extraction and Analysis of DNA from Chitosan or Poly-L-lysine-Coated Alginate Beads

Douglas Quong[†] and Ronald J. Neufeld*[,‡]

Department of Chemical Engineering, McGill University, 3480 University Street, Montréal, Québec, H3A 2A7, Canada, E-mail: neufeld@chee.queensu.ca

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Abstract

Alginate beads containing entrapped DNA were produced using both external and internal calcium sources, and coated with chitosan or poly-L-lysine membranes. The beads were assayed with DNase nuclease to determine formulation conditions offering the highest level of DNA protection from nucleic acid hydrolysis, simulating gastrointestinal exposure. A method was developed to extract and assay intracapsular DNA through a modified agarose electrophoresis system. Both external and internally gelled beads were permeable to DNase (M_w = 31 kDa), indicated by the absence of DNA after nuclease exposure. At low levels of DNase exposure, coated high guluronic content alginate beads offered a higher level of DNA protection compared with coated beads with low guluronic alginate. No apparent correlation was found with chitosan membrane molecular weight and degree of deacetylation; however, increasing poly-L-lysine molecular weight appeared to increase DNase exclusion from beads. At elevated levels of DNase exposure, DNA hydrolysis was evident within all coated beads with the exception of those coated with the highest molecular weight poly-L-lysine (M_w = 197.1 kDa), which provided almost total nuclease protection. Optimal combination then for DNA protection from nucleases is a high guluronic alginate core, coated with high molecular weight poly-L-lysine.

Index Entries: DNA; chitosan; poly-L-lysine; alginate; electrophoresis.

*Author to whom all correspondence and reprint requests should be addressed.
[†]Present address: 3M Canada, P.O. Box 5757, London, Ontario, N6A 4T1, Canada.
[‡]Present address: Department of Chemcial Engineering, Queen's University, Kingston, Ontario, K7L 3N6, Canada.

Introduction

Artificial cells containing DNA were developed for in vivo trapping of environmental carcinogens and mutagens in the gastrointestinal (GI) tract *(1,2)*. The DNA is used as a trap for these compounds to bind and form DNA adducts. Earlier methods included the use of DNA surrogates involving semipermeable polyethyleneimine (PEI) and crosslinked PEI microcapsules *(3–6)* for trapping metabolites in vivo. The advantages of using DNA, as opposed to surrogates, is that the surrogates are poor mimics of DNA structure *(7–9)* and its ability to simulate DNA damage within an artificial cell without the interference of cellular DNA repair mechanisms. The utilization of immobilized DNA for in vivo assay is novel, and is also being considered for other applications such as gene therapy *(10–12)*. DNA is being immobilized within a semipermeable matrix that is intended to provide protection during GI transit. The beads should retain DNA, while allowing lower molecular weight endogenous carcinogens within the colon to diffuse through the beads and be trapped by the target DNA forming DNA adducts *(2)*. DNA alginate beads were dosed to rats through oral administration and magnetically recovered from the feces by coencapsulating iron magnetite. A bead recovery of about 60% *(1)* was achieved, but with low DNA residuals within the beads. The loss of DNA was attributed to the diffusion of GI nucleases into the beads leading to DNA hydrolysis. Therefore, exclusion of nucleases is necessary to protect encapsulated DNA.

Alginate is a polysaccharide composed of mannuronic (M) and guluronic acid (G) residues *(13,14)* and was used as the gel material for immobilizing DNA. Two bead formation techniques were used, involving internal and external calcium sources yielding homogeneous and heterogeneous gels, respectively *(15)*. To reduce bead porosity, various chitosan or poly-L-lysine membranes were formed on the alginate beads and DNA protection levels when exposed to DNase nuclease determined.

Intracapsular DNA was evaluated using a modified agarose gel electrophoresis technique as an extractive procedure that involves a direct separation and assay of the DNA from the bead. The extractive electrophoretic technique is a simple tool for the routine extraction and analysis of encapsulated DNA.

