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Immobilized Cells: Basics and Applications

Gastrointestinal protection of cellular component DNA within an artificial cell system for environmental carcinogen biomonitoring

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Introduction

Environmental factors (i.e. water, air, food) including chemical carcinogens or their precursors enter the body through dietary and environmental pathways and are currently believed to be the leading factors to human carcinogenesis [1]. Among the many important chemical carcinogens, benzo[a]pyrene (B[a]P) have proven to have carcinogenic potential in a variety of species and tissues. Benzo[a]pyrene is frequently isolated and categorized as an important airborne carcinogen and as the best available index of air pollution carcinogenecity from burning of fossil fuel. As a consequence of cigarette smoke, coal gasification, roofing tar, and coke oven effluents, airborne B[a]P bound to particular matter is respirable by the human lung and is also a common contaminant in food and water. B[a]P itself is not carcinogenic to humans until it has undergone metabolic activation with enzymes in the body (i.e. cytochrome P-450 monooxygenase) to form reactive benzo[a]pyrene diol epoxides, ultimate carcinogen. The gastrointestinal tract undergoes one of the highest environmental exposure of and highest incidence of non-tobacco-related cancer. Thus, catching such reactive agents before they are absorbed within the GI mucosa is of paramount importance in relative to human exposure detection [2].

Current dosimetric techniques for monitoring of carcinogens in the body using *Salmonella typhimurium* [3] to detect either genetic changes or DNA damage, but of does not isolate the radicals causing this change, and are complicated by sophisticated gene repair mechanisms functioning within the cells. As an alternative route to determining the toxicology of environmental carcinogens *in vivo*, artificial cells containing DNA could be used as targets. Metabolites diffuse through the porous gels which provide access to the entrapped DNA. These artificial cells are recovered magnetically from feces followed by DNA purification and adduct quantification.

The purpose of the present work was to create articifial cells by immobilizing DNA within an alginate matrix in the form of microspheres, to trap carcinogens (i.e. B[a]P metabolites) within the GI tract. Microspheres were formulated by emulsification/internal gelation and physico-chemico techniques were developed for DNA/alginate separation and quantification. Chitosan membranes are formed on these artificial cells or reduce permeability of nuclease DNAse while offering protection to cellular DNA from simulated gastric conditions.

Materials and Methods

Sodium alginate (Satialgine SG 300: Sanofi Systems Bio-Industries, France), chitosan (SeaCure 123: Pronova, Norway), calcium carbonate (Setacarb 06: Omya, France), carbonyl

iron powder (R-1470: GAF Chemicals Co., New Jersey), Span 80 (Atkemix: Montreal), and canola oil (Maple Leaf: donated by Canada Packers Montreal) were used in the encapsulation procedures. Calf thymus DNA (DNA highly polymerized, sodium salt) and Sepharose CL-2B were purchased from Sigma (St. Louis). All other chemicals used were reagent grade.

DNA immobilization was prepared by emulsification/internal gelation as described by [4]. The formulation involved the emulsification of a solution of alginate (1.8% w/v), DNA (0.1% w/v), sonicated calcium carbonate (0.5% (w/v), and iron magnetite (0.5g) within a continuous oil phase with the addition of an Span emulsifier for 15 minutes using a three-blade propeller. The gelation of alginate microdroplets were initiated by lowering the pH of the slurry using glacial acetic acid (0.14%). The microspheres were then partitioned away from the oil phase into a solution of calcium chloride (50mM) containing surfactant Tween 80. The DNA containing microspheres were then washed periodically with distilled water until clear and free of oil.

The size distribution of microspheres containing iron was measured microscopically by diameter (over 300 beads counted per batch) while those without were measured by volume (volume within each diameter class) using a Malvern 2605-LC particle size analyzer (Malvern Instruments, England), and its density was measured using a Fuchs-Rosenthal haemocytomer. Extraction of DNA from beads first required liquefaction of alginate microspheres in sodium citrate, removal of iron by centrifugation, and DNA/alginate separation by Sepharose column chromatography [5]. DNA was quantified spectrophotometrically (260, 280, and 320nm) according to Warburg and Christian's equation [6] whereas alginate was quantified following the phenol/sulfuric acid procedure [7].

