MICROENCAPSULATION OF DNA WITHIN CROSS-LINKED CHITOSAN MEMBRANES

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ABSTRACT

DNA was microencapsulated by emulsification/interfacial polymerization within semi-permeable cross-linked chitosan membranes. Polar solvents and pH extremes were avoided during microencapsulation by using vegetable or mineral oil as the continuous phase and chitosan as the polymeric backbone. The membrane was cross-linked with glutaraldehyde or hexamethylene diisocyanate and microcapsules varied in diameter from 95 to 325 μm. DNA was visualized within the microcapsules with ethidium bromide stain. Binding of [^{14}C]methyl iodide and [^{14}C]benzo[a]pyrene by microcapsules was demonstrated in vitro and in vivo, respectively, although binding was mostly evident in the chitosan membranes. Magnetic recovery of the microcapsules from rat faeces following GI transit was facilitated by co-encapsulating magnetite. The microcapsule diameter decreased by 60-70% during GI transit due to dehydration in the colon and the recovery was approximately 10%.

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1.0 INTRODUCTION

1.1 CANCER

Environmental factors are currently believed to be responsible for 60-90% of human cancers[9] In particular, studies suggest that compounds found naturally or artificially in food are important risk factors for certain types of cancer Within the intestinal tract there exists a wide range of potentially toxic substances which may be a causative factor in cancer of the lower intestinal tract[6]. Hence, methods to quantify exposure levels to carcinogenic agents are being developed.

Most carcinogenic chemicals can undergo a covalent binding to biological macromolecules either by themselves or after metabolic activation to a chemically reactive form or an electrophile. Electrophiles react to form covalent bonds through the sharing of electron pairs from nucleophilic atoms. Binding to a biological nucleophilic macromolecule can lead to cellular damage, most directly if the target is DNA.

Fecapentaenes, which are unstable direct acting mutagens, have been isolated from human faeces[5]. However, analysis of foods or faeces cannot identify or quantify either the formation of reactive compounds present in the stomach or intestinal tract or their interaction with gastrointestinal cells[1,32]. As a result, the trapping of such species within the intestinal lumen is one possible method for quantifying human exposure.

Reports on the clinical uses of microcapsules as detoxifiers[4] led to the application

of this approach for investigating the in-situ formation of carcinogens within the intestinal tract of rodents.

1.2 MICROENCAPSULATION

Microcapsules are small (1μm to 1mm), membrane bound spheres. The encapsulating membrane may be composed of natural or synthetic polymers with varying thicknesses and degrees of permeability.

Early work in microencapsulation technology resulted in microcapsules with impermeable walls. Hence, the encapsulated material is released once the membrane is ruptured. Impermeable microcapsules were first used for carbonless copy paper[36] and later found applications in the cosmetics industry for perfumes[36], pharmaceutical industry for controlling the rate of drug release[15], and food industry for the protection of flavours and aromas in food[35]. The development of microcapsules with semi-permeable membranes resulted in medical applications as detoxicants[4].

The use of microcapsules for medical purposes introduced the concept of the artificial cell. Artificial cells are microcapsules with semi-permeable membranes containing aqueous solutions or suspensions of biologically active materials such as enzymes, proteins and detoxicants. The activity of the encapsulated material is not dependant on membrane rupture and release. While protected from the external environment, the core material acts on molecules which permeate into the artificial cells

1.2.1 Interfacial Polymerization

Interfacial polymerization is often favoured over several alternate microencapsulation techniques due to its simplicity, ability to control membrane properties and resultant membrane strength. The technique is based on a membrane polymerization reaction at the liquid/liquid interface consisting of the following steps[13]. An aqueous solution of the core material containing a water soluble reactant is dispersed within an organic fluid, facilitated by an emulsifier. Membrane polymerization on the surface of the dispersed aqueous droplets is initiated by the addition of a water-insoluble reactant to the emulsion. Following membrane formation, the microcapsules are separated from the organic phase and washed.

The main advantage of the interfacial polymerization technique is that membrane properties such as strength and permeability, can be modified by selecting appropriate combinations of monomers, reactants or cross-linking agents. However, one important limitation is that the process of emulsification typically yields a broad size distribution. Factors affecting the mean diameter and size distributions include the type of impeller used[22], agitation rate[12,22], concentration of emulsifier[12,22] and other factors such as temperature[31].

1.3 MICROENCAPSULATION IN CANCER RESEARCH

The microencapsulation of cellular macromolecules within semi-permeable membranes (artificial cells) may provide a mechanism capable of trapping reactive intermediates in the intestinal tract. A trapping system must be stable during transit through the intestinal tract, recoverable from the faeces, and permit recovery of the target from the microcapsule core. The semi-permeable membrane should allow reactive carcinogens to pass into the core with little hinderance, but exclude higher molecular weight molecules, such as hydrolytic enzymes which might destroy the target during gastrointestinal transit.

Semi-permeable, cross-linked nylon microcapsules containing polyethyleneimine (PEI) or polyvinylalcohol-triethylenetetramine (PVA/TETA) as DNA surrogates, have been investigated for gastrointestinal cancer research[19-21,25-30]. The microcapsules, rendered magnetic by the incorporation of magnetite, were developed for covalently trapping carcinogenic species within the gastrointestinal (GI) tract of rodents prior to the recovery of the microcapsules from the faeces[25]. It has been shown that these microcapsules are able to trap N-methyl-N-nitrosourea[27] and its electrophilic products[28], as well as metabolites of benzo[a]pyrene[29] within the GI tract. Several million microcapsules, together with the carcinogenic probes were administered intragastrically, thus presenting a high surface area for the diffusion of the various compounds. The carcinogen probes of low molecular weight permeated the membranes while molecules of higher molecular weight, such as enzymes

were excluded.

The control of the mean diameter and size distribution of the microcapsules was important in these studies. Microcapsules were to be sufficiently small to withstand passage through the gastrointestinal tract, yet large enough to avoid being trapped by the intestinal tissues and to facilitate recovery from the faeces. From the point of view of mass transfer, smaller microcapsules were preferred due to the larger specific surface area. The use of PEI, acting through its amine functions, as a DNA surrogate, presented some difficulties. Since PEI and DNA differ significantly in structure, carcinogen binding on PEI may not be indicative of possible DNA damage. It was also found that a significant portion of the core PEI was in fact being incorporated into the membrane during microcapsule formation[30].

Previous problems with PEI as a DNA surrogate, led to the notion of using DNA itself as a target. A collaboration was established between several laboratories, O'Neill (IARC, Lyon), Neufeld (McGill), Poncelet (McGill), Golding and Bleasdale (Guelph), with the general aim of developing non-invasive microencapsulated DNA for trapping DNA damaging agents. The intended application was the identification of gastrointestinal carcinogens and their dietary sources. The overall study is to precede and compliment anticipated human use in 1992.

Liposomes have been prepared containing DNA [33] and erythrocyte 'ghosts' have been filled with DNA by lysing and resealing them [34]. However, these procedures not only

resulted in low yield of encapsulation, but their product would not withstand gastrointestinal transit.

Hence, the present study involves the development and optimization of a technique for the microencapsulation of DNA by interfacial polymerization, providing a system capable of gastrointestinal transit and trapping of carcinogens therein.

2.0 OBJECTIVES

An interfacial polymerization technique will be used to microencapsulate DNA for trapping DNA-damaging agents within the gastrointestinal tract. The objectives of this study are as follows.

1. DNA is to be encapsulated within a cross-linked polymeric membrane. The microcapsules should be recoverable magnetically, resistant to an acidic environment and strong enough to withstand gastrointestinal transit.

2. The presence of DNA within the microcapsules will be confirmed using microphotographic techniques.

3. The carcinogen trapping ability of microcapsules will be tested in vitro and in vivo.

4. The degree of binding of a model carcinogen and recovery of microcapsules after transit through the gastrointestinal tract of rodents will be assessed.

3.0 MATERIALS AND METHODS

3.1 CROSS-LINKED POLYETHYLENEIMINE (PEI) MICROCAPSULES

3.1.1 Preparation of PEI microcapsules

Polyethyleneimine membranes were formed by a polycondensation reaction between the PEI (Aldrich, 50% in water) in a buffered aqueous solution at an initial pH of 8.0 to 9.5, and a di- or trichloride in cyclohexane (A&C Chemical), the organic solvent. The optimum procedure for PEI membrane formation involved emulsifying 50 ml cyclohexane containing 2% (v/v) Span 85 emulsifier (Atkemix), with 10 ml of a 5% (w/w) polyethyleneimine solution. Mixing in a 200 ml beaker with a sheet lattice type impeller[23] at 200 rpm for 2 minutes provided a stable emulsion. Membrane formation was then initiated at the droplet interface by adding 0.94 mmol sebacoyl chloride (Aldrich) in 10 ml cyclohexane. After 3 minutes, the reaction was stopped by dilution with 50 ml cyclohexane and mixed for 1 minute. Some experiments were performed in a system scaled up to 133% (235% in volume).

The suspension was then allowed to settle, the supernatant discarded, and the microcapsules rinsed with 50 ml cyclohexane. The transfer of microcapsules into the aqueous phase was achieved by dispersing the capsules in 50 ml Tween 20 (50% v/v) and gradually adding 250 ml of distilled water. The microcapsules were finally recovered on a buchner filter and rinsed several times with distilled water to remove traces of organic solvent and surfactant.

3.1.2 Membrane weight

Microcapsule membranes were isolated by sonicating (Artek Sonic 300 Dismembrator) to release the soluble core contents, and then washing with water to remove residual soluble PEI. Membrane fragments were filtered (Whatman no. 4), dried at 100 °C, and weighted. Mass of membrane reported is per batch of microcapsule preparation.

3.1.3 Measurements of pH

Bromothymol blue (Sigma), introduced prior to encapsulation, served as a pH probe. Titration showed that Bromothymol blue-PEI solution is blue at a pH higher than 7.6, green between 7.6 and 6.0 and yellow at pH less than 6.0.

3.2 CROSS-LINKED CHITOSAN MICROCAPSULES

DNA, being a highly reactive molecule, must be protected during the microencapsulation process. As a result, polar solvents and high or low pH levels were avoided. The following techniques were investigated.

