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3. THE BIO-POLYMERS

3.1. Alginate

Alginate, a high-molecular-mass polysaccharide, is a naturally occurring biodegradable copolymer of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) and extracted from brown seaweeds (*Phaeophyceae*, mainly *Laminaria*). Species wise M and G content distribution of different alginate is enumerated in the Table below. Alginate is chemically very stable between pH 5 and 10. High acid concentrations cause decarboxylation of alginate (He and Wang, 2002). Alginate has the advantages of being nontoxic orally and having high biocompatibility (Park et al., 1993b). Alginate is used as an entrapment matrix for cells and enzymes as well as for pharmaceutical and food adjuvant.

Table 3.1: Compositions of alginates obtained from different sources (1987).

Species	M content (%)	G content (%)	M/G ratio
Macrocystis pyrifera	61	39	1.56
Ascophyllum nodosum	65	35	1.85
Laminaria digitata	59	41	1.45
Laminaria hyperborean	31	69	0.45
Ecklonia cava	62	38	1.6
Eisenia bicyclis	62	38	1.6

3.1.1. STRUCTURES

Alginate, a high-molecular-mass polysaccharide, is a naturally occurring biodegradable copolymer of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) and extracted from brown seaweeds (*Phaeophyceae*, mainly *Laminaria*) (Figure 3.1). It has been shown that the G and M units are joined together in blocks and as such, three types

of blocks may be found: homo-polymeric G blocks (GG), homopolymeric M blocks (MM) and heteropolymeric sequentially alternating blocks (MG).

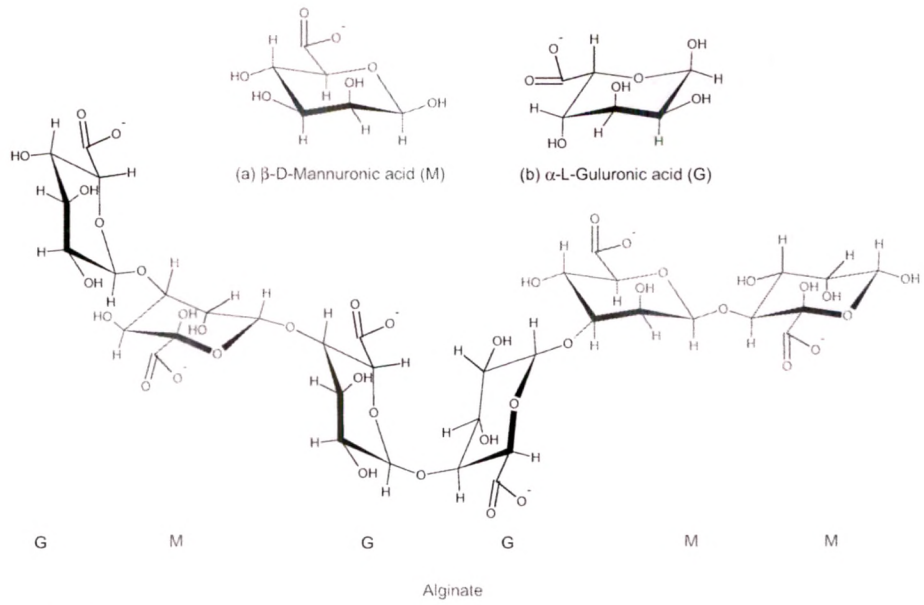


Figure 3.1: Chemical structure of alginate.

(A) Flat, ribbon like polymannuronic acid chain

(B) Buckled, ribbon like polyguluronic acid chain

Figure 3.2: Schematic presentation of conformation of poly(uronic) acids in alginate.

