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Microencapsulation of DNA Within Alginate Microspheres and Crosslinked Chitosan Membranes for In Vivo Application

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ABSTRACT

Calf thymus DNA was microencapsulated within crosslinked chitosan membranes, or immobilized within chitosan-coated alginate microspheres. Microcapsules were prepared by interfacial polymerization of chitosan, and alginate microspheres formed by emulsification/internal gelation. Diameters ranged from 20 to 500 μm, depending on the formulation conditions. Encapsulated DNA was quantified *in situ* by direct spectrophotometry (260 nm) and ethidium bromide fluorimetry, and compared to DNA measurements on the fractions following disruption and dissolution of the microspheres. Approximately 84% of the DNA was released upon core dissolution and membrane disruption, with 12% membrane bound. The yield of encapsulation was 96%. Leakage of DNA from intact microspheres/capsules was not observed. DNA microcapsules and microspheres were recovered intact from rat feces following gavage and gastrointestinal transit. Higher recoveries (60%) and reduced shrinkage during transit were obtained with the alginate microspheres. DNA was recovered and purified from the microcapsules and microspheres by chromatography and differential precipitation with ethanol. This is the first

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report of microcapsules or microspheres containing biologically active material (DNA) being passed through the gastrointestinal tract, with the potential for substantial recovery.

Index Entries: DNA; encapsulation; alginate; chitosan.

INTRODUCTION

Environmental factors are currently believed responsible for 60–90% of human cancers (*1*). Natural or artificial components of food are important risk factors. Most carcinogens form a covalent complex with biological macromolecules, either directly or after metabolic activation to a chemically reactive form. Electrophiles form covalent bonds with nucleophiles such as DNA, potentially leading to cellular damage. Fecapentaenes, which are unstable direct acting mutagens, have been isolated from human feces (*2*). Analysis of food and feces cannot identify or quantify the formation of reactive compounds present in the stomach or intestinal tract or interactions with gastrointestinal cells.

The trapping of fecapentaenes or similar species within the intestinal lumen may be of interest for quantifying exposure. A trapping system must be stable during gastrointestinal (GI) transit, and recoverable from feces. Semipermeable, crosslinked nylon microcapsules containing polyethyleneimine (PEI) or polyvinylalcohol-triethylenetetramine (PVA-TETA) as DNA surrogates have been used (*3–10*) with coencapsulated magnetite to facilitate recovery of the microcapsules. *N*-methyl-*N*-nitro-sourea (*7*) and its electrophilic products (*8*) as well as metabolites of benzo[a]pyrene (*9*) were trapped within the GI tract.

PEI serves as a DNA surrogate by acting through its amine functions, although PEI binding may not be indicative of DNA damage. Many techniques have been described for the immobilization of cells and enzymes, yet little has been reported on that of DNA itself. DNA was entrapped in liposomes for genetic transformation of animals (*11*), and erythrocyte ghosts were filled with DNA by lysing and resealing (*12*). These procedures are limited by low yields of encapsulation and the products would not withstand GI transit.

DNA damage during microencapsulation may result from the formation of covalent adducts with the polymeric membane during polymerization, or owing to fragmentation resulting from shear during microbead formulation. Protective preentrapment of DNA within alginates or other polysaccharide gels prior to membrane formation should minimize membrane complexation. An encapsulating membrane can provide protection from hydrolytic intestinal enzymes while permitting diffusional assess to lower molecular weight carcinogens during gastrointestinal transit.

Chitosan, a water soluble polycation, will form a membrane on polyanionic calcium alginate beads (*13*) by displacing the calcium and forming chitosan-alginate crosslinks. Chitosan may also be crosslinked at an oil/water interface using an oil soluble reagent (*14*). The permeability of the encapsulating membrane may be controlled by its molecular weight, type of crosslinking agent, and degree of crosslinking.

The objective of the present study was to coimmobilize DNA and carbonyl iron powder within alginate microspheres and/or semipermeable membranes. Combined entrapment/microencapsulation techniques were adapted to provide a gentle, protective environment during encapsulation, and to avoid DNA fragmentation or incorporation into the encapsulating membrane. DNA microspheres and microcapsules were evaluated for the yield of encapsulation and the potential for in vivo application and recovery.