3.2.1 Preparation of chitosan membranes cross-linked with glutaraldehyde (GA)

Chitosan membrane bound microcapsules were prepared by interfacial polymerization[7] Calf thymus DNA (0.2% w/v, Sigma) was suspended in an aqueous solution (pH 5 7) containing 4% (w/v) chitosan (Protan), 2.8% (v/v) glacial acetic acid (Anachemia), and 0.738% (w/v) sodium acetate (J.J. Baker Chemical Co., Phillipsburg, NJ) The DNA was homogenized in the chitosan solution for approximately 20 minutes to obtain a uniform suspension. 5% (w/v) carbonyl iron powder (GAF) was then added to the suspension. The organic phase consisted of 50 ml sunflower oil (Sun Queen) with 2% (v/v) Span 85 (Atkemix, Brantford, Ont.) as the emulsifier. The cross-linker was prepared by suspending 0.6 ml glutaraldehyde (25% in water, Aldrich) in 10 ml sunflower oil.

A cylindrical reaction vessel (200 ml) with a sheet lattice impeller[23], operating at 200 rpm, was used to emulsify the aqueous phase with the oil. The aqueous phase was added to the reactor followed by the organic phase, in a ratio of 1 to 5, producing a water in oil emulsion. After 2 minutes of emulsification the glutaraldehyde solution was added and reacted for 3 minutes. The reaction mixture was then diluted with 100 ml of an aqueous solution of 25% Tween 20 (Sigma). The microcapsules were then permitted to settle and the organic phase removed with a vacuum aspirator. The microcapsules were washed with Tween 20 several times to remove all traces of oil.

3.2.2 Preparation of chitosan membranes cross-linked with hexamethylene diisocyanate (HDI)

The formulation of chitosan-hexamethylene diisocyanate (chitosan-HDI) microcapsules is similar to that for preparing chitosan-glutaraldehyde (chitosan-GA) microcapsules. Differences involve the concentrations and types of reagents.

The aqueous phase consisted of 0.4% (w/v) DNA suspended in a solution containing 5% (w/v) chitosan, 3.5% (v/v) acetic acid, 0.738% (w/v) sodium acetate and 5% (w/v) carbonyl iron powder. Mineral oil (American Chemicals Ltd.) was used instead of sunflower oil as the organic phase and 500 μl hexamethylene diisocyanate (American Chemicals Ltd.) in 10 ml mineral oil replaced glutaraldehyde in the cross-linking phase. The emulsi-fication time was kept at 2 minutes, but the reaction time was increased to 15 minutes. After the reaction, 100 ml of 25% Tween 20 was added, the microcapsules were allowed to settle, and the organic phase was removed by vacuum aspiration. The chitosan-HDI microcapsules were then washed several times with Tween 20.

The chitosan-HDI microencapsulation procedure was altered during optimization. A concentrated chitosan solution was prepared, consisting of 8% (w/v) chitosan, 5.6% (v/v) acetic acid (MERCK), and 1.48% (w/v) sodium acetate. 5 ml of an aqueous 0.1% (w/v) DNA solution was added to 5 ml of an 8% chitosan solution. The precipitate was then homogenized for approximately 5 minutes with a homogenizer (Polytron) at setting number 6 or 7. 5% (w/v) carbonyl iron powder was then added. The procedures for the emul-

sification, reaction and washing steps were identical to those used previously.

3.3 IN VITRO EXPOSURE OF MICROCAPSULES TO [^{14}C]METHYL IODIDE

3.3.1 Binding of [^{14}C]methyl iodide

Microcapsule suspensions (3 ml) were incubated with 2.8 μCi (6.2 x 10^7 dpm) ([^{14}C]methyl iodide (sp. act. 55 Mci/mmol, Amersham) in 3 ml of ethanol (Cooperation pharmaceutique francaise) for 15.75 h at 37 °C. Microcapsules were then washed at least ten times with a 50:50 mixture of ethanol and deionized water to remove unbound radiolabel.

3.3.2 Determination of core-to-membrane ratio

Radiolabelled microcapsules were sonicated with an Ultrasonic probe (Bioblock Scientific) and total radioactivity determined on the homogenate. The membrane fragments were separated from the soluble core by centrifugation at 3500 rpm for 30 minutes. The radioactivity of each fraction was then measured.

3.4 IN VIVO EXPOSURE OF MICROCAPSULES TO [^{14}C]BENZO[a]PYRENE ([^{14}C]BaP)

3.4.1 Administration of microca, ıles and [^{14}C]BaP to rodents

F355 rats were obtained from a breeding colony at IARC (Lyon, France) and fed biscuits and tap water.

A 1 ml suspension of microcapsules was administered intragastrically with an animal feeding syringe needle (5 cm long; internal diameter 2.25 mm; Perfektum, Popper and Sons, Inc, NY) to rats starved for 4 hours, followed immediately with 1 ml of [^{14}C]BaP in Sunflower Oil (Casino) (15.8 μCi/ml or 34.8 x 10^6 dpm/ml). The rats were then placed in separate metabolic cages (SAFI type COD 1700; Suresnes, Paris) which allowed for the individual collection of faeces and urine and were given unlimited access to water and rat chow.

Ten rats were available for experimentation. The experimental conditions are presented in Table 3.1.

Table 3.1 Experimental conditions for in vivo study

Number of rats	Experimental Conditions
3 rats	Chitosan-HDI microcapsules and [^{14}C]BaP (no DNA)
3 rats	Chitosan-HDI-DNA microcapsules
4 rats	Chitosan-HDI-DNA microcapsules and [^{14}C]BaP

3.4.2 Extraction of microcapsules from faeces

The faecal and urine samples were collected 48 hours after the treatment of rats. Each faecal suspension was diluted with approximately 200 ml of an aqueous solution of 1% (v/v) Tween 20 and 0.2% (w/v) Natriumazid (Merck), a bacterial growth inhibitor. Uneaten feed that fell through the cage floor was removed to facilitate the extraction of microcapsules. The magnetic micro-capsules were then extracted from the faeces by the repeated gentle stirring of the suspension with a rectangular magnetic plaque (Advanced Magnetics Inc.). The magnet was withdrawn from the mixture and the attached microcapsules were transferred to a beaker by rinsing with a jet of deionized water. The microcapsules were further purified by two more extraction steps using a weaker magnetic bar. The radioactivity of the extracted microcapsules and the urine samples was then determined by

liquid scintillation counting.

3.5 DETERMINATION OF RADIOACTIVITY

Radioactivity was determined in a liquid scintillation spectrometer (Packard, Model Tri-Carb 453) All samples were diluted with 10 ml Biofluor (Dupont), a high efficiency emulsifier.

3.6 MICROCAPSULE SIZE DISTRIBUTIONS AND NUMBER COUNT

Distribution curves for microcapsules containing magnetite were determined microscopically by measuring the diameter of microcapsules with the aid of a graduated ocular. Size distributions were obtained by plotting relative frequencies versus particle diameters. The mean diameter was computed using the following equation.

$$d = \frac{\sum_i n_i d_i}{\sum_i n_i}$$

where n_i is the number of microcapsules having a diameter d_i.

The size distribution of microcapsules without magnetite (PEI microcapsules) were determined with a 2604 LC Particle Size Analyzer Malvern Instruments, Malvern, England),

using the volume distribution[24].

3.7 PHENOL EXTRACTION OF DNA

A phenol solution containing 1 kg phenol, 150 ml cresol, 1 g 8-hydroxyquinoleine and 110 ml water was kindly supplied by Dr. Mironov at IARC. The precipitate which formed following the addition of 100 μl 8% chitosan solution to 5 ml 0.1% DNA solution was contacted with 5 ml phenol solution for 3 hours. The supernatant (aqueous phase) was removed and treated with 1 volume of ether for 20 minutes to remove dissolved phenol.

To precipitate DNA for the aqueous solution, one tenth volume of 4M sodium acetate and 2.5 volumes of cold ethanol were added and held at -20 °C overnight.

3.8 MICROPHOTOGRAPHY

Microphotography was performed on microcapsules stained with ethidium bromide (Sigma). This compound binds specifically to double-stranded DNA resulting in fluorescence of DNA. A 1 ml microcapsule suspension was treated with 10 μl ethidium bromide aqueous solution (10 μg/ml). The suspensions of treated microcapsules were mixed by gentle shaking at room temperature for 1.5 hours. The microcapsules were then observed under a light microscope with fluorescence facility (Olympus Vanox microscope, Japan)

4.0 RESULTS

4.1 CROSS-LINKED PEI MICROCAPSULES

Spherical polyethyleneimine membrane bound microcapsules were formed with a mean diameter of 100 μm in a log-normal distribution (Figure 4.1) with a standard deviation of 64 μm. The smooth thin membranes appeared rigid when examined microscopically with micro-manipulators. An increase in the concentration of sebacoyl chloride (SC) from 0.47 to 1.4 mmol produced microcapsules which resisted washing and filtration (Table 4.1). Higher concentrations of terephthaloyl chloride (TC) were required in comparison to the SC for microcapsule formulation. The branching of the cross-linked PEI by use of a trifunctional cross-linking agent such as TMC both with or without SC, resulted in the formation of intact microcapsules with less rigid membranes.

Bromothymol blue was used to monitor pH during membrane formation. For an initial pH higher than 8.0, with 0.94 mmol of SC, the core pH remained above 6 during formulation, even in the absence of an acid buffer. When using TC and TMC, the pH reduction was more significant (Table 4.1), leading to pH values lower than 6.0 even when 0.45 M of tris was used as buffer.