It has been shown that in aqueous solutions, alginates are highly hydrated polyelectrolytes in the extended ribbon conformation (1987). X-ray diffraction studies and polarized infrared spectroscopy have provided information on the crystalline structure of polymannuronic acid and polyguluronic acid segments. Polymannuronic acid is a flat ribbonlike molecule (Figure 3.2A), the conformation of which appears to be stabilized by the formation of an intramolecular hydrogen bond between the hydroxyl group on C3 of one unit and the ring oxygen of the next unit (Atkins et al., 1971). The shape of the

polyguluronic acid chain is quite different from that of polymannuronic acid. Polyguluronic acid is a buckled, ribbon like molecule (Figure 3.2B). This conformation is stabilized by an intramolecular hydrogen bond between the hydroxyl group on C2 and the oxygen atom of the carboxyl group in the next unit (Atkins et al., 1971).

3.1.2. GELLING MECHANISM

The reactivity with calcium and the subsequent gel formation capacity is a direct function of the average chain length of the G blocks. Hence, alginates containing the highest GG fractions possess the strongest ability to form gels. This initially arises from the ability of the divalent calcium cation to fit into the guluronate structures like eggs in an "egg box junctions". Consequently, this binds the alginate chains together by forming junction zones, and sequentially leading to gelling of the solution mixture and bead formation. When an aqueous solution of sodium alginate is added dropwise to an aqueous solution of calcium chloride, a spherical gel with regular shape and size results, also known as ''alginate bead''. These gels which are similar to solids in retaining their shape and in resisting stress, are 99-99.5% water with the rest being alginate. It was initially suggested that cross-links were caused either by simple ionic bridging of two carboxyl groups on adjacent polymer chains with calcium ions or by chelation of single calcium ions by hydroxyl and carboxyl groups on each of a pair of polymer chains (Rees, 1969). It was also suggested that a cooperative association of either polymannuronic acid or polyguluronic acid segments is involved in the formation of the cross-linked network of polymer chains. This current proposed structure of alginate in which calcium ions are bound between the associated segments of polymer chains is shown in Figure 3.3. Circular dichroism studies showed that the calcium ions react preferentially with the

polyguluronic acid segments (Morris et al., 1973). It was also suggested that the alternating segments likely play no role in the gelation except to join the associated segments to form a three dimensional network. Based on coordination geometry, Grant et. Al. (Grant et al., 1973) suggested the "egg box model", in which the polyguluronic acid segments associate into aggregates with interstices into which the calcium ions fit.

