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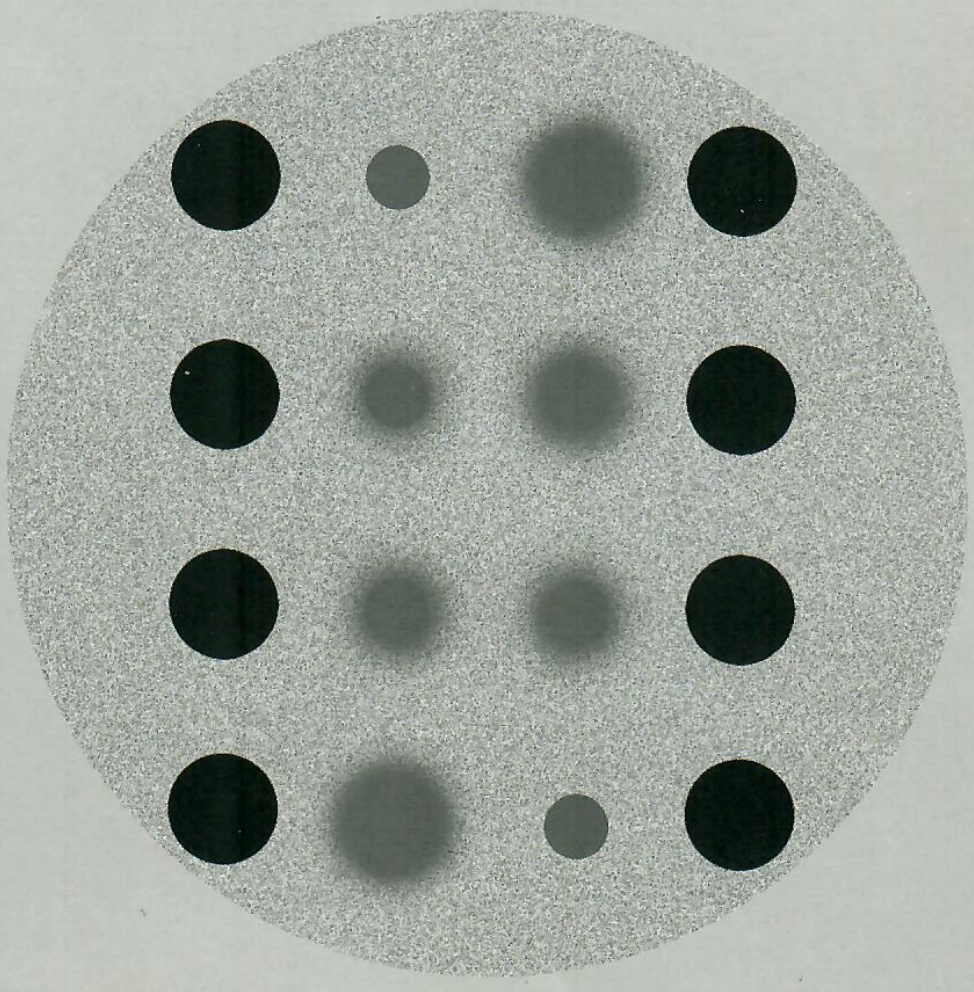

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Microencapsulation within cross-linked chitosan membranes

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DNA (Alexakis *et al.*, 1995) and lactic cultures (Groboillot *et al.*, 1993) have been encapsulated within cross-linked chitosan membrane-bound microcapsules for biological protection during gastrointestinal transit with DNA, and enhanced productivity in dairy fermentations with lactic acid bacteria. A related technique involves the entrapment of the DNA or cells within an alginate microsphere,