Figure 4.1 **Size distribution of cross-linked PEI microcapsules (5% PEI, 0.94 mmol SC, pH 8.5, 200 rpm)**

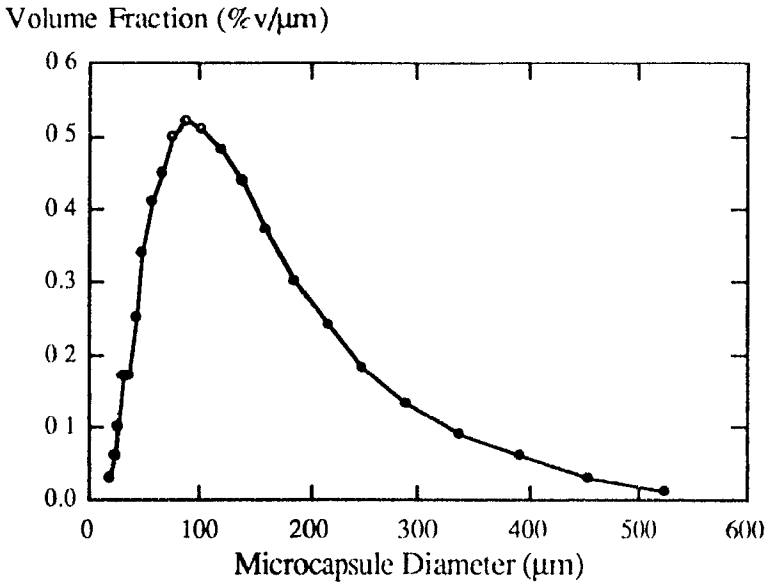


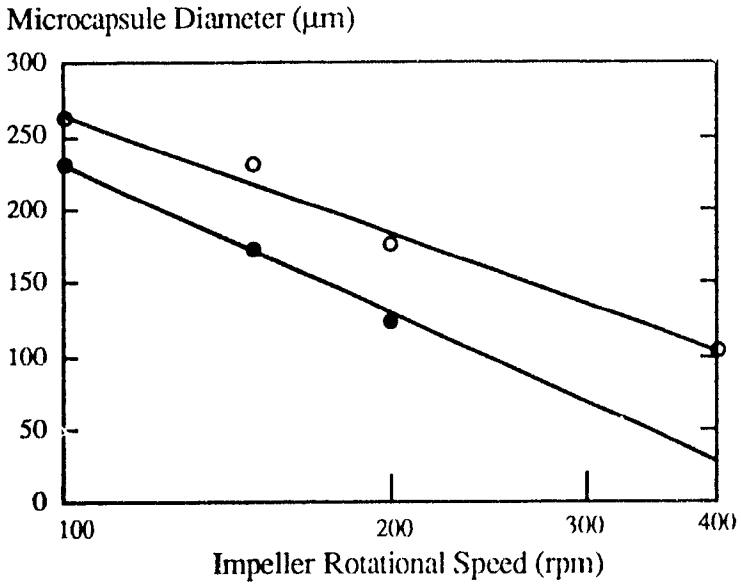
Table 4.1 Selection of a cross-linking agent for the preparation of PEI microcapsules

Amount of cross-linker (mmol)	Observations	pH	Classification*
Sebacoyl chloride			
0.47	many broken, spherical, pliable, smooth	>7.6	++
0.94	some broken, spherical, rigid	>7.6	+++
1.40	intact, spherical, rigid, strong	7.6	+++
Trimesoyl chloride			
0.38	many broken, fragile membrane	<6.0	–
0.76	many broken, non-spherical, fragile	<6.0	-
1.50	some broken, non-spherical, pliable	<6.0	+
3.20	some broken, spherical	7.6	++
Terephthaloyl chloride			
3.9	some broken, irregular	<6.0	+
2.0	aggregation, irregular	>7.6	+
Combination			
TMC 0.38 SC 0.47	some broken, spherical	6.0-7.6	+
TMC 0.38 SC 0.94	some broken, strong	7.6	++
TMC 0.76 SC 0.47	some broken, fragile, aggregation	-	++

* qualitative evaluation of microcapsules in degrees of desirable (+) or undesirable (-) qualities

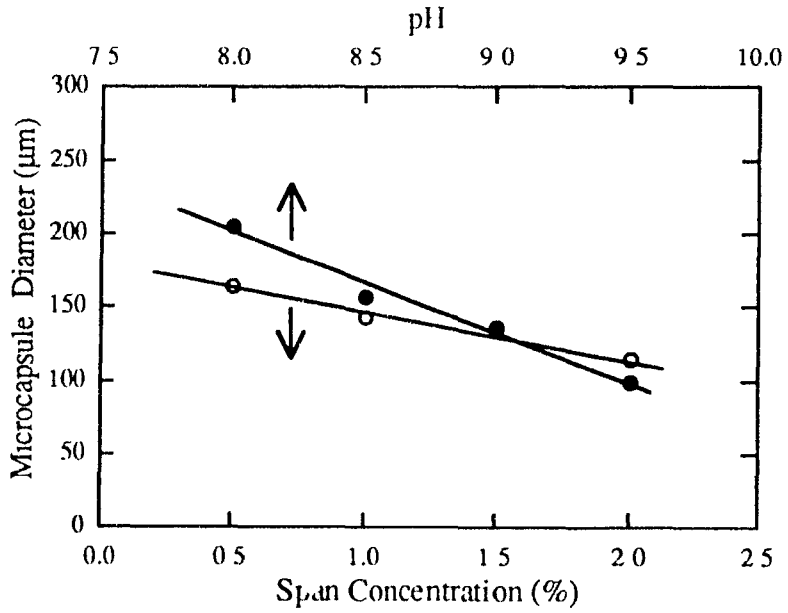
The mean diameter of the microcapsules decreased as the rotational speed of the impeller was increased as seen in Figure 4.2. The size was also affected by doubling the scale of the system (reactor, impeller, solution volume; Figure 4.2).

Figure 4.2 impact of impeller rotational speed and reactor scale on mean diameter of cross-linked PEI microcapsules (5% PEI, 0.94 mmol SC, pH 8.5, 200 rpm, rt = 3 min)



Furthermore, the microcapsule size decreased as the emulsifier concentration was increased (Figure 4 3). However, strong, intact and spherical microcapsules were formed only in the presence of an emulsifier at concentrations from 1 to 2%. At lower concentrations, weak membranes were obtained with a high proportion of ruptured capsules. At higher concentrations, microcapsules tended to aggregate and the membrane was weak and wrinkled. The size of the microcapsules decreased as the pH of the initial PEI solution was increased (Figure 4.3). The microcapsule size was not affected by the nature or concentration of the cross-linking agent.

Figure 4.3 **Impact of emulsifier concentration (Span 85) and pH on mean diameter of cross-linked PEI microcapsules (5% PEI, 0.94 mmol SC, pH 8.5, 200 rpm, rt =3 min)**



Figures 4.4 and 4.5, present the impact of reaction time as well as PEI and sebacoyl dichloride concentrations on the membrane mass. Membrane formation was primarily influenced by the concentration of cross-linking agent, (Figure 4.5) while both PEI concentration (Figure 4.5) and reaction time (Figure 4.4) appeared to have little effect on the membrane weight.

Figure 4.4 Impact of reaction time on membrane weight (5% PEI, 0.94 mmol SC, pH 8.5, 200 rpm)

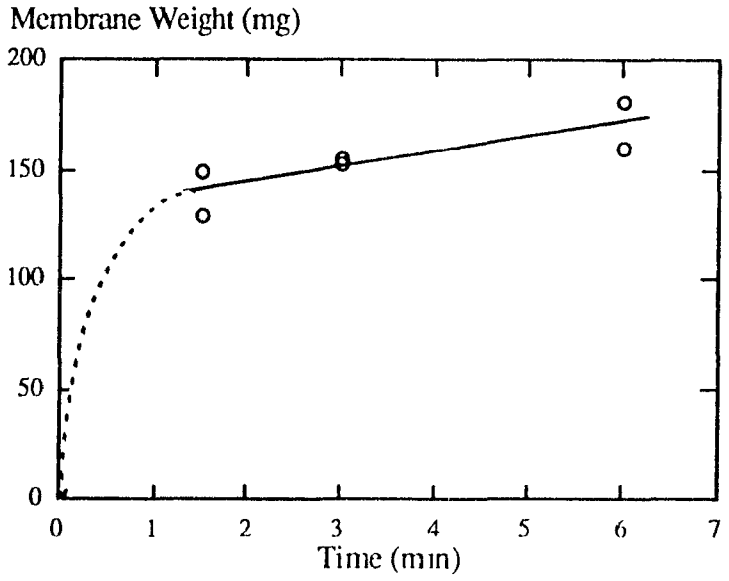
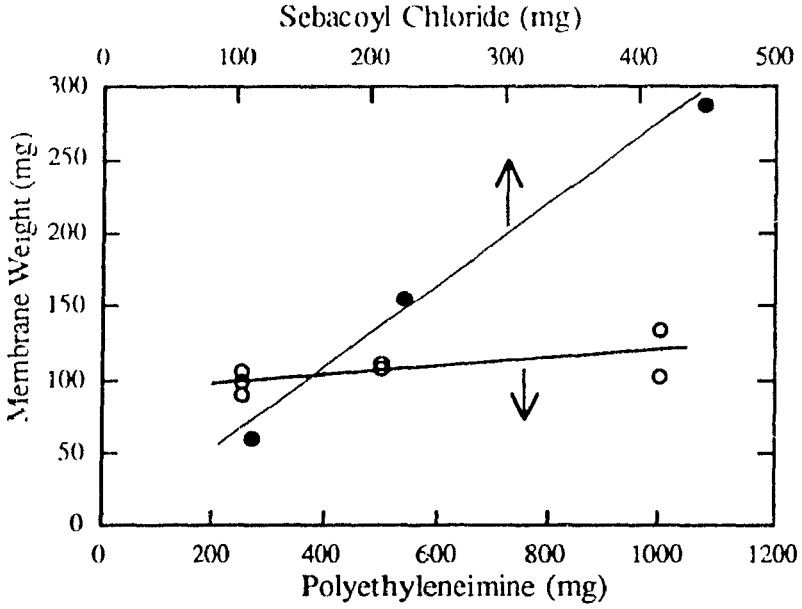


Figure 4.5 **Impact of SC and PEI concentrations on membrane weight (pH 8.5, 200 rpm, rt = 3 min)**



4.2 CROSS-LINKED CHITOSAN MICROCAPSULES

Cross-linked chitosan microcapsules were prepared by interfacial polymerization The suitable conditions for the dispersion of DNA and magnetite in chitosan solution and the optimum concentration of chitosan were determined as part of this study. Previous work[7] provided the optimum conditions with regard to the nature of the continuous phase, type and concentration of cross-linkers, and emulsification and reaction time. The optimized procedures were used for the preparation of chitosan-GA and chitosan-HDI microcapsules.

4.2.1 Dispersion of DNA in chitosan solution

Calf thymus DNA (5 mg) was suspended overnight in 5 ml of a 4% chitosan solution. Although the solubility of DNA in water is 0.1% (w/v), the DNA remained in suspension. The addition of salts (0.2-1M NaCl) or increasing the pH from 5.0 to 6.4 did not facilitate the dissolution of DNA.

The chitosan solution was acidified with phosphoric acid to prevent the precipitation of DNA by adding excess phosphate ions. In addition to the variation of pH, the concentrations of chitosan and DNA were varied from 2% to 4% and 0.025 to 0.05%, respectively. The results in Table 4.2 show that DNA was only soluble in very acidic solutions (pH 1.1), at a pH which is not compatible with membrane formation or possibly the DNA itself.