Materials and Methods

Alginates

Commercial samples of sodium alginate SG300 isolated from stipes of *Laminaria hyperborea* (designated high guluronic acid or high G) and S550 obtained from *Laminaria digitata* or *Ascophyllum nodosum* (designated as high mannuronic acid or high M) were obtained from Systems Bio-Industries (Mississauga, ON). The alginate composition was analyzed by ^{1}H-nuclear magnetic resonance spectroscopy and the average molecular weight by low-angle laser light scattering (LALLS) (*see* Table 1).

Table 1
Alginate and Polycationic Biopolymer Characteristics

Type	Lot number	Average mol wt (kDa)	Guluronic content (F_G)	Degree of deacetylation (%)	Degree of polymerization
Alginate	SG300	645.0	0.61	—	—
	S550	1000.0	0.30	—	—
Chitosan	060308G	378.0	—	87	—
	740447RG	495.0	—	97	—
	741037G	644.0	—	89	—
	720627G	1250.0	—	83	—
Poly-L-lysine	14H5526	2.7	—	—	13
	49F5560	19.5	—	—	93
	72H5536	197.1	—	—	943

Membrane-Coating Materials

Chitosan SeaCure 143 lot no. 060308G, 740447RG, and 720627G and chitosan Profloc 143 lot no. 741037G were donated by Pronova Biopolymers (Washington, DC). Degree of deacetylation was determined by ultraviolet (UV) and carbon/hydrogen/nitrogen elemental analysis and the average molecular weight by viscosity and LALLS, as shown in Table 1.

Poly-L-lysine hydrobromide of various average molecular weights and degrees of polymerization was purchased from Sigma (St. Louis, MO).

Bead Preparation

For the production of calcium alginate beads using the external gelation method, a solution of 2% (w/v) alginate and 0.02% (w/v) calf thymus DNA (highly polymerized) (Sigma) was extruded through a droplet generator (1000XL Automatic Liquid Dispenser; EFD, Montreal) using a 0.2-mm (internal diameter) syringe needle (EFD) at a pressure of 20 psi into a gelation bath consisting of 50 m*M* calcium chloride for 3 h. To produce beads using internal gelation, a solution of 2% alginate, 0.02% DNA, and 0.5% (w/v) calcium carbonate (sonicated for 30 min) was extruded through the droplet generator into canola oil containing 0.1% (v/v) glacial acetic acid and was reacted for 3 h. A 1% (v/v) Tween-80 in 50 m*M* calcium chloride solution was used for washing.

Membrane Coating

Forty alginate beads were placed in 2 mL of 0.4% (w/v) chitosan solution or 0.05% (w/v) poly-L-lysine solution, both at pH 6.0. The beads were agitated on an orbital shaker at 300 rpm for 1–3 and 0.2–1 h for chitosan and poly-L-lysine, respectively. The beads were then washed several times with distilled water and stored in 50 m*M* calcium chloride.

DNA Extraction and Analysis

DNA content within beads was analyzed by extraction and electrophoresis on a 2% (w/v) 25.4 × 29.0 cm agarose gel (Ultra Pure, electrophoresis grade; Gibco BRL, Burlington, Ontario) containing Tris-acetate EDTA (40 m*M* Tris base, 1.0 m*M* EDTA [pH 8.0]), 20 m*M* glacial acetic acid). A 16-well comb was used to form 0.4 × 0.1 cm wells to hold single beads. DNA-free beads and DNA markers (100 bp; Gibco BRL) were used as controls. For liquid samples, 10 µL of sample were initially mixed with 4 µL of loading buffer (50% v/v glycerol, 100 m*M* $Na_2EDTA \cdot 2H_2O$, 1% w/v sodium dodecyl sulfate, 0.1% w/v bromophenol blue) prior to well insertion. The electrophoresis unit (Model MPH; Interscience, Ontario) was operated at 100 V for 3 h with a Tris-acetate EDTA running buffer. Ethidium bromide (1 µg/mL) was used to stain the agarose gel for 15 min followed by a destaining with distilled water for 20 min. The gel slab was photographed under UV illumination.