MATERIALS AND METHODS

Microencapsulation of DNA

For the preparation of chitosan-glutaraldehyde microcapsules, calf thymus DNA (0.2% w/v, Sigma, St. Louis, MO) was dispersed in an aqueous solution of 4% (w/v) chitosan (Protan, Portsmouth, NH), 2.8% (v/v) glacial acetic acid (Anachemia, Toronto, Ont.), and 0.74% (w/v) sodium acetate (J. J. Baker Chemical Co., Montreal, Que.) containing 5% (w/v) carbonyl iron powder (GAF, Wayne, NJ), then emulsified in sunflower oil (Sun Queen, Montreal, Que.) aided by 2% (v/v) Span 85 (Atkemix, Brantford, Ont.). The membrane was formed by interfacial polymerization (*15*) of chitosan following addition of glutaraldehyde (0.6 mL of 25% aqueous solution, Eastman, Rochester, NY) as crosslinking agent in 10 mL sunflower oil. The emulsion was formed in a 200-mL reaction vessel, operating at 200 rpm with a sheet lattice impeller (*16,17*) at a ratio of 1:5 aqueous solution to oil. The emulsification and reaction times were 2 and 3 min respectively, and the reaction was quenched by dilution with 100 mL of 25% Tween 20 (Sigma) solution. The supernatant solutions were removed by aspiration, and the microcapsules were washed several times with Tween 20 solution.

Chitosan-hexamethylene diisocyanate microcapsules were formed by dispersing 5 mL of an 0.1% (w/v) DNA solution into a solution of 8% chitosan, 5.6% acetic acid, 1.48% sodium acetate containing 5% carbonyl iron powder. The DNA-chitosan solution was dispersed in mineral oil (American Chemicals Ltd., Montreal, Que.), and the membrane formed by adding 500 μL hexamethylene diisocyanate (American Chemicals Ltd.) as crosslinking agent in 10 mL mineral oil. The emulsification and reaction times were 2 and 15 min, respectively.

Entrapment of DNA

DNA was immobilized in alginate microspheres by emulsification/internal gellation (*18*). The gelation mixture containing 0.08% DNA, 1.5% sodium alginate (Sanofi, Paris), 1% (w/v) calcium carbonate, and 0.6% carbonyl iron powder was dispersed in pure canola oil (Canada Packers, Montreal, Que.) containing 1% Span 80 (Sigma). The gelation mixture was emulsified within the organic solution (1:5) for 15 min at 300 rpm. Gelation was initiated by addition to 0.4% (v/v) glacial acetic acid (BDH, Toronto, Ont.) dissolved in a small aliquot of oil. After 5 min reaction, the DNA-alginate microspheres were partitioned into 125 mL of 0.05 m*M* calcium chloride (Anachemia) solution, the supernatant solutions were removed by aspiration and the microspheres washed with distilled water. DNA-alginate microspheres were added to 200 mL of 0.5% (w/v; pH 6) chitosan solution (Seacure 123 low viscosity chitosan, lot 10037RG supplied by Protan) for 60 minutes to form the chitosan membrane, then washed with distilled water. The membrane was crosslinked by suspending the microspheres in 200 mL of 0.02 mmol/mL glutaraldehyde or benzenetetracarboxylic dianhydride (Aldrich, Montreal, Que.) for 30 min, then washed and suspended in distilled water.

Quantification of Encapsulated DNA

DNA was measured directly in the microcapsule or microsphere suspension at 260 nm using a Varian CARY 2 double beam spectrophotometer. The reference cell contained microcapsules without DNA present.

DNA was also quantified by suspending 2 g of beads in 0.15 mg/mL ethidium bromide solution (Sigma) for 20 min. Samples were irradiated (900–200 nm) and the intensity of the fluorescent emission (530 nm for DNA-ethidium bromide) measured, perpendicular to the excitation light, using a Varian CARY 2 spectrophotometer with fluorescence accessory.

Size Distribution

Volumetric (volume within each diameter class) and cumulative size distributions of microspheres and microcapsules were obtained with a Malvern 2605 LC Particle Size Analyzer (Malvern Instruments, Malvern, England). This provides a mean value estimate of the diameter at 50% of the cumulative volume fraction (CVF). The mean diameter and arithmetic standard deviation were calculated from the cumulative volume distribution curve as described previously (*16*).