The affinity of these artificial cells, containing both DNA and iron, to bind to carcinogens was tested *in vitro* by incubation in radiolabelled methyl iodide solution for 16 hours at 37 °C. Afterwards, the beads were washed thoroughly with a solution of ethanol/water (50/50) as to remove excess unbound radiolabelled material. For *in vivo* testing, Fischer male rats (F344) were dosed, by oral gavage, with radiolabelled B[a]P and some DNA microspheres containing iron. The rat feces were collected for 48 hours which afterwards the microspheres were magnetically extracted out. In both cases, *in vitro* and *in vivo*, the beads were liquefied, centrifuged to remove the iron, and fractionated using the Sepharose column. The radiolabelled carcinogens (i.e. [14C]methyl iodide, [14C]benzo[a]pyrene) were quantified by liquid scintillation counting.

Chitosan membrane around alginate microspheres were formed by reacting the artificial cells containing DNA in a 0.08% (w/v) chitosan solution containing 40 µl glacial acetic acid for a period of 0, 5, 40, and 840 minutes. In vitro analysis for DNA protection from simulated gastric juice (pH 1.2) and nuclease DNAse was performed independently for 3 hours at 37 °C. These artificial cells containg membranes were then thoroughly rinsed and analyzed qualitatively using horizontal agarose gel electrophoresis for the presence of DNA.

Results and Discussion

Good reproducibility in the size distribution of the microspheres was achieved, with mean diameters typically ranging from 80 to 90 µm. Dimensions of the calcium carbonate grains introduced to alginate solution, were critical in the formulation of alginate microspheres: During dissolution of the grains, calcium carbonate agrregates (30 µm diameter) leave residual pores within the alginate matrix, resulting in leaky cells. Calcium carbonate was sonicated in aqueous suspension to disperse aggregates and to form grains with a size range of 2-2.5 µm. The resultant microspheres were rigid, spherical in shape, and contained no visible residual grains due to a more complete dissolution of the calcium salt.

Table 1
In vitro binding of [14C]methyl iodide to DNA-alginate microspheres.

	Radioactivity * (DPM/million microspheres)	Binding of [14C]CH3I * (nmoles/million microspheres)
Alginate Microspheres	719	6.2
DNA/Alginate Microspheres	1313	11.3

* values represent mean of 4 replicates

An encapsulation yield of 94 % was achieved, the balance lost during handling and washing operations. The presence of DNA was visible microscopically using ethidium bromide as a fluorescent marker. DNA was quantified by liquefying microspheres in sodium citrate and measuring for DNA absorbance or DNA ethidium bromide fluorescence. Elution of DNA and alginate from a Sepharose CL-2B gel column reduced the alginate/DNA ratio from 20:1 to approximately 2:1 facilitating DNA quantification.

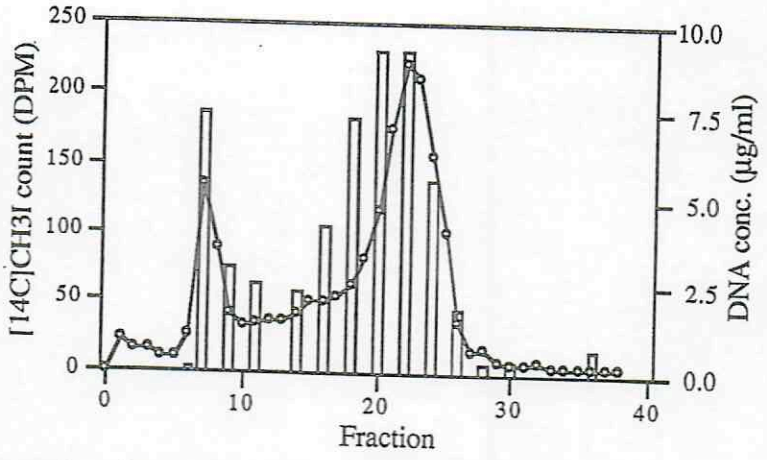

Figure 1. Separation of methylated DNA on a sepharose CL-2B gel column. Methylation was conducted at 37 °c for 16 hours, the microspheres were liquefied in 1 % sodium citrate solution. Measured fractions for DNA at 260 nm (o) and radioactivity using scintillation counter (bars).