Calf thymus DNA molecular size range was characterized using 1 to 2% (w/v) agarose electrophoresis with 100 bp and λ *Hind*III (Gibco BRL) DNA markers.

DNase Treatment

Ten beads were incubated with 200 µL of either low (1 µg/mL) or high (10 µg/mL) levels of pancreatic DNase I (Pharmacia Biotech, Montreal) solution for 0 to 1 h in an Eppendorf tube with continuous agitation on a Vortex mixer (level 3). After digestion, the supernatant was aspirated and the beads were washed with distilled water to eliminate DNase.

Results

Calf thymus DNA covers a wide range of molecular size, ranging from 66 to 15,173 kDa, as characterized through agarose electrophoresis (*see* Table 2). High molecular weight fragments (370–15,173 kDa) were separated in 1% agarose using λ *Hind*III DNA marker whereas 2% agarose and 100-bp DNA marker were used to characterize the lower molecular weight region (66–1359 kDa). Agarose electrophoresis was used to extract DNA from calcium alginate beads following encapsulation. The agarose gel along with the Tris-acetate EDTA buffer were optimized for high separation and resolution of DNA fragments.

Alginate beads were produced using either an internal or an external source of calcium, with both high G and low G alginates. Following encapsulation, the chromatogram of extracted DNA ranged from 394 to 15,173 kDa for internally and externally formed alginate gels, and both high G and low G alginates, suggesting loss of the lowest molecular weight fractions during encapsulation (66–395 kDa), as summarized in Table 2.

Following 1 h of low DNase (1 µg/mL) exposure, absence of extractable DNA demonstrated nuclease diffusion into the gel, resulting in DNA hydrolysis, as shown in Table 3. Nuclease with a mol wt of 31 kDa appears

Table 2
DNA Molecular Size Range Before and After Encapsulation Within Low G and High G Alginate

DNA type	Alginate (F_G)	DNA size range (kDa)
DNA solution	—	66–15,173
Encapsulated DNA	0.61	394–15,173
	0.30	394–15,173

Table 3
Level of Protection of Encapsulated DNA in Presence of Low Levels (1 µg/mL) of DNase[a]

Bead type	Alginate (F_G)	DNase reaction time (h)	DNA size range after DNase exposure (kDa)[b]
Internal gelation DNA beads	0.61	0.5	262–984
		1.0	ND[b]
	0.30	0.5	66–1358
		1.0	ND
External gelation DNA beads	0.61	0.5	ND
		1.0	ND
	0.30	0.5	ND
		1.0	ND

[a]DNA was subsequently extracted from beads and characterized by gel electrophoresis.
[b]ND, none detected.

able to permeate the porous gel bead and hydrolyze the DNA. With a shorter exposure time (0.5 h), extractable DNA was evident only in beads formed using the internal gelation method (Table 3). Extractable DNA showed evidence of hydrolysis owing to the absence of high molecular weight segments and the presence of low molecular weight DNA portions ranging in size from 66 to 1358 kDa.

Beads coated with chitosan were also exposed to low levels of DNase. Internally gelled and coated beads offered no protection from DNase, indicating that the beads were accessible to the nuclease (Table 4). Coated beads formed by external gelation provided a degree of protection since extractable DNA was present after low nuclease exposure. Coated high G alginate provided higher protection of DNA than did low G alginate. Nevertheless, appearance of low molecular weight fragments indicated hydrolysis in both high G and low G alginates. With low G alginate, the level of DNA protection was enhanced through increased membrane formation time; however, only low molecular weight fragments remained (<984 kDa).