When iron powder interfered with the size analysis based on laser light diffraction (Malvern), microcapsules were sized microscopically using a graduated ocular. Diameter distributions were obtained by plotting relative frequency versus particle diameter, with the mean diameter obtained by $d = \Sigma n_i d_i / \Sigma n_i$, where n_i is the number of microcapsules with diameter d_i.

In Vivo Assays

A 1 mL suspension of microcapsules or microspheres was administered intragastrically to F355 rats that had previously been starved for 4 h. Separate metabolic cages permitted individual collection of feces for 48 h. The fecal material was diluted with 200 mL of 1% Tween 20 and 0.2% (w/v) sodium azide (Merck) as bacterial growth inhibitor. Microcapsules or microspheres were extracted by stirring gently with a rectangular magnetic plaque (Advanced Magnetics Inc.), and washed with deionized water.

Microcapsule/Sphere Disruption and DNA Recovery

A batch of chitosan-coated, alginate microspheres containing DNA was suspended in 200 mL of 1*M* (11%) calcium chloride for 12 h with shaking, washed with distilled water, filtered, and resuspended in 200 mL of 1% sodium citrate (pH 7) for 12 h to dissolve the ALG core. The membranes were broken by homogenization for 25 min at maximum speed with a tissue homogenizer (Tekmar tissumizer), or using a tissue grinder (Glas-col, 40 mL capacity) operating at 1500 rpm. Membrane debris was removed by centrifugation at 6000 rpm (5860*g*) for 20 min. The supernatant was eluted on a Sepharose CL-2B (Sigma) column (1.6 × 40 cm; packing to 30 cm), using phosphate buffer (pH 7). Fractions (3.0 mL) were scanned at 260, 280, and 320 nm and DNA quantified using the Warburg/Christian equation (*19*), and ALG determined colorimetrically following phenol reaction (*20*). Residual ALG in pooled DNA-rich fractions was precipitated with 1/4 to 1/3 vol ethanol.

Terminology

The term "microcapsule" is used to describe a membrane bound sphere with a liquid core, whereas the term "microsphere" is used for a gel bead. The prefix "micro" implies a submillimeter diameter range. The expression "coated microsphere" will be used to describe a particle consisting of a gel core enveloped within a membrane coat.

RESULTS

Calf thymus DNA was entrapped within alginate microspheres (DNA-ALG) by emulsification/internal gelation. Chitosan (CHT), a cationic polymer was applied to the polyanionic microspheres (DNA-ALG-CHT), and in some cases crosslinked with glutaraldehyde (DNA-ALG-CHT-GLU), hexamethylene diisocyanate (DNA-ALG-CHT-HDI) or benzene tetracarboxylic dianhydride (DNA-ALG-CHT-BCA). Chitosan, being water soluble, may also be crosslinked at an aqueous/oil interface, using an oil soluble

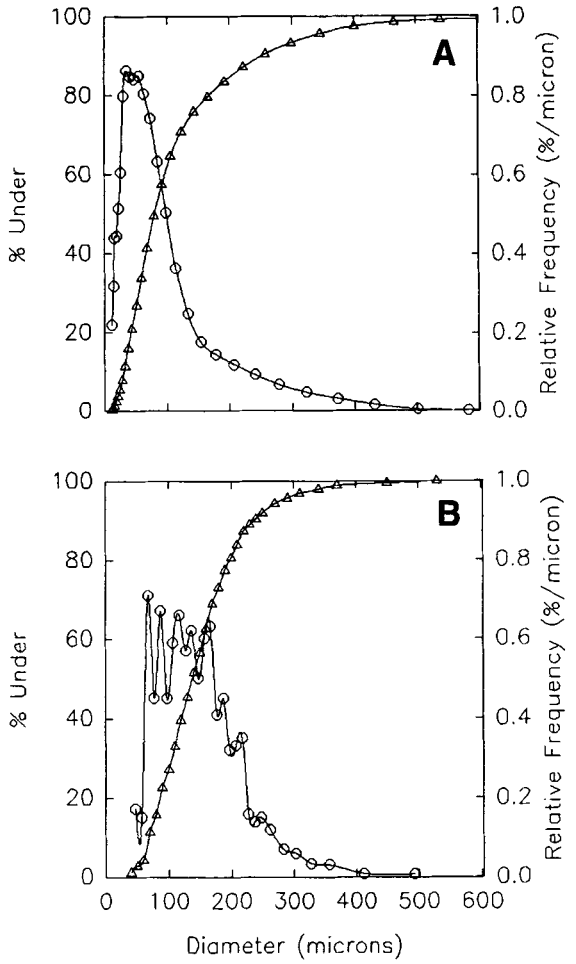

Fig. 1. Volumetric (○) and cumulative (Δ) size distributions of DNA-ALG microspheres produced by emulsification/internal gelation (**A**) and DNA-CHT-GLU microcapsules formed by interfacial polymerization (**B**). The microcapsule diameters (B) were measured microscopically owing to interference of the carbonyl iron powder when measuring by laser light diffraction. Fewer particles were measured (typically 400) resulting in the appearance of greater scatter in the data.