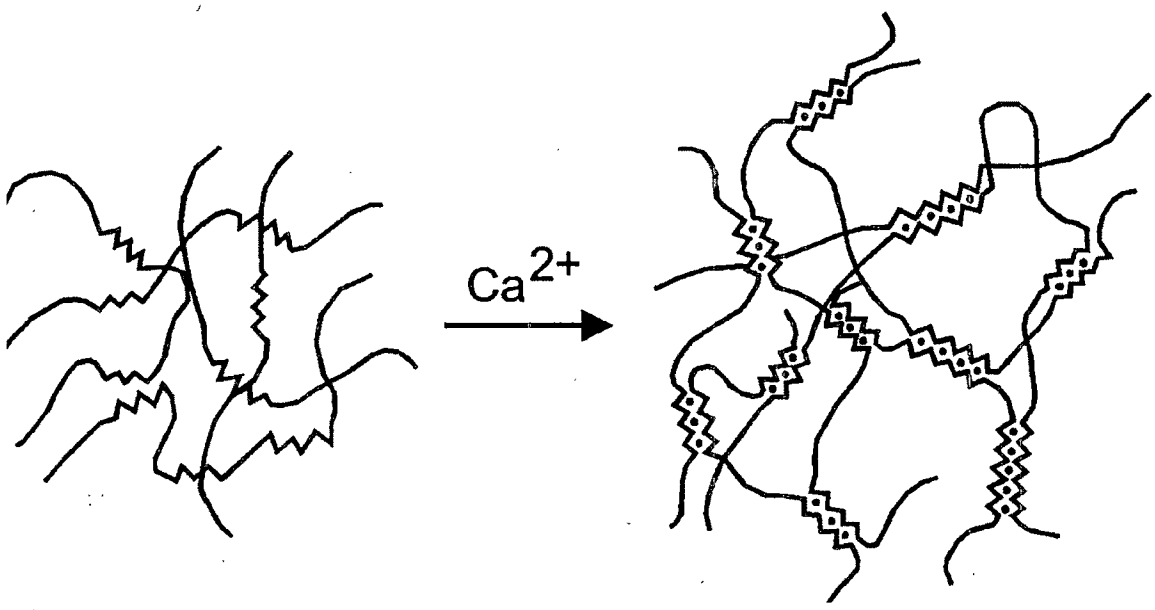


Figure 3.3: Schematic representation of calcium induced gelation of alginate in accordance with the "egg-box" structure.

In principle, any multivalent cation can cause gelation of alginate. Aluminum, barium (Zekorn and Bretzel, 1999) and zinc (Lee et al., 1996) have been used to form alginate gels. Magnesium is one exception to this rule. The differences in affinity of cations to alginates has been studied by Haug (Haug, 1964). The mechanical rigidity of the gels reflected the degree of affinity (Smidsrod and Haug, 1972). The affinity of cations to alginates is in the following order:

Pb > Cu > Cd > Ba > Sr > Ca > Co, Ni, Zn > Mn

Alginates also form strong complexes with polycations such as chitosan, polyamino acids, polyethyleneimine or polyacrylamide. Numerous studies have shown that the

chemical structure, molecular size, as well as the gel forming kinetics and the cat-ion have a significant impact on several of its functional properties including porosity, swelling behavior, stability, biodegradability, gel strength and the gel's immunological characteristics and biocompatibility (Martinsen et al., 1989; Martinsen et al., 1992). The pore size of hydrogels formed by cross-linking alginates can vary greatly. Proteins of small molecular weight diffuse easily out of calcium alginate microcapsules (Martinsen et al., 1989). Only very high molecular weight enzymes and whole cells are completely immobilized in calcium alginate gels (Tanaka et al., 1984).

3.1.3. BIOCOMPATIBILITY OF ALGINATES

Alginate is used extensively in the food industry as a thickener, emulsifier and as a stabilizer. Alginates are included in a group of compounds that are generally regarded as safe (GRAS) by the FDA . The biocompatibility of alginates has been studied by several investigators. Alginate beads have the advantages of being nontoxic orally and having high biocompatibility. (Park et al., 1993a) Another advantageous property is their inability to reswell in acidic environment while easily reswells in alkaline environment, so acid-sensitive drugs incorporated into the beads would be protected from gastric juice. (Yotsuyanagi et al., 1987) Therefore, alginate is used as an entrapment matrix for cells and enzymes as well as for pharmaceutical and food adjuvants.

3.1.4. ALGINATE IN DRUG DELIVERY AND CELL ENCAPSULATION

The gelation method for alginate involves dropping alginate solution into an aqueous solution of multivalent cations. This process can be carried out under an extremely mild

environment and uses non-toxic reactants. For this reason, alginate has been extensively studied for protein and enzyme delivery and in fact is the polymer of choice for cell encapsulation. Alginate hydrogels have been the focus of several studies including delivery of drugs such as diclofenac sodium (Pillay and Fassihi, 1999; Gursoy and Cevik, 2000; Mitrevej et al., 2001), flurbiprofen (Oh et al., 2005; Pillay et al., 2005), prednisolone (Sugawara et al., 1994), vancomycin (Lin et al., 1999; Ueng et al., 2004), theophylline (Efentakis and Buckton, 2002; Nokhodchi and Tailor, 2004) and proteins such as melatonin (Hua et al., 2003), heparin (Ohta et al., 2004; Liao et al., 2005), bovine serum albumin (Shi et al., 2005; Zheng et al., 2005), different types of growth factors including nerve growth factor and vaccines. There has also been some work on developing conjugates of alginate with drugs such as daunomycin (Bouhadir et al., 2000). Due to the mild encapsulation method, alginate has become the polymer of choice for cell encapsulation. In the past, conventional crosslinked calcium–alginate beads have been investigated for the development of a multiple unit drug delivery system. (Pillay et al., 1998; Arica et al., 2002; Murata et al., 2004; Rastello De Boisseson et al., 2004; Talukder and Fassihi, 2004) However, not even a single reference could be cited in literature till date for entrapment of enzymes in alginate beads for improvisation of shelf-life.