Variation in chitosan membrane molecular weight and degree of deacetylation resulted in different levels of DNase exclusion from coated

Table 4
Degree of Protection of Encapsulated DNA
Within Chitosan-Coated Alginate Beads
in Presence of Low Levels (1 µg/mL) of DNase[a]

Bead type	Alginate (F_G)	Chitosan coating time (h)	DNA size range after DNase exposure (kDa)[b]	Protection level[c]
Internal gelation DNA beads	0.61	1.0	ND	– –
		3.0	ND	– –
	0.30	1.0	ND	– –
		3.0	ND	– –
External gelation DNA beads	0.61	1.0	66–15,173	+ +
		3.0	66–15,173	+ +
	0.30	1.0	ND	– –
		3.0	197–984	+

[a]DNA was extracted from beads following DNase exposure and characterized by gel electrophoresis.

[b]ND, none detected.

[c]The extracted DNA is compared to DNA in solution (control) (*see* Table 2). The protection level refers to a qualitative assessment of the extent of DNA hydrolysis. No double-stranded DNA detected (– –), DNA is detected in low amounts within the chromatogram (+), chromatogram contains both high (>1358 kDa) and low molecular weight DNA and the intensity is strong (+ +), and contains both high (>1358 kDa) and low molecular weight DNA and the chromatogram is very strong (+ + +) compared to controls.

beads depending on the DNase concentration level. For a low level of DNase exposure, a higher degree of protection was observed within high G alginate regardless of membrane coating time and chitosan type, as shown in Table 5. Coated low G alginate was more permeable to DNase as evident by extensive DNA hydrolysis. For high DNase exposure (10 µg/mL), no DNA was detected in coated high G alginates, suggesting that the membrane is permeable to the 31-kDa mol wt nuclease.

Poly-L-lysine was also used to coat alginate beads formed using the external gelation method. Results shown in Table 6 illustrate the size range of extracted DNA after low and high levels of DNase exposure. As poly-L-lysine molecular weight increased, a higher level of protection was evident for both high G and low G alginate. High G alginate offered better protection as shown by the DNA molecular weight size range. High molecular weight DNA (>1359 kDa) was extracted from high G (F_G 0.61) alginate coated with 197 kDa poly-L-lysine following exposure to both low and high concentrations of DNase.

Discussion

A standard horizontal agarose gel electrophoresis unit was adapted to electrophoretically extract and analyze encapsulated DNA. Previously, DNA content within coated alginate beads after in vivo testing required

Table 5
Extent of DNA Protection from Low DNase Exposure
of Chitosan-Coated Beads Formed Using External Gelation Method
as a Function of Chitosan Average Molecular Weight,
Degree of Deacetylation, and Coating Time[a]

Chitosan degree of deacetylation (%) and mol wt (kDa)	Alginate (F_G)	Chitosan coating time (h)	DNA size range after low[b] DNase exposure[c] (kDa)	Protection level
83%; 1250 kDa	0.61	1.0	66–15,173	+ + +
		3.0	66–15,173	+ + +
	0.30	1.0	197–787	+
		3.0	197–787	+
87%; 378 kDa	0.61	1.0	66–15,173	+ + +
		3.0	66–15,173	+ + +
	0.30	1.0	ND	– –
		3.0	197–984	+
89%; 644 kDa	0.61	1.0	131–1358	+ +
		3.0	131–1358	+ +
	0.30	1.0	ND	– –
		3.0	ND	– –
97%; 495 kDa	0.61	1.0	66–15,173	+ +
		3.0	66–15,173	+ +
	0.30	1.0	197–787	+
		3.0	197–787	+

[a]High DNase exposure resulted in no DNA detected for all the chitosan-coated beads listed.
[b]Low DNase concentration, 1 μg/mL.
[c]ND, none detected.

bead liquefaction in sodium citrate followed by membrane disruption and removal, and DNA precipitation and separation from residual alginate solute through size exclusion chromatography *(2)*. Extractive electrophoresis involves DNA extraction from beads by applying electrical potential combined with size fractionation in agarose gel. DNA with a negatively charged phosphate backbone will migrate toward the anode and separate along the agarose gel based on its fragment size. Small DNA fragments migrate faster, appearing farther down the gel. The concentration of agarose affects the porosity of the separation medium and thus determines the resolution of the electrophoresis unit. For partitioning low molecular weight DNA (100–2000 bp; 66–1359 kDa), a 2% agarose was adequate. The addition of EDTA to the Tris buffer inhibited nuclease activity during extraction and electrophoresis. Double-stranded DNA is visualized with ethidium bromide, which intercalates within stacked bases of the double-helical DNA and fluoresces under UV illumination. By comparison to DNA standards (i.e., 100 bp or λ *Hind*III), DNA size can be estimated.