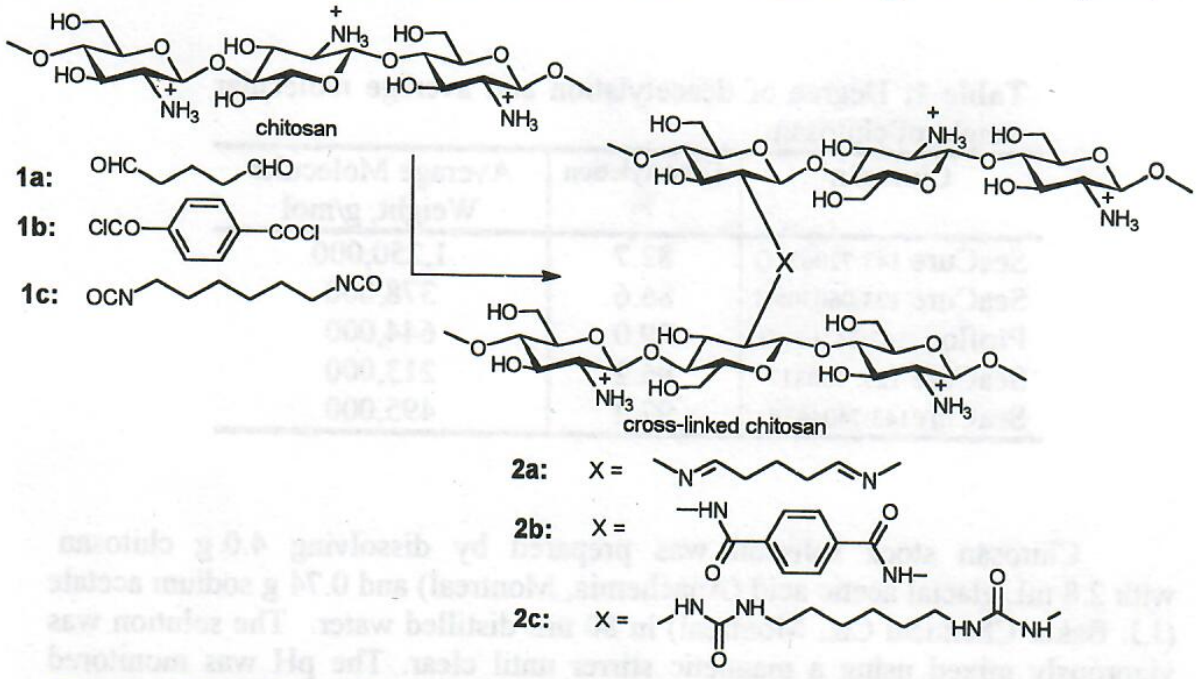

Figure 1: Deacetylation of chitin to chitosan and the subsequent cross-linking with different bi-functional agents: (1a) glutaraldehyde (GA), (1b) terephthaloyl chloride (TC), (1c) hexamethylene diisocyanate (HDI). Structures (2a), (2b), (2c) are formed when GA, TC and HDI are respectively used to cross-link chitosan. (Groboillot *et al.*, 1993. Reprinted with permission.)

followed by application of a chitosan membrane and optional cross-linking of the membrane with bi-functional reagents.

Our approach to microencapsulation involves three steps: emulsification, membrane formation via interfacial polymerization or deposition and cross-linking, and microcapsule recovery via phase partitioning. In the case of chitosan, cross-linkers have been selected for their reactivity with amino groups as shown in Figure 1, resulting in liquid droplets or gel microspheres enveloped within a strong, thin, semi-permeable polymeric membrane. The membrane offers a higher retention of biological material because of the smaller membrane pores and minimizes mass transfer limitations due to a potentially smaller diameter sphere. Control over permeability may be exercised by appropriate choice of polymer molecular weight and membrane thickness, degree of deacetylation, and type of cross-linker and extent of cross-linking.

Encapsulation procedures and results

Chitosans (Table 1), were donated by Pronova Biopolymers (Washington, U.S.A.) and varied in molecular weight and degree of deacetylation. Chitosans were characterized using low angle laser light scattering and intrinsic viscosity for molecular weight and CHN elemental and UV analyses for degree of deacetylation.

Table 1. Degree of deacetylation and average molecular weight of chitosan.

Chitosan	Deacetylation %	Average Molecular Weight, g/mol
SeaCure 143:720627G	82.7	1,250,000
SeaCure 123:060308G	86.6	378,000
Profloc 143:741037G	89.0	644,000
SeaCure 123: 100317	96.2	213,000
SeaCure143:740447R	97.2	495,000

Chitosan stock solution was prepared by dissolving 4.0 g chitosan with 2.8 mL glacial acetic acid (Anachemia, Montreal) and 0.74 g sodium acetate (J.J. Baker Chemical Co., Montreal) in 80 mL distilled water. The solution was vigorously mixed using a magnetic stirrer until clear. The pH was monitored during dissolution and adjusted to a final pH of 5.6 or 6.0 using 1.0 N sodium hydroxide (Fisher Scientific, Montreal). The final volume was adjusted to 100 mL and residual solids removed by filtration with Whatman No. 1 paper.

Table 2: Possible organic fluids used for emulsification during microcapsule formation.