3.1.5. CHARACTERIZATION

3.1.5.1. Fourier Transform Infra-Red Spectroscopy (FTIR)

IR transmission spectrum of sodium alginate was obtained using a FTIR spectrophotometer (FTIR-8300, Shimadzu, Japan) and is shown in Figure 3.4. Sodium alginate showed various distinct peaks: broad strong band at around 3600–3000 cm^{-1} due

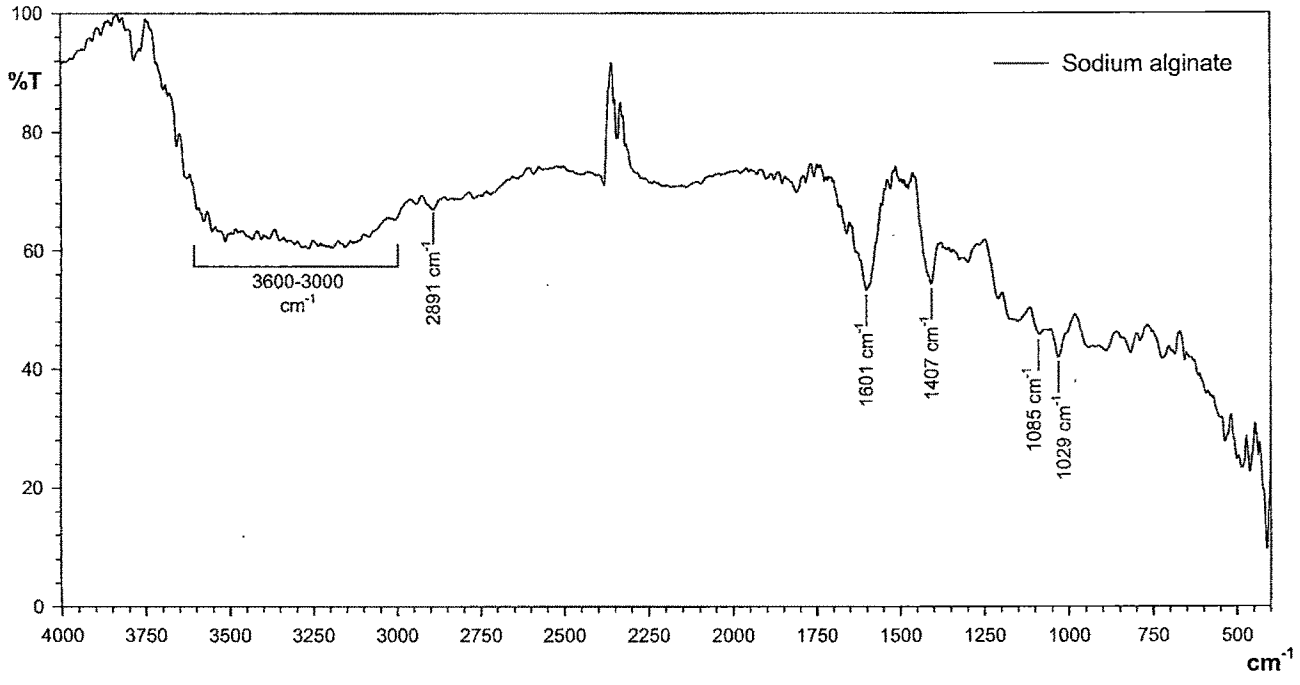


Figure 3.4: FTIR spectrum of sodium alginate alginate.