The precipitate was washed several times and resuspended in distilled water.

Although pure DNA dissolves in distilled water, the precipitate was insoluble, suggesting that it may result from the formation of a DNA-chitosan complex. Several attempts were made to break apart the DNA-chitosan complex using concentrated phenol. However, it was found that no DNA was present in the aqueous phase after the extraction step and the precipitate remained unchanged in the phenol solution.

Table 4.2 Effect of phosphoric acid addition to chitosan solution

pH	Percent Chitosan	Percent DNA	OBSERVATIONS after addition of DNA
5.5	4	0.05	precipitate
5.1	3	0.05	precipitate
4.0	2	0.05	precipitate
3.4	2	0.05	precipitate
1.1	4	0.025	no precipitate
1.1	4	0.035	no precipitate

DNA was finally dispersed in chitosan solution by homogenizing (24 000 rpm) the DNA/chitosan mixture for 30 minutes at 10 minute intervals, so as to minimize damage to the DNA due to an increase in temperature. The procedure was then optimized by introducing a 0.1% (w/v) aqueous solution of DNA to an equal volume of an 8% chitosan

solution. The precipitate which formed was easily dispersed by 5 minutes of homogenizing at a lower speed.

4.2.2 Characterization of cross-linked chitosan microcapsules

Spherical, magnetic microcapsules with and without DNA were prepared using glutaraldehyde or hexamethylene diisocyanate as cross-linking agents. Chitosan-GA microcapsules were prepared by dispersing 20 mg of DNA in chitosan solution. In contrast, 5 mg DNA (in suspension) were dispersed in chitosan solution according to the optimized procedure for the preparation of chitosan-HDI microcapsules.

Figures 4.8 and 4.9 show the linear size distributions of DNA microcapsules, as well as control microcapsules without incorporated DNA. Table 4.3 presents the mean diameters and microcapsule number concentration for all types of microcapsules. Chitosan-GA microcapsules were used in the in vitro studies, whereas chitosan-HDI microcapsules were also used in the in vivo experiments with rats. An attempt was made to obtain an appropriate size range of chitosan-HDI microcapsules by sieving. Capsules with diameter less than 50 μm were unacceptable because of possible trapping within intestinal tissues. Diameters greater than 400 μm were also undesirable due to probable breakage during gavage of the rats. Figure 4.9 shows that approximately 30% of the microcapsules were greater than 400 μm after several sieving operations,indicating that sieving of large microcapsule batches was inefficient.

As seen in Table 4.3 the incorporation of DNA only affected the size of chitosan-GA microcapsules. The mean diameter of chitosan-HDI microcapsules remained unchanged. This may be explained by the fact that the insoluble DNA aggregates were sufficiently small to be suspended in the 325 μm aqueous droplets (chitosan-HDI), whereas they forced an increase in diameter of the 95 μm droplets (chitosan-GA) during emulsification.

Figure 4.8 Size distribution of control and DNA chitosan-GA microcapsules

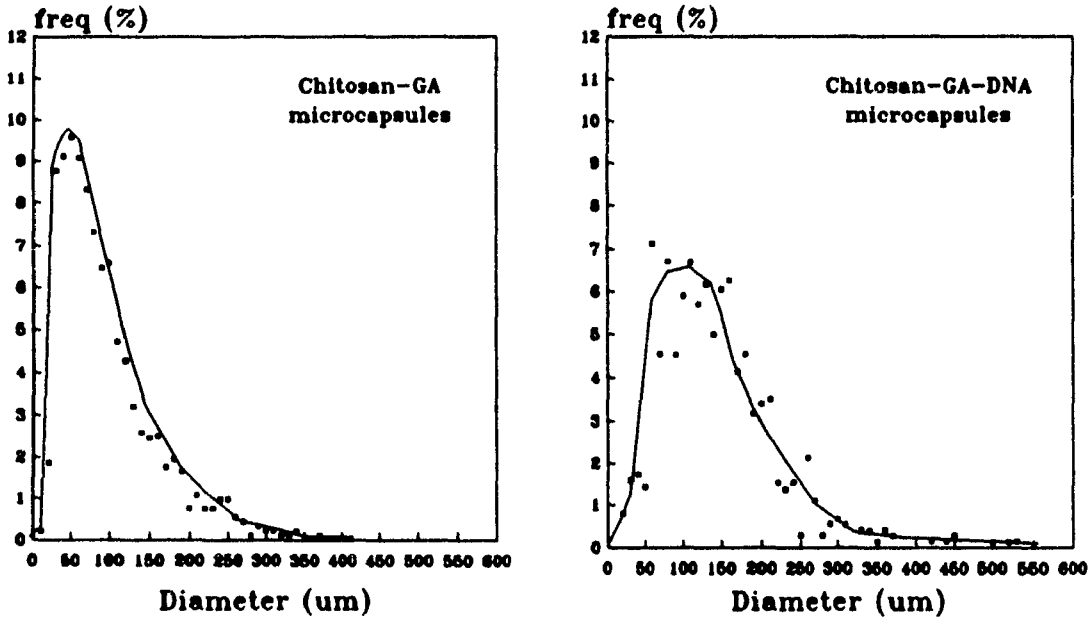


Figure 4.9 Size distribution of control and DNA chitosan-HDI microcapsules

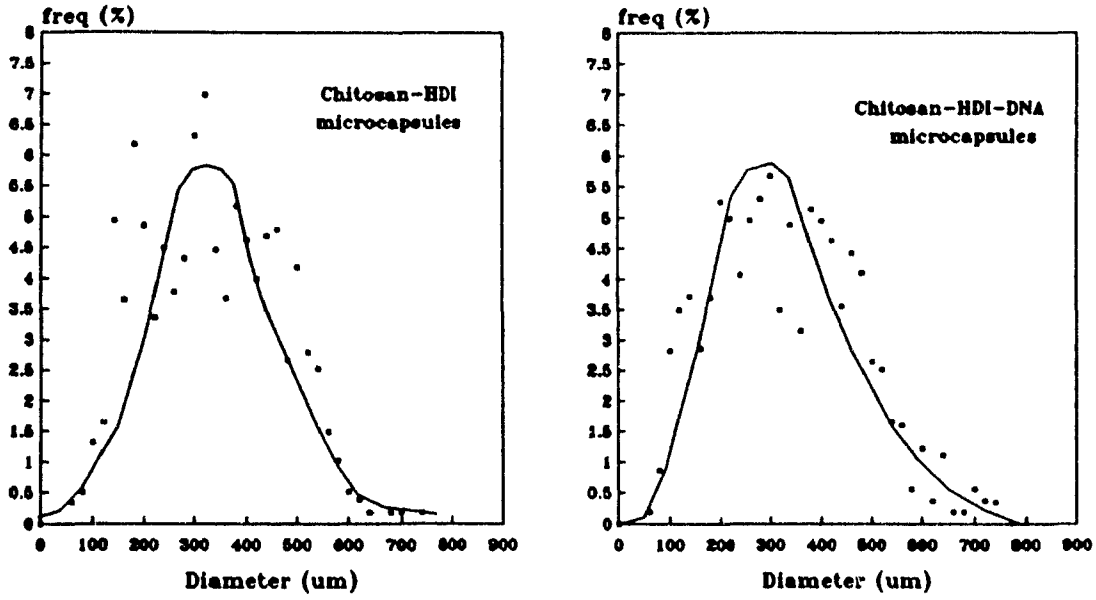


Table 4.3 Size analysis and number concentration of chitosan microcapsules

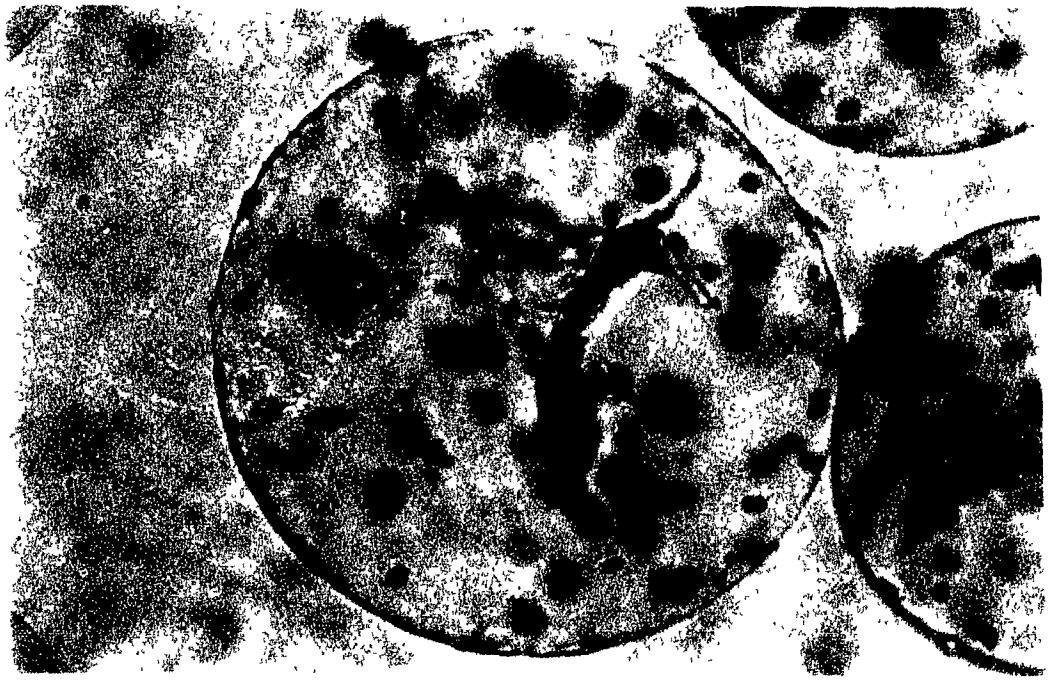
Microcapsule Sample	Mean Diameter (μm)	Microcapsule Concentration (μcaps/ml)	μg DNA per thousand microcapsules
Chitosan-HDI	326	203 600	0
Chitosan-HDI-DNA	325	181 200	5.5
Chitosan-GA	95	30 600	0
Chitosan-GA-DNA	144	46 000	7.2

A micrograph of chitosan-GA-DNA microcapsules is presented in Figure 4.10. Carbonyl iron powder or magnetite is seen in the microcapsules as dark specs, as well as insoluble DNA.