Organic fluids	Supplier	Viscosity at 23°C cP
Cyclohexane	American Chemicals	1
Sunflower seed oil	Queen	63
Canola oil	Crisco	58
Mineral oil	American Chemicals	68
Carnation mineral oil	Witco	20
Klearol mineral oil	Witco	12.5

For the preparation of microcapsules, calf thymus DNA (0.2 % w/v, Sigma, St. Louis, MO) or cell (*Lactococcus lactis cremoris*) pellet was dispersed in an aqueous solution of 4 % (w/v) chitosan and acetate buffer (Groboillot *et al.*, 1993). 10 mL of this chitosan mixture was mixed into 50 mL of an organic phase containing 2 % (v/v) sorbitan trioleate (Span 85, Atkemix Inc., Bradford, Ontario) for about 2-5 min. The continuous organic phase could be selected from a wide range of biocompatible fluids as illustrated in Table 2. The emulsification step involved use of a flat or round-bottomed cylindrical reactor with an impeller (six blade turbine, double flat blades of wire mesh or marine impeller) as shown in Figure 2. The rpm selected depended on the choice and scale of mixer, to provide control over the mean diameter and size distribution of the resultant microcapsules (Poncelet De Smet *et al.*, 1989, 1990).

Once the emulsion had stabilized, membrane formation is initiated by interfacial cross-linking of the chitosan. The cross-linker was dissolved in 10 mL of organic fluid and added to the reaction mixture for the reaction time shown in Table 3. The reaction was stopped by dilution with 50 mL of the respective organic phase or 100 mL of 25-50 % (v/v) polysorbate (Tween 20, Sigma) aqueous solution. The supernatant solution was removed by aspiration and the microcapsules washed with either Tween 20, sodium chloride (0.39 g/L) or peptone (1 g/L) aqueous solutions.

Table 3: Summary of cross-linkers and the total reaction time required to set the membrane.

Cross-linkers	Supplier	Amount used in organic	Reaction time (min)
Terephthaloyl chloride	Aldrich	0.6g	3
Glutaraldehyde	Aldrich	0.3ml	3
Hexamethylene diisocyanate	American Chemicals	0.5ml	15

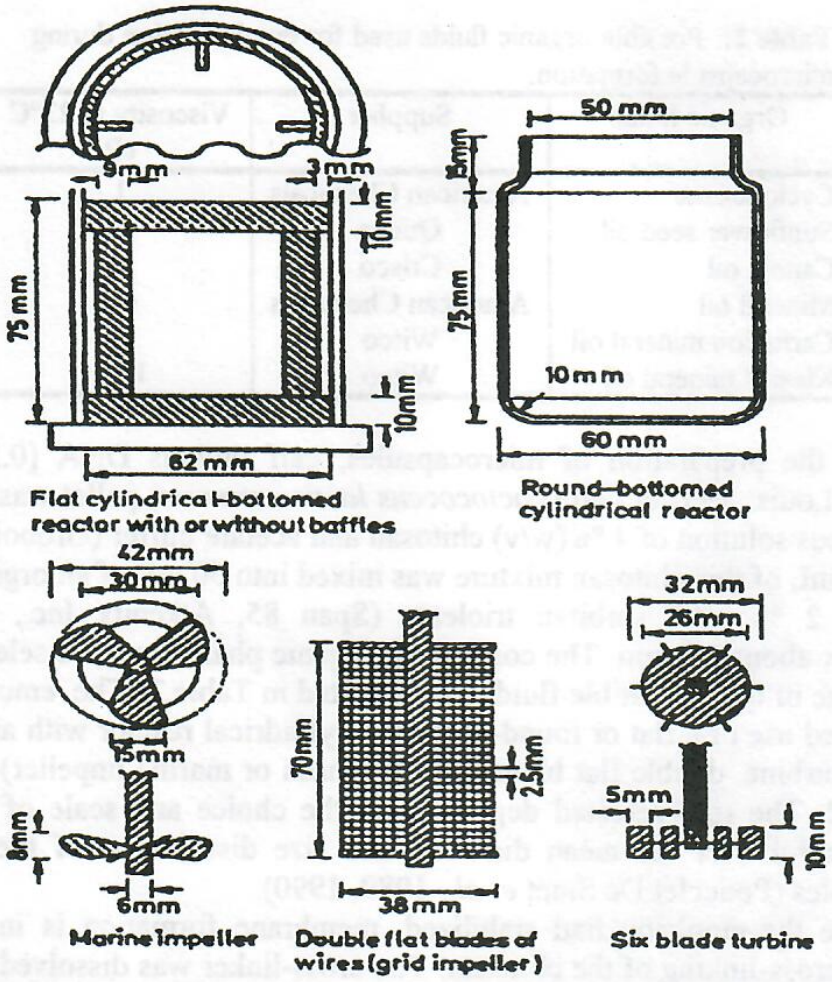

Figure 2: Design of reactors and impellers. (Poncelet *et al.*, 1992. Reprinted with permission.)