In vitro experiments were conducted with radiolabelled [14C]methyl iodide at 37 °C (Table 1). Binding of label to the DNA microspheres was almost double that of the control. Liquefied and column fractionated DNA/[14C]methyl iodide and alginate separation was achieved and the results of the scintillation count of the label and DNA quantification of the eluted volumes is shown in Figure 1. The extent of binding of the radiolabel to DNA was found to be 33.1 µg [14C]CH3I per gram of DNA. Previous *in vitro* studies have been done using semi-permeable cross-linked nylon magnetic polyethyleimine (PEI) microcapsules with PEI acting through its amine functions as a surrogate for DNA; showed similar specific levels of entrapment. PEI microcapsules were also shown to trap [14C]N-[methyl-N-nitrosourea] *in vitro* [8]. In other studies, PEI and copper phthalocyanine tetrasulphonic acid (CPTS) microcapsules were shown

to trap 3-hydroxy B[a]P, B[a]P 3,6-dione, and 7,8-diol-9,20 epoxide *in vitro* from a crude B[a]P metabolite mixture [9]. Assessment from *in vitro* studies alone cannot demonstrate the extent of trapping of GI metabolites *in vivo*.

Table 2
In vivo binding of [14C]benzo[a]pyrene to DNA-alginate microspheres.

	Radioactivity * (DPM/million microspheres)	**Binding of [14C]BaP *** (nmoles/million microspheres)
Alginate Microspheres	5804	44.0
DNA/Alginate Microspheres	30458	230.9

* values represent mean of 3 replicates

Artificial cells containing magnetite, with and without DNA, were introduced to rats by gavage together with radiolabelled [14C]benzo[a]pyrene. After GI transit, microspheres were extracted magnetically from fecal suspension and counted using a haemocytometer and sized using a microscope mounted image analyzer. Microsphere recovery from rat feces was 50% of the microspheres administered. A symmetrical size distribution, before and after GI transit was observed with mean diameters of 64 and 36 μm, respectively, attributed to the dewatering in the lower intestine and possibly due to acidic conditions in the stomach. Microspheres prior to GI transit were spherical, clear, transparent with visible magnetite in the alginate core. Magnetically recovered microspheres were dark yellow with some beads irregular in shape and showing damage due to GI transit.

Table 2 presents binding of [14C]B[a]P following GI transit in rats. Results show that 0.32% of total administered label was trapped within recovered beads. DNA microspheres were able to trap 5.7 times as much B[a]P adduct as the control which contained no DNA. Weight-for-weight, DNA was comparably more specific to binding to B[a]P than alginate by a factor of 158. DNA was also more specific to binding benzo[a]pyrene metabolites than to PEI [10] by 30 fold on a weight-to-weight basis.

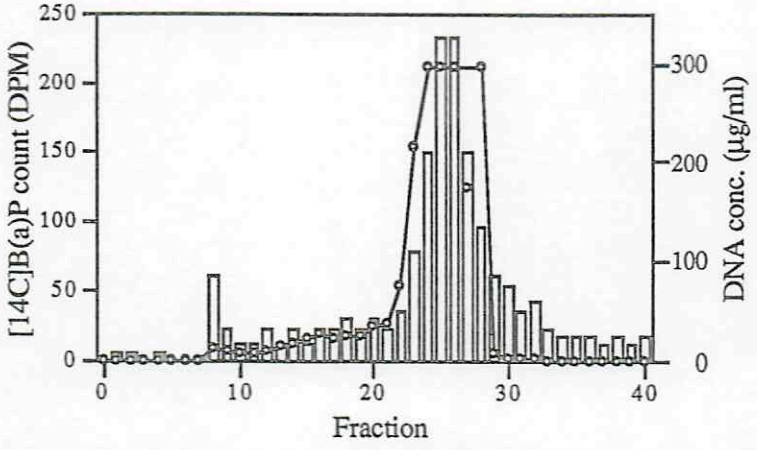

Figure 2. Separation of DNA microspheres after GI transit. Microspheres were liquefied in 1 % sodium citrate and fractionated using a sepharose CL-2B gel chromatography column. DNA (o) and bound radiolabelled benzo(a)pyrene metabolites (bars).

Liquefying alginate using sodium citrate and separating using a Sepharose CL-2B column showed direct correspondence between label activity with DNA (Figure 2). DNA would elute betzeen fractions 10-12 and between fractions 21-26 before and after gavage, respectively (Figure 3). The DNA was fragmented during transit as gastric conditions or GI nucleases appeared to have reduced high molecular weight DNA from 2.0 million to 500 base pairs.