The microcapsules contained sufficient magnetite (5% w/v), to be recovered with magnetic plaques and bars. Furthermore, the microcapsules resisted acid (HCl, pH 1.2, 2 hours, 37 °C) treatment and could be disrupted by homogenization at high speed.

When increasing the concentration of DNA to be encapsulated, it was observed that magnetite was being excluded from the microcapsules. Since excluded magnetite may be harmful to the animals because of its small size (<40 μm), it was eliminated by sieving. The sieving of small diameter particles was satisfactory.

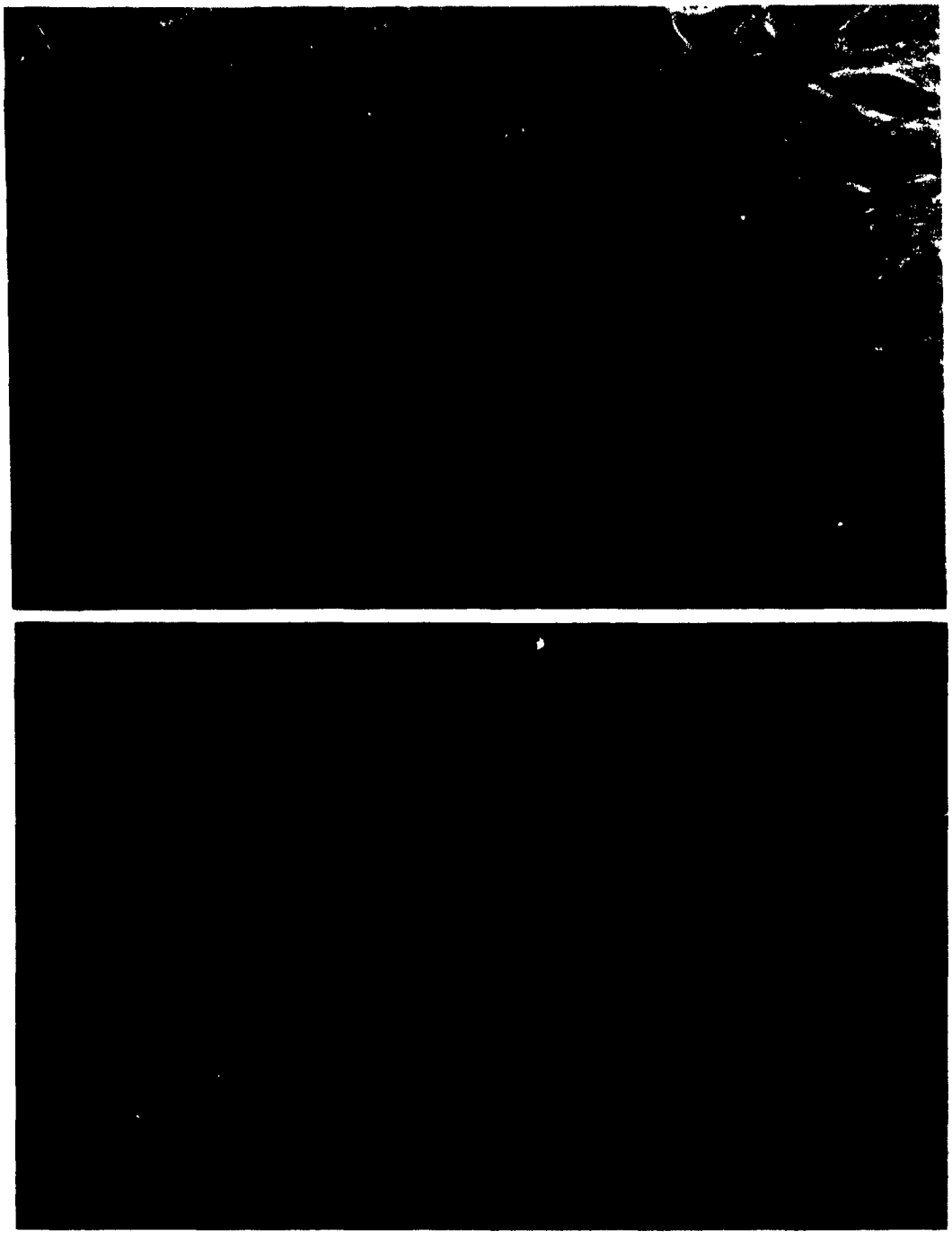
Figure 4.10 **Micrograph of chitosan-GA-DNA microcapsules under light microscope**



4.2.3 Detection of DNA

Ethidium bromide was used as a marker to verify the presence of DNA in chitosan microcapsules. This dye has a molecular weight of 394, is a carcinogen, and fluoresces when complexed with double-stranded DNA. Figure 4.11 is a micrograph of calf thymus DNA treated with ethidium bromide in which fluorescence indicates the presence of DNA.

Figure 4.11 **Micrograph of calf thymus DNA under light microscope and fluorescence**



Figures 4.12 and 4.13 are micrographs of control and DNA-containing chitosan-HDI microcapsules under light and fluorescent microscopy. Only chitosan-HDI-DNA microcapsules containing DNA showed fluorescence, as is seen in Figure 4.13. The degree of fluorescence varied from one microcapsule to another, with a few not exhibiting fluorescence at all. Fluorescence was observed throughout the entire volume of the microcapsule, suggesting that some DNA was in solution. Some microcapsules also contained fibrous strands which fluoresced strongly.

Figure 4.12 **Chitosan-HDI microcapsules under light microscope and fluorescence**

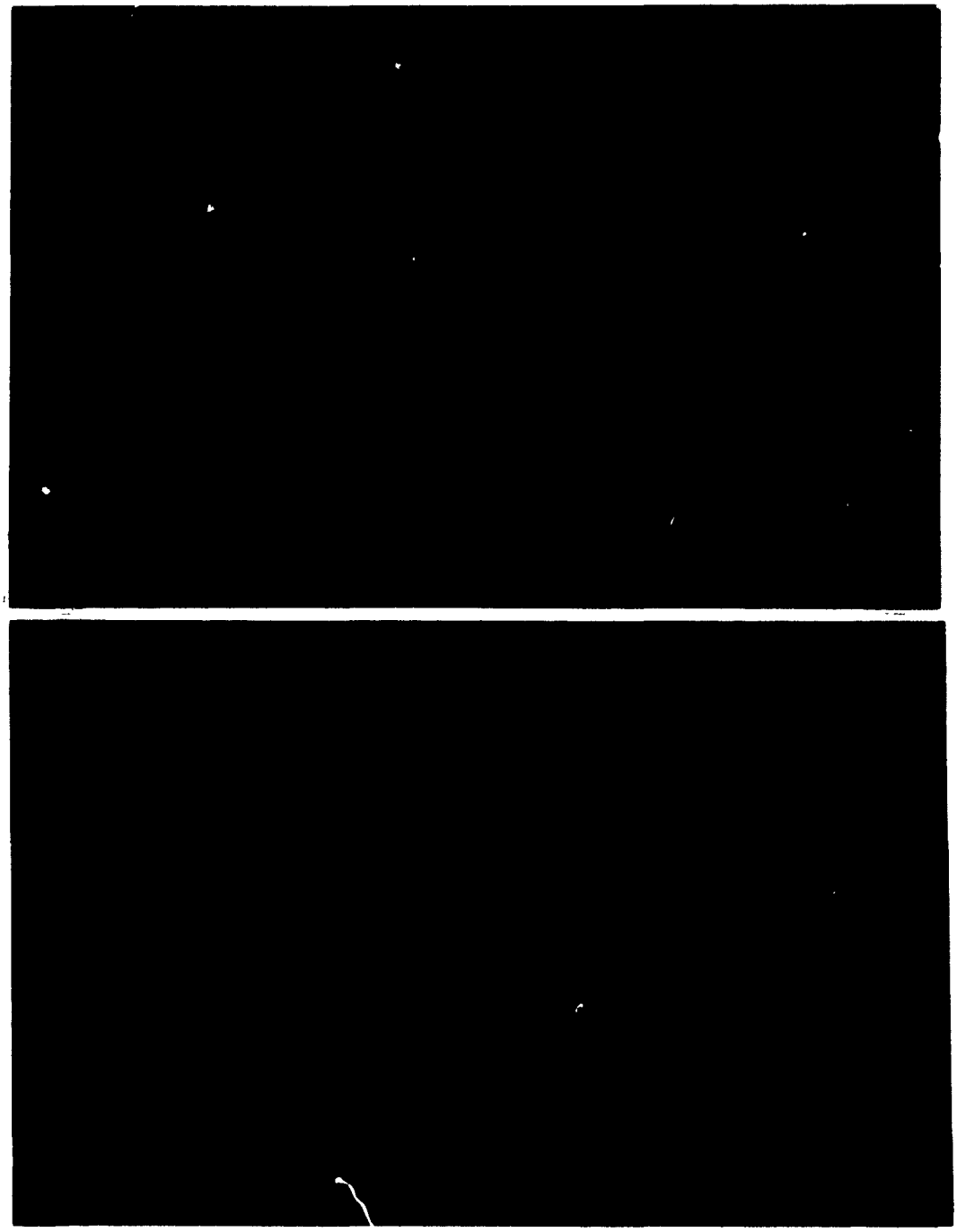
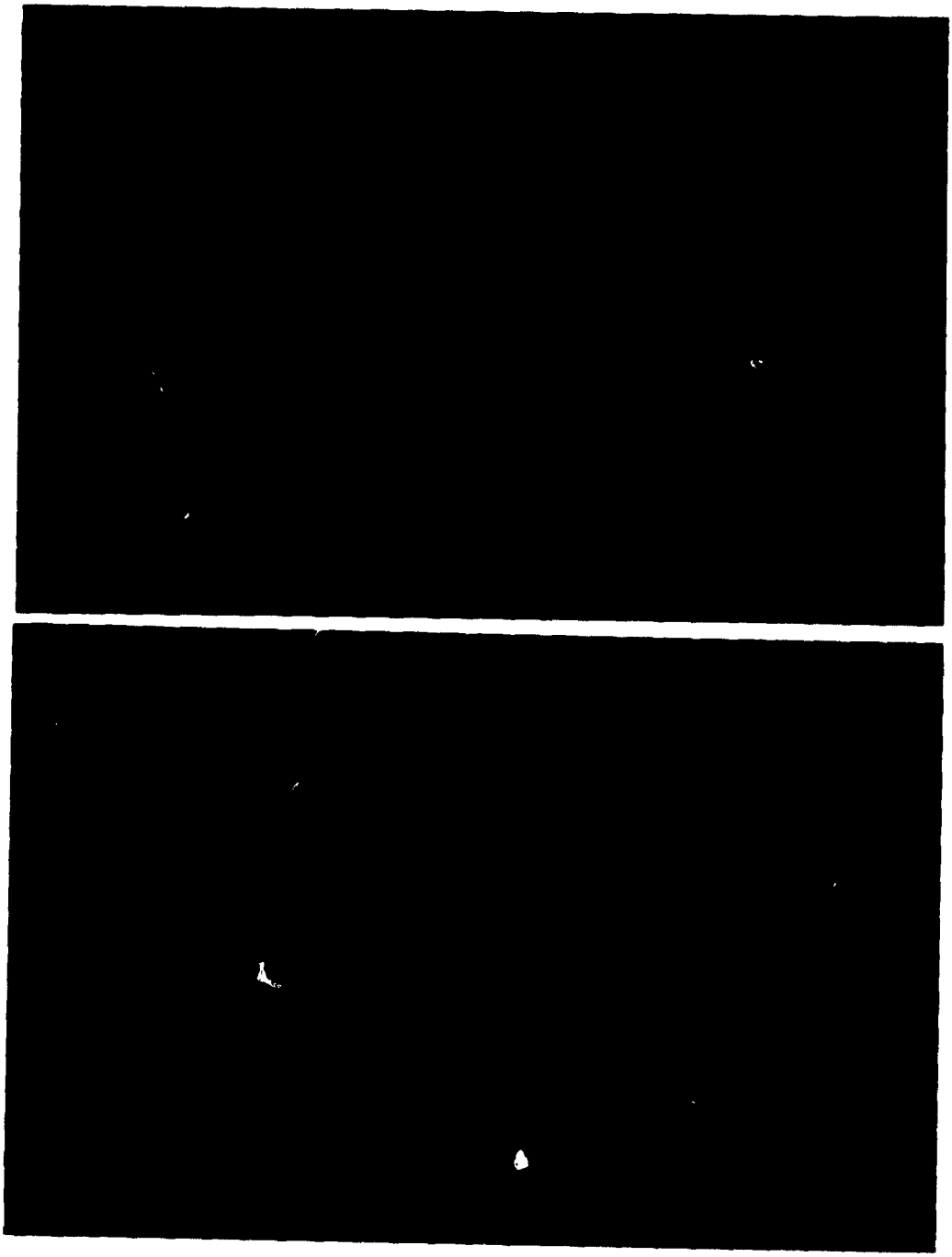