Activity of lactic cultures encapsulated within chitosan membranes, cross-linked with hexamethylene diisocyanate, is shown in Figure 3 during repeated fermentations involving acidification of milk. Increased activity with successive fermentations was probably due to continuing microbial growth within the microcapsules.

DNA was also immobilized in an alginate core, coated with a cross-linked chitosan membrane. The alginate core was prepared via an emulsification/internal

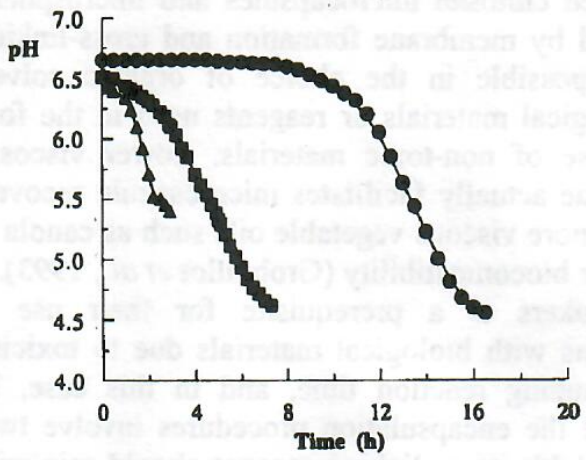

Figure 3: Milk acidification profile during successive fermentations with chitosan/HDI encapsulated cells during first (●), second (■) and third fermentations (▲). Cells were encapsulated in 4% chitosan solution cross-linked with 0.5 ml HDI in A&C mineral oil. (Groboillot *et al.*, 1993. Reprinted with permission.)

gelation procedure involving the dispersion of a mixture of 0.08% (w/v) DNA, 1.5% (w/v) alginate (Systems Bio-Industries, Paris, France) and 1% (w/v) calcium carbonate into canola oil containing 1% (v/v) Span 80 (Alexakis *et al.*, 1995). After emulsification, gelation was initiated by acidifying with 0.4% (v/v) glacial acetic acid. After 5 min, microspheres were partitioned into 50 mM calcium chloride, filtered and washed. The chitosan membrane was then applied to the microspheres by suspending them in 200 mL of 0.5% (w/v) chitosan solution (Seacure 123, lot 10036RG, Pronova Biopolymers) for 60 min followed by washing with 50 mM calcium chloride and cross-linking by suspension in 300 mL of 20 mM glutaraldehyde or benzenetetracarboxylic dianhydride (Aldrich, Montreal) for 30 min.

Yield of DNA encapsulated within chitosan-coated alginate microspheres was 96% (Alexakis *et al.*, 1995). Intact microspheres were recovered from rat feces following gavage and gastrointestinal transit. Recovery levels ranged from 31 to 58%, depending on the cross-linker used. DNA was subsequently recovered and purified from the microspheres, following core liquefaction in 1% (w/v) sodium citrate, membrane disruption in a tissue homogenizer, differential precipitation with ethanol and chromatography (Alexakis *et al.*, 1995).

Discussion

The term microcapsule has been used in reference to a membrane coated liquid droplet, while microsphere has been used to describe a gel bead in a submillimeter size range. Chitosan membranes have been applied in both cases, and the addition of a cross-linker improves the integrity and strength of the membrane (Alexakis *et al.*, 1995). In all cases, when using the procedures described, smooth and resilient membranes were formed and the resultant microcapsules and microspheres were fully spherical. Diameters typically ranged from 20 μm to 1 mm, and control over the mean diameter was exercised through appropriate selection of formulation and mixing conditions.

The formation of cross-linked chitosan microcapsules and microspheres involves a dispersion step, followed by membrane formation and cross-linking. While considerable flexibility is possible in the choice of organic solvent systems, the encapsulation of biological materials or reagents used in the food and drug industries necessitates use of non-toxic materials. Lower viscosity organic solvents such as cyclohexane actually facilitates microcapsule recovery by phase partitioning, however the more viscous vegetable oils such as canola or corn oil are safer choices due to their biocompatibility (Groboillot *et al.*, 1993).

High reactivity of cross-linkers is a prerequisite for their use in polymerization, but present problems with biological materials due to toxicity. The effect is moderated by minimizing reaction time, and in this case, by selecting oil soluble reagents. Since the encapsulation procedures involve two-phase systems, the use of an oil soluble cross-linking reagent should minimize contact with the active biological material, and restrict any reaction to the interface, the site of membrane formation. Glutaraldehyde is a common choice, but is both oil and water soluble, possibly cross-linking the DNA contained within the particle core, or contributing to the loss in viability of the lactic cultures (Groboillot *et al.*, 1993). Hexamethylene diisocyanate, being oil soluble may be a better choice, although other alternatives are under active investigation.

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