In previous alginate-DNA separations using chromatography *(2)*, a weight ratio reduction from 20:1 to approx 2:1 was achieved, facilitating

Table 6
Extent of DNA Protection from Nuclease Hydrolysis
Within Poly-L-Lysine-Coated Beads as a Function
of Poly-L-Lysine Average Molecular Weight and Coating Time[a]

Poly-L-lysine mol wt (kDa)	Alginate (F_G)	Poly-L-lysine coating time (h)	DNA size range after low DNase exposure[b,c] (kDa)	DNA size range after high DNase exposure[d,e] (kDa)	Protection level
2.7	0.61	0.2	ND	ND	– –
		1.0	ND	ND	– –
	0.30	0.2	ND	NA	– –
		1.0	ND	NA	– –
19.5	0.61	0.2	66–1358	ND	+ +
		1.0	66–1358	ND	+ +
	0.30	0.2	ND	NA	– –
		1.0	ND	NA	– –
197.1	0.61	0.2	66–15,173	66–15,173	+ + +
		1.0	66–15,173	66–15,173	+ + +
	0.30	0.2	66–984	NA	+
		1.0	66–984	NA	+

[a]Alginates were formed using the external gelation method.
[b]Low refers to DNase concentration of 1 µg/mL.
[c]ND, none detected.
[d]High refers to DNase concentration of 10 µg/mL.
[e]NA, not available.

DNA quantification by absorbance. Alginate residuals remaining in the DNA fractions may be acceptable in some cases, but purity levels required for molecular biological assays (i.e., absorbance ratio 260/280 nm of 1.8) may be achieved by extractive electrophoresis. Pure DNA can also be recovered from the agarose gel *(16)*.

DNA chromatograms offered a qualitative assessment as to the extent of DNA hydrolysis. DNase hydrolyzes DNA randomly by forming single-stranded cuts in the presence of magnesium and calcium *(17)*. DNA extraction from beads exposed to DNase, followed by electrophoretic separation, indicated whether immobilized DNA was protected from nuclease diffusion and hydrolysis. The presence of extractable high molecular weight DNA (>1359 kDa) demonstrated that the bead offered protection, whereas the evolution of low molecular weight fragments (<394 kDa) indicated some degree of hydrolysis. The extractive electrophoretic protocol is therefore a simple, direct, and quick means of assessing DNA content within beads.

Electroporation is an analytical technique to transform cells genetically by using electrical potential to increase membrane porosity for facilitated insertion of chromosomal material *(18–20)*. By contrast, extractive electrophoresis draws DNA out from the artificial cell using electrical potential, combined with direct assay for molecular size.