Figure 4.13 Chitosan-HDI-DNA microcapsules under light microscope and fluorescence



4.3 IN VITRO EXPOSURE OF MICROCAPSULES TO [^{14}C]METHYL IODIDE

The ability of DNA microcapsules to trap methyl iodide in vitro is an indication of the ability to trap carcinogens in vivo.

4.3.1 Binding per thousand microcapsules

The results of [^{14}C]methyl iodide contacting experiments are summarized in Table 4.4. Chitosan microcapsules cross-linked with hexamethylene diisocyanate or glutaraldehyde were contacted with [^{14}C]methyl iodide. Chitosan-HDI microcapsules containing DNA trapped approximately the same quantity of [^{14}C]methyl iodide as controls, 65 and 55 dpm/thousand microcapsules, respectively. When glutaraldehyde was used as the cross-linking agent, the microcapsules containing DNA showed twice the binding of [^{14}C]methyl iodide (380 dpm/thousand microcapsules) as control chitosan-GA microcapsules (190 dpm/thousand microcapsules). In addition, chitosan-GA microcapsules showed greater binding per thousand microcapsules than chitosan-HDI microcapsules.

4.3.2 Core-to-membrane ratio

The core-to-membrane ratio is defined as the ratio of [^{14}C]methyl iodide bound to the core material to that bound to the membrane. The core-to-membrane ratio of chitosan-HDI microcapsules containing DNA was 0.348, indicating that 25.8% of the trapped [^{14}C]methyl iodide was found inside the capsules. In the case of the control chitosan-HDI microcap-

sules, the core-to-membrane ratio was 0.022 resulting in only a 2.2% recovery of the radiolabel in the core. Although this result suggests that the radiolabel enters the DNA-containing microcapsules more readily than the control microcapsules, three points must be considered. Firstly, the control microcapsules were difficult to break, as can be seen in Table 4.5, resulting in an inadequate release of the core material for radioactive counting. Secondly, incorporation of undissolved DNA into the membrane may have resulted in membrane irregularities, facilitating penetration of [^{14}C]methyl iodide. Finally, the core fraction also contained insoluble DNA, which was separated with the membrane fraction during centrifugation. [^{14}C]methyl iodide bound to insoluble DNA would result in a lower core-to membrane ratio.

For the chitosan-GA microcapsules, the radiolabel was found mostly on the membrane, as can be seen from the low core-to-membrane ratios for both control and DNA containing microcapsules. Although Table 4.5 shows that DNA-containing microcapsules were easier to break than control microcapsules, both control and DNA chitosan-GA microcapsules were sufficiently broken for the determination of core-to-membrane ratio.

The choice of cross-linker not only has an effect on the total binding per thousand microcapsules but also on the core-to-membrane ratio. The core-to-membrane ratio of bound [^{14}C]methyl iodide is higher for chitosan microcapsules cross-linked with hexamethylene diisocyanate than for those cross-linked with glutaraldehyde.

4.3.3 Binding per milligram DNA

The quantity of [^{14}C]methyl iodide per milligram of DNA was calculated, assuming that all of the DNA was encapsulated during the microencapsulation procedures. Results show that 7 600 dpm [^{14}C]methyl iodide per mg DNA was trapped for chitosan-HDI microcapsules, while 68 900 dpm/mg DNA was obtained for chitosan-GA microcapsules. These values, however, do not represent direct binding to DNA, since the chitosan membranes play a significant role in the binding of [^{14}C]methyl iodide.

Table 4.4 In vitro binding of [^{14}C]methyl iodide by chitosan microcapsules

Sample	Radioactivity per thousand Micro-capsules (dpm)	Core-to-Membrane Ratio	Radio-activity per mg DNA (dpm/mg)
Chitosan-HDI microcapsules	65	0.022	N/A
Chitosan-HDI-DNA microcapsules	55	0.348	7 600
Chitosan-GA microcapsules	190	0.006	N/A
Chitosan-GA-DNA microcapsules	380	0.003	68 900

N/A not applicable

Table 4.5 Observations after sonication of chitosan microcapsules

Sample	Setting	Time (50% cycle) (s)	Observations
Chitosan-HDI microcapsules	moderate high	120 120	very few broken (20%) very few broken (20%)
Chitosan-HDI-DNA microcapsules	moderate	120	all broken
Chitosan-GA microcapsules	moderate high	240 240	some broken (40%) most broken (80%)
Chitosan-GA-DNA microcapsules	moderate high	240 240	almost all broken almost all broken

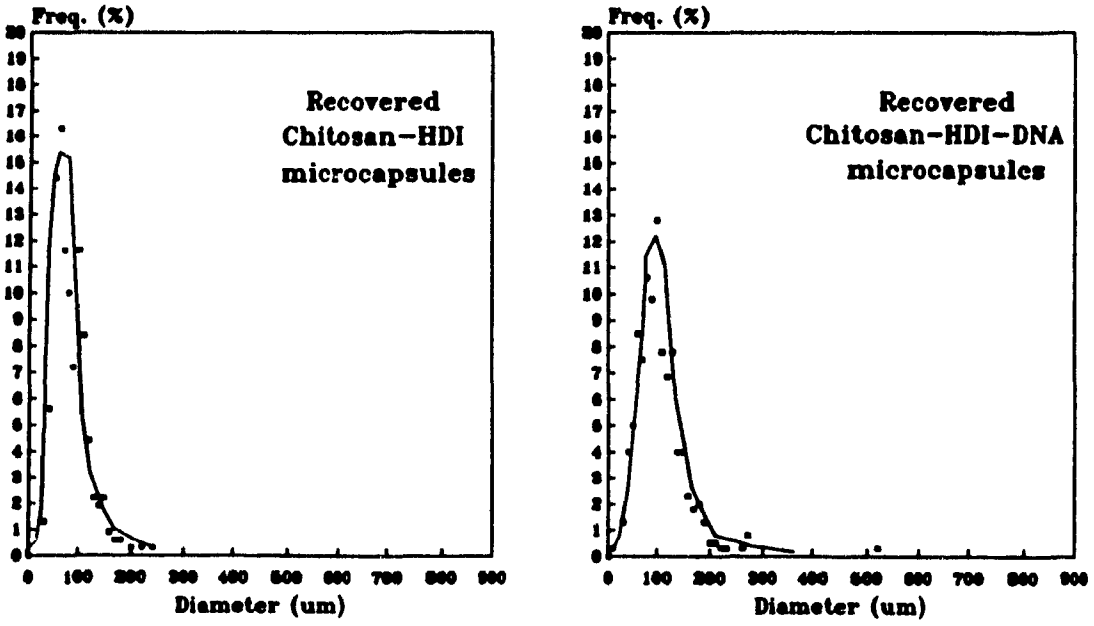
4.4 IN VIVO EXPOSURE OF MICROCAPSULES TO [^{14}C]BENZO[a]PYRENE

4.4.1 Recovery of magnetic chitosan microcapsules after gastrointestinal transit

Magnetic chitosan-HDI microcapsules, with and without incorporated DNA, were fed to rats and extracted magnetically from faecal suspensions. Figure 4.14 shows the size distribution of microcapsules recovered from rats. The size distribution of DNA containing microcapsules was similar to that of control microcapsules. Comparing these size distributions to those in Figure 4.9 it is seen that there was a decrease in the size of

microcapsules after gastrointestinal transit. The mean diameter of DNA microcapsules decreased from 325 μm to 103 μm. Chitosan microcapsules having undergone gastrointestinal transit were dark and irregularly shaped, compared to the spherical, transparent microcapsules that were administered.

Figure 4.14 Size distribution of recovered control and DNA chitosan-HDI microcapsules



The recovery of encapsulated core DNA can be estimated from the numerical recovery of intact microcapsules and their size distributions, assuming that DNA was not lost during transit and that the amount of DNA within the core is proportional to the microcapsule volume.

Table 4.6 presents the percent recovery of microcapsules. Control microcapsules show a 13% recovery, while 8% of the DNA containing microcapsules were recovered. Weaker membranes due to DNA incorporation as shown previously, may increase the probability of rupture during GI transit, resulting in a reduced recovery of intact microcapsules.