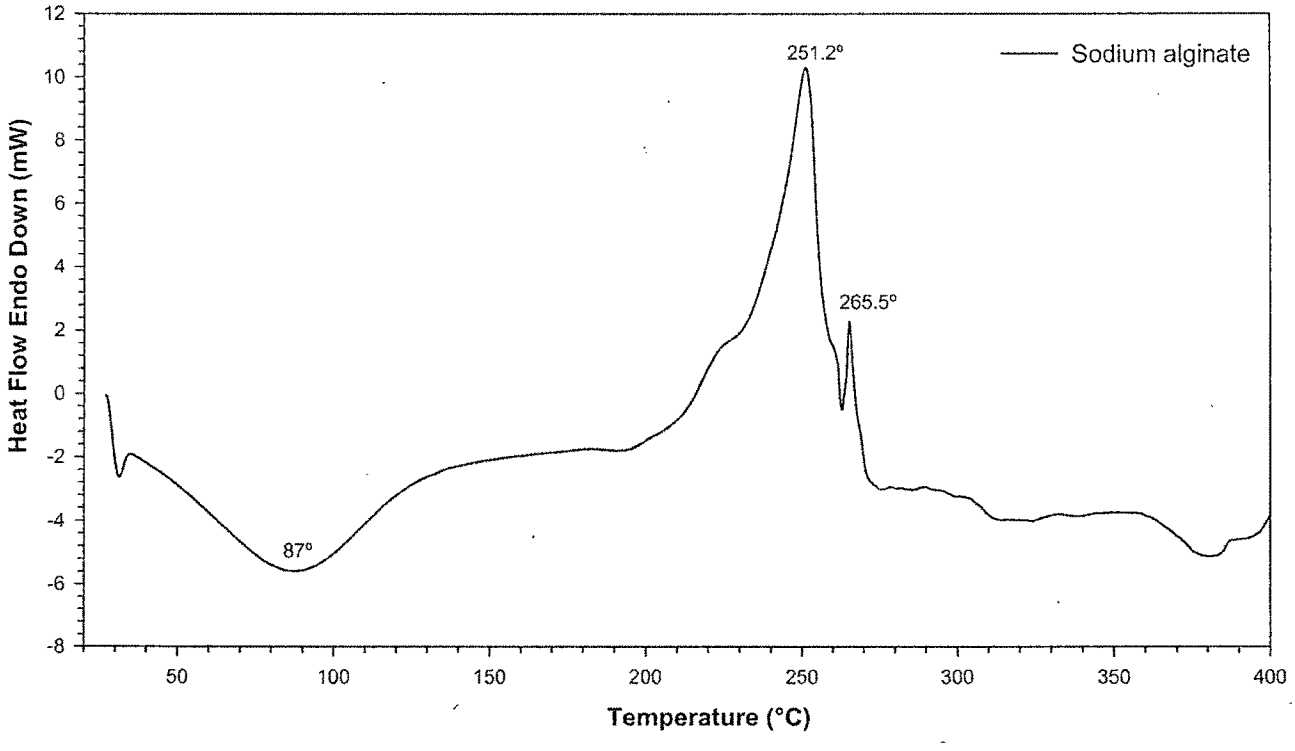


Figure 3.5: DSC thermogram of sodium alginate.

to O–H stretching and strong peak at 1029 cm^{-1} due to C–O stretch of polyhydroxy $(OH)_n$ carbohydrate; strong peak at 1085 cm^{-1} due to C–O–C stretch of cyclic ether (carbohydrate). Sodium alginate as a carboxyl salt showed strong absorption bands at 1601 and 1407 cm^{-1} due to carboxyl anions (asymmetric and symmetric stretching vibrations). The frequency of carbonyl absorption is lowered compared to the value found for the parent carboxylic acid due to a resonance phenomenon.