reagent. GLU and HDI were used to successfully form crosslinked, chitosan membrane-bound, microcapsules containing DNA (DNA-CHI-GLU or DNA-CHI-HDI). The mean diameter and size distribution of DNA microspheres and microcapsules were characterized by laser light diffraction. Typical size distributions are presented in Fig. 1. In general, the size distributions followed the log-normal law, with diameters ranging from 20–500 μm. The microspheres and microcapsules were spherical and clear, with carbonyl iron powder distributed within the microcapsule, as observed microscopically (Fig. 2). Dissolution of the internal alginate gel

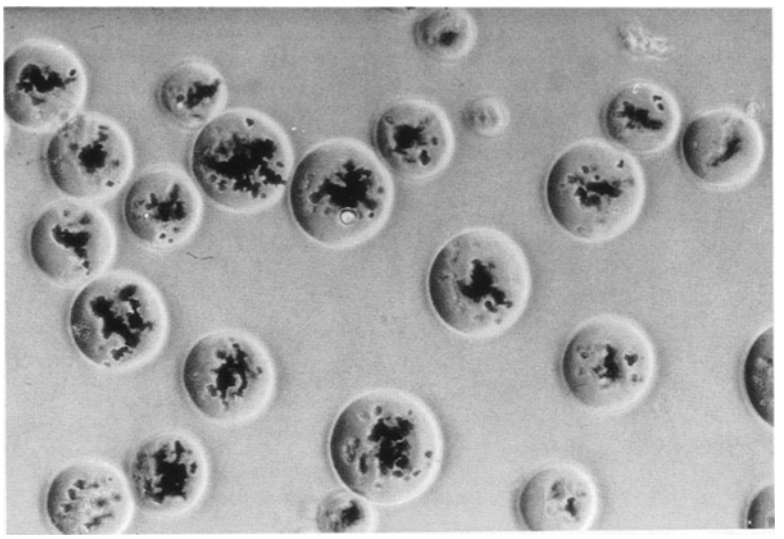

Fig. 2. Photomicrograph of ALG microspheres containing iron powder (dark spots) and DNA.

structure of the microspheres using sodium citrate, caused the iron particles to cluster and interact in a magnetic field. Treatment of the intact microcapsules and microspheres with ethidium bromide caused them fluoresce more brightly than the background, as expected for intercalation of the dye into double-stranded DNA. The capsules/spheres resisted acid treatment (pH 1.2, 2 h, 37°C), and could be collected from liquid suspension magnetically. Since GLU was both water and oil soluble, its use as a crosslinking agent was discontinued owing to possible damage to DNA within the microcapsule core, thus oil-soluble HDI was the reagent of choice.

Absorption spectra of DNA-ALG beads are illustrated in Fig. 3 with the spectrum of free DNA for comparison. The apparent disappearance of 210 nm absorption upon encapsulation may be owing to strong ALG end absorption in both beams not providing any detection of that due to DNA. Absorbance at 260 nm increased linearly with DNA concentration, thus was used to directly quantify the DNA, using blank beads in the reference cell. The extinction coefficients were 0.0109 and 0.0056 mL/μg, for DNA in solution, and in alginate beads, respectively. Differences in extinction coefficient may be owing to altered pH within the beads, compared to free solution.