Table 3
Influence of the chitosan coating on the DNA protection against nuclease DNAse (*in vitro*).

		Reaction time (min)	**DNA protection** against Nuclease (*in vitro*)*
Chitosan I	(MW 260 000) (DA 82 %)	5	- - -
		40	+ - -
		840	+ + +
Chitosan II	(MW 350 000) (DA 91 %)	5	- - -
		40	+ - -
		840	+ - -
Chitosan III	(MW 160 000) (DA 95 %)	5	- - -
		40	- - -
		840	- - -

* cumulated result of 3 tests; MW molecular weight; DA deacetylation degree

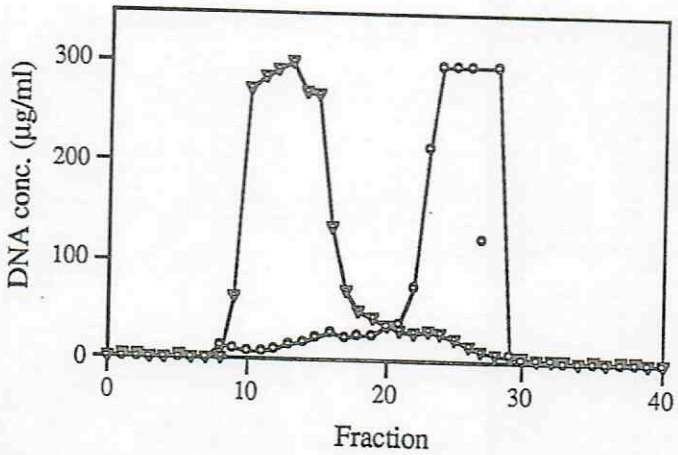

Figure 3. Separation of calf thymus DNA before (∇) and after (o) gastrointestinal transit. Microspheres were liquefied in 1 % sodium citrate solution fractionated using a sepharose CL-2B gel chromatography column.

Three different chitosans of varying molecular weight and percent degree of deacetylation were applied to the artificial cells with varying reaction times. Table 3 summarizes the

protection offered by a membraned artificial cell system under nuclease DNAse. In every case study, cellular DNA was protected from simulated gastric pH 1.2 and the protease pepsin. All cellular DNA within an artificial cell coated with chitosan III was totally hydrolyzed by DNAse, while chitosan I and II offered some protection when membrane was formed for more than 40 minutes. Reproducibilty of results for chitosan I (40 minutes) and chitosan II (40 and 840 minutes) showed some uncertainty, however, chitosan I (840 minutes) with the lowest degree of deacetylation offered best protection from DNAse.

Conclusions

DNA was successfully immobilized within an alginate bead using the emulsification/internal gelation technique. Dispersion of calcium carbonate aggregates resulted in 2-2.5μm grains which improved bead strength and sphericity with a 80-90 μm diameter range. Incorporation of iron powder within an alginate matrix allowed microspheres to be magnetically recoverable. Alginate beads were shown to withstand gastrointestinal transit along with protection of the target. Purification of DNA from alginate was accomplished using a Sepharose CL-2B gel column. Elution separation reduced alginate/DNA ratio by a factor of 2 which eliminated large alginate interference when quantifying DNA using absorbance. Trapping carcinogens/metabolites *in vitro* and *in vivo* was assessed using radiolabelled methyl iodide and benzo(a)pyrene, respectively. Methylation showed that DNA containing alginate microspheres trapped 2 times as much label as alginate microspheres. Iron containing microspheres, with and without DNA were fed intragastrically to rats with [14C]benzo(a)pyrene. Microspheres containing DNA trapped 5 times as much as those without DNA. Microspheres were magnetically retrieved from feces and underwent size reduction from 64 to 37 μm due to dewatering in the lower GI tract. Microsphere recovery from gastrointestinal transit was 50% and 0.32% of total dosed carcinogen was trapped. Recovered DNA was fractionated using a Sepharose CL-2B gel column and the DNA elutions corresponded to label activity. Low molecular weight (MW 260 000) with low degree of deacetylation (DA 84%) offered the best protection under simulated gastric conditions as well as from the nuclease DNAse.

Acknowledgements

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