Table 4.6 **Numerical recovery and size analysis of excreted chitosan microcapsules**

Treatment	Microcapsules Administered		Microcapsules recovered		Percentage of microcapsules recovered
	Number	Size (μm)	Number	Size (μm)	
Chitosan-HDI microcapsules and [^{14}C]BaP	30 600	326	3 950	89*	13
Chitosan-HDI-DNA microcapsules and [^{14}C]BaP	46 000	325	3 660	103**	8
Chitosan-HDI-DNA microcapsules	46 000	325	3 090	88*	7

* average of 3 rats
** average of 4 rats

4.4.2 Binding of radiolabelled BaP

The results of the in vivo study on rats are summarized in Tables 4.7 and 4.8. Rats were divided into three groups, and administered different treatments of microcapsules and [^{14}C]BaP.

Table 4.7 shows the percentage of radioactivity recovered in the urine and excreted

microcapsules after 24 hours. The total radioactivity recovered in the urine and microcapsules between rats treated with control and DNA-containing chitosan-HDI microcapsules was found to be the same. Both control and DNA microcapsules trapped 0.2% of the radiolabelled BaP. The radioactivity that is not accounted for was lost by exhalation and in the faeces.

Table 4.7 Recovery of [^{14}C]benzo[a]pyrene in urine and excreted chitosan microcapsules

Treatment	Percentage of radioactivity in urine	Percentage of radioactivity in microcapsules
Chitosan-HDI microcapsules and [^{14}C]BaP	3.9 *	0.2 *
Chitosan-HDI-DNA microcapsules and [^{14}C]BaP	4.0 **	0.2 **
Chitosan-HDI-DNA microcapsules	N/A	N/A

* average of 3 rats
** average of 4 rats
N/A not applicable

Table 4.8 presents the average radioactivity recovered per thousand microcapsules for each treatment group. Results show that DNA-containing chitosan-HDI microcapsules trap about the same amount of [^{14}C]BaP as control chitosan-HDI microcapsules. Assuming that 100% of the DNA was incorporated within the chitosan microcapsules, results show that 2.6 x 10^6 dpm [^{14}C]BaP is bound per milligram DNA.

Table 4.8 *In vivo* binding of [^{14}C]benzo[a]pyrene by chitosan microcapsules

Treatment	Radioactivity per thousand microcapsules (dpm)	Radioactivity per mg DNA (dpm/mg)
Chitosan-HDI microcapsules and [^{14}C]BaP	21 000*	N/A
Chitosan-HDI-DNA microcapsules and [^{14}C]BaP	19 000**	2.6 x 10^6
Chitosan-HDI-DNA microcapsules	N/A	N/A

* average of 2 rats
** average of 4 rats
N/A not applicable

5.0 DISCUSSION

Tumours may result from the presence of carcinogens in the environment, specifically in air and food. The affinity of known carcinogens such as methyl iodide, and benzo[a]pyrene for DNA could provide a mechanism or tool for monitoring, detection or trapping of carcinogens within the intestinal tract. The ability to encapsulate DNA within ultrathin, semi-permeable membranes, provides a mechanism by which DNA is immobilized and protected during intestinal transit, facilitating recovery and providing access via membrane diffusion to lower molecular weight carcinogens. The objective of the present study was to develop microencapsulated DNA for trapping DNA-damaging agents within the gastrointestinal tract.

Biologically active compounds may be microencapsulated within a variety of polymeric materials. In the present study, two techniques were considered based on the ability to form membranes via a process of interfacial polymerization. A technique was developed to microencapsulate DNA within cross-linked polyethyleneimine or chitosan. Polymers, solvents and cross-linking agents were selected in order to minimize damage to the DNA. Alternative techniques such as that used in nylon membrane formation[23] were rejected due to potential solvent damage (chloroform/cyclohexane) and extremes of pH required for membrane formation.

An optimization of each procedure was largely based on qualitative evaluations. A

microscopic analysis was performed to assess membrane strength and determine if the microcapsules were spherical with smooth membranes.

5.1 CROSS-LINKED PEI MICROCAPSULES

Microencapsulation by interfacial polymerization was first developed for nylon membranes in 1964[3]. Nylon-6,10 polymerization is a result of the polycondensation reaction between 1,6-hexanediamine and sebacoyl chloride and is limited by the diffusion of diamine through the forming membrane to the organic side of the interface. This transfer rate is reduced when the polarity of an organic solvent and pH of the diamine solution are decreased[18,23]. Although, a mixture of chloroform and cyclohexane is generally used as the organic solvent phase in nylon membrane preparation, the activity of *Streptococcus cremoris* was negatively affected when contacted with both solvents, which may be replaced with butylacetate[14]. The subsequent use of a less polar solvent, with reduced toxicity[2,8], was not successful in microcapsule formation.

PEI membranes were produced by the cross-linking of the PEI polymer. PEI is insoluble in organic solvents, thus the reaction tended towards the aqueous side of the interface. As a result, PEI microcapsules may be prepared in a variety of solvents of different polarity including biocompatible solvents such as mineral, silicon or perfluoro-carbon oils. Another advantage of PEI microcapsules is that preparation is possible at an

initial pH lower than that required for the formulation of polyamide membranes. The pH drop due to the release of acid chloride during the cross-linking reaction, imposes a limit on the extent to which the initial pH can be reduced. An initial buffered pH between 8.0 and 8.5 maintained the final pH between 6.5 to 7.0, compatible with most enzymes, biological cells and natural compounds, including DNA.

5.1.1 Membrane strength

Nylon and PEI membranes have very different characteristics. Nylon is an elastic and deformable membrane[11], whereas the PEI membrane appeared more rigid. In a previous study[23], it was observed that the mechanical resistance of nylon membranes was decreased strongly when the temperature was lowered below the glass transition, since the membrane became more rigid. The rigidity of the PEI membrane could then be a limiting factor for applications requiring high shear hydrodynamic conditions.

Polyethyleneimine includes a large spectrum of water-soluble polyamines of variable molecular weight with varying degrees of modification. All PEI's produced by the ring-opening cationic polymerization of ethyleneimine are believed to be highly branched, containing primary, secondary and tertiary amine groups in the ratio of approximately 1:2:1[9]. Cross-linking of PEI forms a three dimensional network. In contrast, nylon is formed by the polymerization reaction between a diamine and a dichloride, forming mostly linear chains, most likely interlaced to form a net. At high pH values (11), the nylon membrane thickness

is sufficiently large (approximately 1 micron) to ensure good mechanical resistance. However, at lower values of pH, the membrane is sufficiently thin (200 nm) that compounds such as proteins or PEI are required to ensure good mechanical resistance. The membrane is then composed of a network of pure nylon chains, cross-linked PEI and PEI linked by nylon bridges. This structure ensures a high resistance and elasticity of the membrane. At lower pH levels (8.5), the contribution of the nylon to the membrane becomes negligible, and the membrane becomes brittle. The resistance of the membrane may be improved by appropriate selection of cross-linking agent, use of lower molecular weight PEI or introduction of preformed linear chains. Present results showed that strong microcapsules may be prepared by using SC as the cross-linker and a 40,000 MW PEI.

5.1.2 Size distribution

The present results concerning the control of size distribution were similar to that of previous results obtained with collodion and nylon microcapsules[22,24]. Size distribution curves followed the log-normal law. The mean diameter may be controlled by adjusting the emulsifier concentration and the rotational speed of the turbine.

The pH of the PEI aqueous phase also affected the microcapsule size distribution. PEI is a positively charged polymer, the charge increasing by protonation of the amine groups with lowered pH (Figure 4.3). The increased charge on the polymer resulted in a change in the solution viscosity, affecting the size of the droplets formed during

emulsification.

5.1.3 Control of pH

Assuming that the acid release is proportional to the microcapsule surface area during formulation, the drop of pH is more important in small rather than large microcapsules, as confirmed by experimental observations. Increasing the size of the microcapsules would then help maintain the pH at a higher level. However, expecting a relatively constant thickness of membrane[23] as a function of the size, the resistance to shear will drop quickly[22]. A smaller size dispersion would also be an important improvement in pH control.

5.1.4 Membrane formation process

Nylon membrane formation was first described in 1959[37]. The process involves the transfer of diamine to the organic side of the membrane, reaction with the dichloride and precipitation of the nylon polymer thus formed. PEI membrane formation is slightly different. PEI being a polar compound is insoluble in the organic phase[10]. Acid dichloride, on the other hand, is hydrolysed in water. The reaction takes place at the organic/aqueous interface, likely more extensively on the aqueous side due to the hydrophillic properties of cross-linked PEI.

Figure 4.4 showed that most of the membrane mass is formed after 1 minute or less, indicating a very fast reaction. However, this does not exclude a persistent reaction inside

the membrane beyond this initial period. This maturation may lead to stronger microcapsules.

Figure 4.5 also showed that membrane formation is a stronger function of SC concentration than that of PEI concentration. It can be concluded that both components are in excess as the membrane weight is always lower than the total weight of each reactant added. This was also partially confirmed by titration from which only 20 to 40% of the SC was shown to be consumed.

PEI membrane formation likely proceeds due to the penetration of the cross-linker into a PEI layer which forms near the organic/aqueous interface. The cross-linker either reacts with the PEI or is hydrolysed. For thicker membranes, the cross-linking agent must diffuse further through the membrane in order to reach a reactive site. Since the chance for its hydrolysis is greater, the resulting membrane thickness should be the smallest value between the maximum distance from the interface that the cross-linker can diffuse without being hydrolysed, and the thickness of the layer formed by PEI accumulation.

Under the conditions tested, the diffusion of the cross-linker seems to be the limiting factor. The PEI concentration may then be lowered and, furthermore, the use of a higher concentration of cross-linker or a cross-linker that is more stable in water will lead to stronger membranes.

5.1.5 Selection of cross-linker

Results showed that the selection of a cross-linker has a very strong impact on the final product. Membrane properties, final pH, and settling characteristics were all affected by this selection. Sebacoyl chloride appeared to be the most appropriate cross-linker among those tested, since it yielded strong, free flowing microcapsules which settled quickly in water, while maintaining a pH between 6.5 and 8.5 during formulation.