The yield of encapsulation was also estimated by measuring fluorescence of encapsulated DNA, treated with ethidium bromide. The fluorescence emission spectra of DNA-ALG beads is illustrated in Fig. 4, with the peak at 530 nm dependent on the concentration of DNA within the

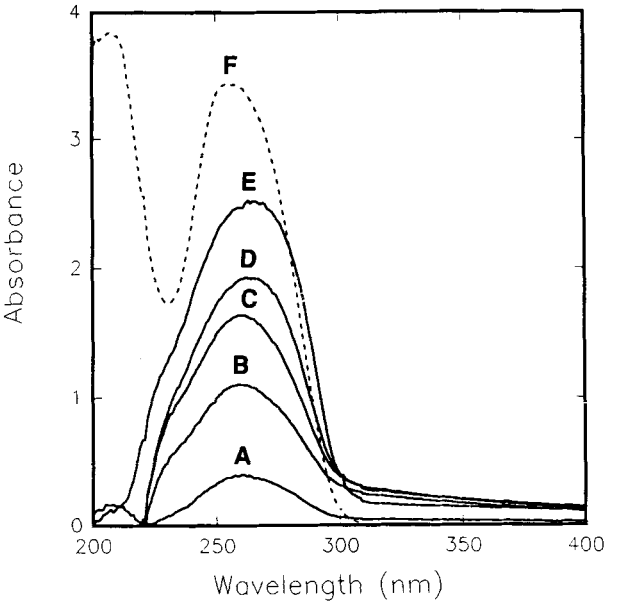

Fig. 3. Absorption spectra of ALG beads at DNA concentrations of 66.7 (A), 133 (B), 200 (C), 333 (D), and 1000 (E) μg/mL. ALG concentration for the preparation of the beads was 2% (w/v). Soluble non-encapsulated DNA absorbance (597 μg/mL) is included (f) for comparison.

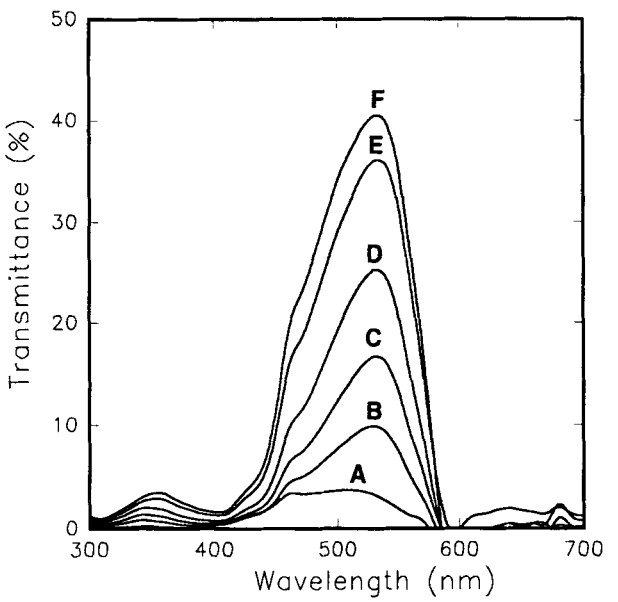

Fig. 4. Fluorescence emission spectra of ethidium bromide complexed with calf thymus DNA entrapped within ALG beads at concentrations of 0 (A), 66.7 (B), 133 (C), 267 (D), 530 (E) and 1200 μg/mL (F) DNA.

Table 1
Yield of Encapsulation of DNA
During DNA-ALG-CHI Entrapment[a]

Sample	DNA recovered, %
Supernatant following ALG microsphere formation	3
Chitosan solution following membrane formation	0.1
DNA recovered after bead dissolution	84
Membrane bound fraction	12
Total DNA recovered	99

[a]The data represents the fraction of DNA recovered, relative to the initial amount of DNA encapsulated. The CHI membrane coat was applied by immersing a 50-mL suspension of microspheres in 50 mL of 0.8% (w/v) chitosan for 40 min.

beads. A plateau within this region, observed with the control, was identical to that measured with a solution of ethidium bromide at a similar concentration. Visually, the ethidium bromide/DNA-ALG microspheres were pink colored, with a deepening of color observed with increasing DNA concentration. ALG microspheres without DNA were orange in color, similar to that of the dilute ethidium bromide solution. DNA was not observed in the supernatant solution, as measured by absorbance or fluorescence emission, indicating that the ALG microspheres fully retained encapsulated DNA, while permitting access to the lower molecular weight marker. The encapsulation yield was estimated to be greater than 80% of the initial charge of DNA.

The actual yield of encapsulation was determined following dissolution of the ALG core in sodium citrate and ultrasonic disruption of the CHI membrane. A DNA mass balance (Table 1), demonstrated 99% recovery of the initial DNA encapsulated. Losses were negligible as was diffusional loss of DNA from the microcapsules once formed (3%). Approximately 84% was released from the core upon dissolution and disruption, with 12% CHI membrane bound.