There are two methods of forming calcium alginate beads, one requiring an external and the other an internal calcium source. Quong et al. *(15)* showed that the internal gelation technique produces beads with a more homogeneous calcium, alginate, and DNA distribution when compared with beads formed using the external calcium source. For the external gelation method, both the alginate and DNA migrate toward the droplet interface, where a high level of crosslinking occurs with the available calcium ions. For the internal gelation model, calcium in the form of nonsoluble microcrystals is homogeneously distributed internally within the sodium alginate solution, and gelation is triggered by a pH reduction from 7.0 to 6.5 *(21)*. Gelation of alginate is predominantly through guluronic acid residues owing to ionic interactions with divalent cations such as calcium *(22)*. Calf thymus DNA, ranging from 66 to 15,173 kDa in molecular weight, was successfully encapsulated using both formulation methods. Diffusional loss of low molecular weight DNA was owing to bead permeability, suggesting a molecular weight cutoff of 394 kDa for linear molecules in alginate beads. The lower molecular weight DNA (<394 kDa) was assumed to have been released during bead formulation. For globular molecules such as proteins, the molecular weight cutoff in alginate beads was found to be within a range of 69 *(23)* to 100 kDa *(24,25)*. Gelation of alginate beads is not instantaneous and requires saturation with calcium so that lower molecular weight DNA may diffuse out of the bead during gelation. DNA may be entrapped such that the strands are prevented from outward diffusion owing to its linear configuration in a lattice gel matrix. Short DNA strands diffuse out of porous alginate beads more readily than long DNA strands. Charge interactions between calcium cations and the anionic phosphate backbone of DNA may help increase retention of DNA within alginate gels similar to the affinity DNA has with hydroxylapatite *(26,27)*. Thus, a combination of charge interactions and gel permeability may determine the extent of DNA retention within calcium alginate beads.

Alginate beads formed by external and internal gelation, using high G and low G alginate were accessible to nucleases as seen by intracapsular DNA hydrolysis. At low DNase levels, higher DNA recovery was possible with alginate beads formed by internal gelation owing to the higher DNA concentration within the core. A lowered rate of DNase diffusion through higher alginate gradients was found for beads formed using external gelation; however, elevated levels of DNA near the bead periphery exposed more DNA to the inward diffusing nucleases, reducing the level of protection. DNase may more easily diffuse into internally formed gels with a lower alginate concentration profile, but the DNA is better protected within the gel core. Longo et al. *(28)* measured the permeability of proteases produced from *Bacillus subtilis* and *Serratia marcescens* through uncoated alginate beads and found that proteins of mol wt 35 and 25 kDa diffused out easily. Goosen et al. *(29)* and Huguet et al. *(30)* demonstrated the permeability of hemoglobin (mol wt 68 kDa) within poly-L-lysine-alginate microcapsules and chitosan-coated alginate beads, respectively. Coated gels of these types were permeable to proteins with mol wt <68 kDa.

At pH 7.0, DNase is negatively charged (p*I* 4.7; *[31]*), and thus subject to charge interactions within alginate gels. Attraction between the nuclease and positively charged calcium bound to nongelling mannuronic and guluronic acid residues may lead to facilitated diffusion within the alginate bead. The application of chitosan or poly-L-lysine membranes on high G alginate beads showed an increase in DNA protection from nuclease. The membranes formed by ionic interaction reduced bead permeability by creating smaller pores. During membrane coating, the chitosan or poly-L-lysine initially forms on the bead surface. Subsequent growth of the membrane is then dependent on diffusion of the polymer through the pores within the gel as well as polymer molecular weight. Goosen et al. *(29,32)* and King et al. *(33)* found that increasing poly-L-lysine concentration and reaction time along with reduced molecular weight increases bead strength owing to formation of thicker membranes. Poly-L-lysine and chitosan membranes combined with dense polymer packing near the bead periphery may be a method of reducing pore size, resulting in enhanced exclusion of DNase. Coated beads formed by external gelation were superior to coated beads formed using internal gelation because they exhibited higher DNA protection. Higher levels of DNA protection were also evident with higher molecular weight poly-L-lysine membranes. A 197.1 kDa poly-L-lysine membrane, coating beads formed by external gelation, provides effective exclusion of a 31 kDa nuclease.

In summary, it has been shown that electrophoretic extraction of DNA directly from beads was an effective technique for assaying intracapsular DNA and its distribution size range. Uncoated beads do not exclude DNase; however, both chitosan- and poly-L-lysine-coated beads protect DNA from DNase to some degree. The protection level from low levels of DNase showed that coated high G alginate beads have a higher DNase exclusion than high M alginate beads. When coating externally formed beads with poly-L-lysine, the level of DNA protection increases with poly-L-lysine molecular weight. Thus, the highest protection of DNA was achieved using high G alginate with a 197.1-kDa molecular weight poly-L-lysine membrane.

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