5.1.6 Microencapsulation of DNA

Microencapsulation of DNA within cross-linked PEI membranes was not attempted for several reasons. Previous work involving the microencapsulation of PEI as a DNA surrogate within nylon microcapsules raised some concerns. It was found that PEI was incorporated with a range of 16 to 30% into the membrane[26]. Consequently the trapping of carcinogens in vitro and in vivo occurred mostly on the membrane. Furthermore, since PEI and DNA differ significantly in structure, binding to PEI may not be indicative of possible DNA damage.

In view of the above, a less reactive polymeric agent, chitosan, was found and was investigated for the microencapsulation of DNA.

5.2 CROSS-LINKED CHITOSAN MICROCAPSULES

Chitosan is a polysaccharide derived from the deacetylation of chitin. Chitin is extracted from crustacea shells and thus is abundant in nature. Chitosan is positively charged, with fewer amine groups than PEI, which participate in the polymerization reaction.

Chitosan, being water soluble, may be cross-linked at an interface using an oil soluble reagent. Two cross-linking agents were investigated, glutaraldehyde (GA) and hexamethylene diisocyanate (HDI). Unlike HDI, GA is both oil and water soluble and thus may diffuse into the aqueous droplet during the microencapsulation process. Since GA itself damages DNA, HDI was the preferred cross-linking agent. Other cross-linking agents, such as terephthaloyl chloride were investigated[7], but it was observed that stronger chitosan microcapsules were obtained with glutaraldehyde or hexamethylene diisocyanate.

5.2.1 Solubility of DNA in chitosan solution

Several attempts were made to solubilize DNA in chitosan solution. Contact of the DNA and chitosan solutions resulted in the formation of what appeared to be a water insoluble DNA-chitosan complex. Attempts to separate the chitosan-DNA complex, including concentrated phenol used to separate DNA from polyamines and proteins in tissues, were unsuccessful.

5.2.2 Detection of DNA

Ethidium bromide was used as a marker for the detection of DNA within microcapsules. Ethidium bromide treatment of chitosan-HDI microcapsules revealed that double-stranded DNA was present in most microcapsules. Non-fluorescent microcapsules may have contained damaged single-stranded DNA or undetectable amounts of DNA.

Soluble encapsulated DNA was exposed with ethidium bromide and observed as fluorescence emitted throughout the entire volume of the microcapsules. Insoluble fibrous strands of DNA inside the core were also seen to bind ethidium bromide.

5.2.3 Incorporation of magnetite

The incorporation of magnetite within the chitosan microcapsules facilitated their recovery from faecal suspensions by stirring the faecal suspension with a magnetic plaque. The presence of magnetite also aided in the settling and separation of microcapsules from the oil phase following membrane formation, during subsequent washing operations.

Increasing concentrations of DNA to be encapsulated, resulted in magnetite being excluded from the capsules. Excluded magnetite was eliminated by sieving prior to gavaging the animals. In a previous study with nylon PEI microcapsules it was observed that incorporation of magnetite decreased from 80% to approximately 50% at higher polymer concentrations[25]. Competition between DNA and magnetite for available space during droplet formation was suggested.

5.2.4 Membrane incorporation of DNA

Previous studies showed that nylon encapsulated macromolecules, such as PEI[26] and enzymes[17] are partially incorporated into the encapsulating membrane, resulting in an overall alteration of the membrane properties. In studies where PEI was used as a DNA surrogate, membrane incorporation of PEI was significant[26]. The PEI core-to-membrane ratio varied from 1.0 to 6.4. Although it was not investigated, DNA incorporation into the membrane in the present study may be important. In previous studies, protein incorporation yielded stronger membranes, whereas microcapsules containing DNA were found to be more fragile than controls. Differences in membrane strength were observed during sonication and by differences in yield after gastrointestinal transit. Weaker chitosan membranes may be due to membrane irregularities caused by the incorporation of insoluble DNA, as compared to the incorporation of soluble enzymes and proteins in the previous studies resulted in stronger membranes.

5.2.5 Microencapsulation of DNA

One application for microencapsulated DNA is as a system for trapping food related carcinogens, provided the DNA is capable of withstanding gastrointestinal transit. The microencapsulation of DNA by interfacial cross-linking of chitosan provided protection for the DNA during transit.

Liposomes have been prepared containing DNA[33] and erythrocyte 'ghosts' have

been filled with DNA by lysing and resealing them[34]. These procedures were limited by low yields of encapsulation and the immobilized system would not withstand gastrointestinal transit.

5.3 BINDING OF RADIOLABELLED CARCINOGENS IN VITRO

Binding of carcinogens in vitro is indicative of the ability to trap carcinogens in vivo. DNA encapsulated within chitosan-GA showed greater binding of [14C]methyl iodide than within chitosan-HDI. Several differences between the two types of microcapsules may explain their differences in binding. The mean diameter of chitosan-HDI microcapsules was 325 μm, compared with 144 μm for the chitosan-GA microcapsules representing a larger specific surface area. Furthermore, chitosan-HDI microcapsules were estimated to contain 5.5 μg DNA/10^3 microcapsules, while chitosan-GA contained 7.2 μg DNA/10^3 microcapsules.

Chitosan-HDI-DNA microcapsules showed similar binding of [14C]methyl iodide as the controls. Although, chitosan-GA-DNA microcapsules seemed to trap twice as much [14C]methyl iodide than controls one cannot conclude that there is a real difference since the levels of trapping are very low (less than 400 dpm per thousand microcapsules).

Chemical carcinogens have been trapped previously in vitro by magnetic nylon microcapsules containing PEI as a DNA surrogate. Nylon-PEI microcapsules were capable

of covalently trapping N-methyl-N-nitrosourea and fluoresceine isothiocyanate and ionically trapping eosin and tetrasodium copper phthalocyanine tetrasulfonic acid (CPTS)[26]. Differences in the site and quantity of binding were ascribed to several factors: core-to-membrane ratio of PEI, probe molecular weight, reaction or adsorption of probe with the microcapsule membrane, probe stability in aqueous solution and amount of probe used. In another study[30], binding of probe substances [14C]N-methyl-N-nitrosourea and eosin varied with the microcapsules preparative conditions used and seemed to be dependent on the membrane characteristics, especially the incorporation of PEI into the membrane.

Although in vitro studies provide important information on the potential trapping ability of microcapsules, in vitro techniques alone cannot be used to assess the magnitude of human risk.

5.4 BINDING OF RADIOLABELLED CARCINOGENS IN VIVO

5.4.1 Recovery of magnetic chitosan microcapsules after gastrointestinal transit

Magnetic DNA-containing chitosan microcapsules were fed to rats and extracted magnetically from faecal material. Recovered microcapsules were dark and irregularly shaped and a significant reduction in the size of microcapsules after passage through the gastrointestinal tract was observed due to dehydration within the colon. Shrinkage was not observed when using PEI encapsulated within nylon membranes[27]. The mean diameter

of the recovered nylon-PEI microcapsules was similar to the mean diameter of the microcapsules administered. This difference between chitosan and nylon bound microcapsules may be explained by the fact that the nylon-PEI microcapsules were 25 to 55 μm in diameter, whereas the chitosan microcapsules were greater than 300 μm prior to administration. Furthermore, PEI is a hygroscopic polymer providing greater water retention than chitosan.

After intragastric administration, the numerical recovery of intact microcapsules from rat faeces was 13% for control microcapsules and 8% for DNA-containing microcapsules. This low recovery may either be due to the magnetic extraction technique used or loss during transit. Chitosan-HDI microcapsules were sieved to obtain a size range between 50 and 400 μm prior to administration. However, since 30% of the microcapsules were greater than 400 μm there may have been a great loss in yield due to breakage by the gavage needle or during transit. The recovery of nylon-PEI microcapsules was found to be 21-48%[27].

5.4.2 Binding of radiolabelled benzo[a]pyrene

Previous work has shown that nylon microcapsules containing PEI can trap electrophilic species from radiolabelled n-methyl-N-nitrosourea in the stomach and colon[27], and from radiolabelled 1,2-dimethylhydrazine within the intestine[28]. N-nitrosating species have also been trapped within the stomach[19].

The use of microcapsules for binding carcinogens within the intestinal tract was tested using benzo[a]pyrene by virtue of its proven carcinogenic potency in a variety of species and tissues. This model carcinogen was chosen since BaP is largely excreted by the bile and eliminated from the body in the faeces. Also, the gastrointestinal tract is one of the principal exposure routes to BaP for humans and the biliary metabolites of BaP are well known.

In a previous study[28], microcapsules administered intragastrically to rats bound up to 0.006% of [^{14}C]dimethyl-hydrazine (DMH) and 1.4% of [^{14}C]N-methyl-N-nitrosourea administered intrarectally. There were no detectable metabolites from [^{14}C]DMH trapped within the colon, whereas binding of [^{14}C]N-methyl-N-nitrosourea indicated that microcapsules could bind transient species present within the colon.

Chitosan microcapsules trapped approximately 0.2% of the intragastric dose of [^{14}C]BaP. In a similar experiment nylon-PEI microcapsules trapped 0.5% of [^{14}C]BaP[29]. Nylon-PEI microcapsules were also shown to trap BaP 3,6-dione and BaP 7,8-diol, two metabolites of BaP. From these results it seems that carcinogen-binding microcapsules can be used to investigate the <u>in situ</u> formation of carcinogen metabolites within the intestinal tract.

6.0 CONCLUSIONS

In the present study two techniques were considered for the microencapsulation of DNA based on the ability to form membranes via a process of interfacial polymerization.

Cross-linked polyethyleneimine microcapsules were investigated, however, due the reactive nature of PEI, it would compete with DNA for potential carcinogen binding and was thus rejected as a polymeric material.

Calf thymus DNA was successfully immobilized within cross-linked chitosan membranes. The present study was preliminary in that it is the first report of immobilization of DNA using an interfacial polymerization technique, producing magnetic microcapsules able to withstand gastrointestinal transit. Double-stranded DNA was detected inside the microcapsules through the use of ethidium bromide, a marker for DNA.

The trapping ability of microcapsules was verified in vitro and in vivo. The in vitro study showed that DNA and control microcapsules trapped similar quantities of [^{14}C]methyl iodide. For the in vivo study, magnetic chitosan-HDI microcapsules, with and without incorporated DNA, as well as [^{14}C]benzo[a]pyrene were fed intragastrically to rats. The size of the microcapsules, recovered by magnetic extraction, decreased by 60% due to dehydration in the colon and their recovery was 8%. Both control and DNA microcapsules trapped 0.2% of the radiolabelled BaP and showed the same degree of trapping per thousand microcapsules.

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