DNA-CHT-HDI microcapsules introduced to rats by gavage were seen to decrease in mean diameter from 325 to 96 μm after gastrointestinal (GI) transit, probably because of dewatering (Table 2). The recovered

Table 2
Change in Mean Diameter and Recovery of Microcapsules from Feces of Rats Following Gavage and GI Transit[a]

Microcapsule composition	Number administered	Mean diameter, μm, initial	Mean diameter, μm, after GI transit	Recovery %, after GI transit	Diameter redn., %, during transit
CHI-HDI	30,600	326	89	13	73
DNA-CHI-HDI	46,000	325	96	7	70

[a]Control consisted of microcapsules without DNA. Results represent the means of three rats (control) and seven rats (DNA-CHI-HDI microcapsules).

Table 3
Recovery and Change in Mean Diameter of ALG Microspheres Recovered Magnetically from Feces of Rats Following Gavage and GI Transit[a]

Microcapsule composition	Number administered	Initial mean diameter, μm	Mean diameter, μm, after GI transit	Recovery (%) after GI transit	Diameter redn. (%) during transit
ALG-CHI-BCD	152,000	182	104	31	43
ALG-CHI-BCD	n.d.	117	89	n.d.	24
DNA-ALG-CHI-BCD	158,000	161	120	32	25
DNA-ALG-CHI-BCD	654,000	126	92	59	27
DNA-ALG-CHI-GLU	181,300	105	98	38	7
DNA-ALG-CHI-GLU	667,000	77	64	58	17

[a]Controls did not contain DNA (n.d.: not determined).

microcapsules were dark and irregularly shaped, compared to the spherical, transparent microcapsules that were administered. Approximately 10% of the microcapsules administered by gavage were recovered magnetically from the feces, with losses probably because of strong adhesion of microcapsules to faecal detritus as reported previously (*8,21*).

A higher recovery of microcapsules resulted when the encapsulating membrane was formed around an ALG core as seen in Table 3. Magnetic recovery of the coated ALG microspheres from feces ranged from 26–59% of that administered by gavage, compared to 7–13% recovery of CHI microcapsules. Also the reduction in diameter of the ALG microspheres was less than that observed with CHI microcapsules following GI transit. Furthermore, the recovered crosslinked DNA-ALG-CHI microspheres were spherical in contrast to the irregularly shaped DNA-CHI microcapsules. The ALG core appears to confer enhanced strength and shape retention of the capsule, even when subject to a significant degree of dewatering.

DNA was recovered from coated ALG microspheres following dissolution of the alginate core in citrate and homogenization to disrupt the encapsulating membrane. DNA was purified chromatographically from alginate solution as seen in Fig. 5. Residual ALG remaining in pooled fractions 5 and 6 was removed by differential precipitation with ethanol.

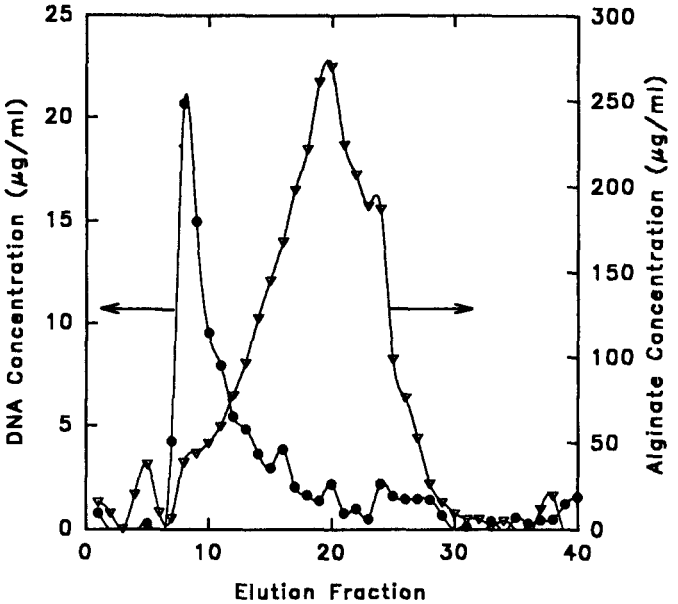

Fig. 5. Separation of DNA-ALG on sepharose column. DNA (○) was quantified at 260 nm and ALG (●) determined colorimetrically using phenol reagent.

DISCUSSION

DNA was coencapsulated with carbonyl iron, within CHI membranes or ALG microspheres coated with CHI membranes. A variety of crosslinking agents was examined, and techniques developed and optimized for each of the procedures. Encapsulation of DNA involving crosslinked CHI membranes is novel, and the procedures were developed specifically for in vivo application of DNA. GI transit and recovery of microcapsules or microspheres containing biologically active materials such as DNA has not been described previously. Application for in vivo trapping of carcinogens to the DNA target will be described in a separate report.

The function of the encapsulating membrane is to retain DNA within the core of the particle, protect DNA from hydrolytic enzymes within the intestinal tract, and provide diffusional access to lower molecular weight carcinogens. Coencapsulation of magnetic carbonyl iorn powder enabled recovery during washing and handling operations and following intestinal transit.

The crosslinking agents selected were oil soluble so as to minimize damage to DNA and to facilitate membrane formation on the bead or droplet surface. GLU formed strong membranes with CHI but was judged undesirable since it is also water soluble, and may thus gain access to the core of the microcapsule. Microscopic examination of CHI-GLU membranes revealed thin, smooth membranes demonstrating that membrane

formation occurred only at the interface, and not within the core of the microcapsule (data not shown).

The microspheres and microcapsules were formed following dispersion of the DNA solution within an oil phase. The emulsified droplets yield microcapsules or microspheres following interfacial membrane polymerization or internal gelation, respectively. Microencapsulation of DNA involves a single step operation while entrapment follows a two-step procedure of ALG microsphere formation, followed by the application and crosslinking of a chitosan membrane. The ALG core promoted retention of DNA, enhanced the strength to the particle, and minimized size reduction owing to dewatering during GI transit. The size distribution of microcapsules and microspheres typically follow a log-normal law, and the diameters were controlled by varying mixing speed and surfactant concentration during formulation, and by using alginates of different viscosities (*16,17*). For in vivo application, diameters ranging from 60–200 μm were desired.

Quantification of DNA following immobilization was possible by absorbance at 260 nm and fluorescence with ethidium bromide. The benefit of the procedures developed are that the encapsulated DNA could be directly measured. A similar procedure was developed to quantify microencapsulated urease within nylon membrane bound microcapsules (*22,23*). In the present study, the yield of encapsulation was 96%, with 12% of the DNA complexed with the membrane. Previous studies showed that nylon encapsulated macromolecules, such as PEI (*6*) and enzymes (*22*) are partially incorporated into the membrane, resulting in overall alteration of the membrane properties. Urease incorporation into nylon membranes resulted in a strengthening of the membrane, whereas microcapsules containing DNA were found to be more fragile than those in which DNA was absent (unpublished data). Differences in membrane strength were observed during sonication of the microcapsules, and in yield after GI transit. Weaker chitosan membranes may be owing to membrane irregularities caused by the incorporation of partially insoluble, high molecular weight DNA, in comparison to the incorporation of soluble proteins used in the previous study. The yield of encapsulation of urease within nylon membranes was 92% with 6% of the enzyme complexed to the membrane itself. Membrane-bound urease was enzymatically active, thus it is possible that membrane complexed DNA may be available to intestinal carcinogens. Only 3% of DNA entrapped within ALG was released from the microspheres in vitro. Thus, the presence of the encapsulating membrane may serve more as a protective barrier to the DNA, than a means to prevent release of the high molecular weight material. Microcapsules and microspheres were recovered intact following GI transit, though recovery of microcapsules was low. Shrinkage was observed due to the dewatering action of the colon, and the presence of an alginate core appeared to minimize shrinkage and provide for the improved retention of capsule

shape. The highest recovery of CHI coated microspheres following gavage and GI transit was approximately 60%.

The use of encapsulated DNA for in vivo application will require subsequent dissolution and disruption of the beads for DNA recovery. Citrate is often used to liquify and disrupt alginate beads by chelation of calcium from alginate complex. Encapsulating membranes may then be disrupted by sonication or homogenization, and the DNA separated from alginate solution by electrophoresis or chromatography. The later procedure was used successfully in the present study to purify the DNA.

The ability of encapsulated DNA to serve as a target for intestinal carcinogens serves as the basis for a subsequent report.

ACKNOWLEDGMENTS

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