3.1.5.2. Differential Scanning Calorimetry (DSC)

DSC thermogram of sodium alginate was obtained using an automatic thermal analyzer system (DSC-60, Shimadzu, Japan) and is shown in Figure 3.5. A broad endothermic peak at 87°C in the thermogram of sodium alginate was similarly attributed to the presence of water molecules in the sample. It also showed two exothermic peaks at 251.2°C and 265.5°C.

3.2. Carrageenan

Carrageenan has been used increasingly in pharmaceutical formulation studies (Picker, 1999), for example, microcapsules for sustained delivery (Suzuki and Lim, 1994), or crosslinked spheres for controlled release (Garcia and Ghaly, 1996; Sipahigil and Dortunc, 2001). Carrageenans are naturally occurring high molecular weight polysaccharides extracted with water or alkaline water from red seaweed (*Rhodophyceae*). It is a hydrocolloid consisting mainly of the potassium, sodium, magnesium, and calcium sulfate esters of galactose and 3,6-anhydro-galactose copolymers. Carrageenan is recovered by alcohol (methanol, ethanol or isopropanol) precipitation, by drum drying, or by freezing.

3.2.1. MANUFACTURE OF CARRAGEENAN

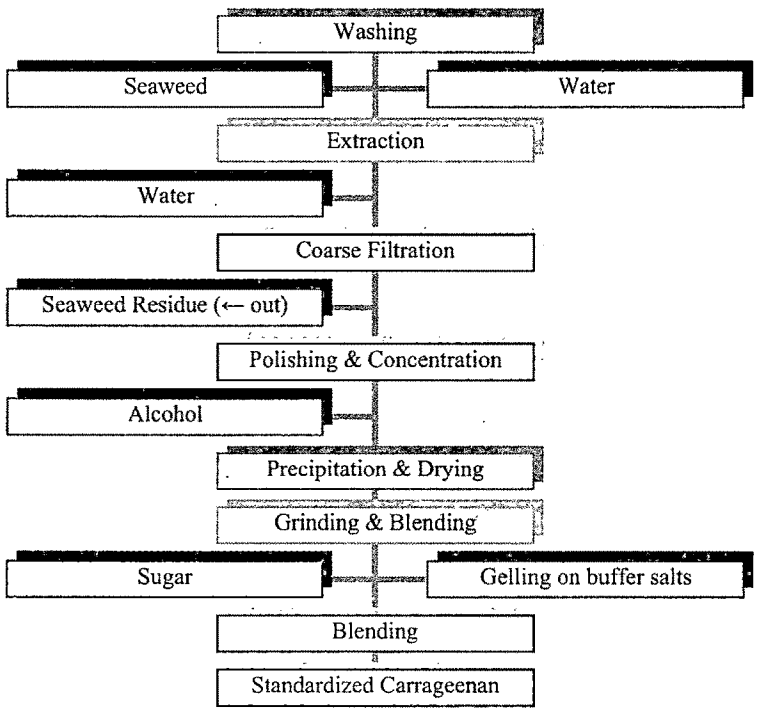


Figure 3.6: Schematic representation of carrageen preparation from red seaweed.

Carrageenan is extracted from the raw material with water at high temperatures. The liquid extract is purified by centrifugation and/or filtration. The liquid extract may be converted into a powder by simple evaporation of water to yield the so called drum dried carrageenan.

3.2.2. MOLECULAR STURCTURE & SOLUBILITY

Carrageenans are made up of alternating copolymers of 1,3-linked β-D-galactose and 1,4-linked 3,6-anhydro-α-D-galactose. The units are joined by alternating α-1,4 and β-1,4 glycosidic linkages. Depending on the algae from which they are extracted and the preparative technique, three main types of carrageenans (Figure 3.7) available; kappa (κ), lambda (λ), and iota (ι).

In a kappa-type seaweed extract some of the D-galactose containing 6-sulfate ester groups and some of the 3,6-anhydro-D-galactose containing 2-sulfate ester groups. 6-sulfate ester groups reduce the gelling power considerably, but by alkali treatment it is possible to eliminate 6-sulfate groups, which results in the

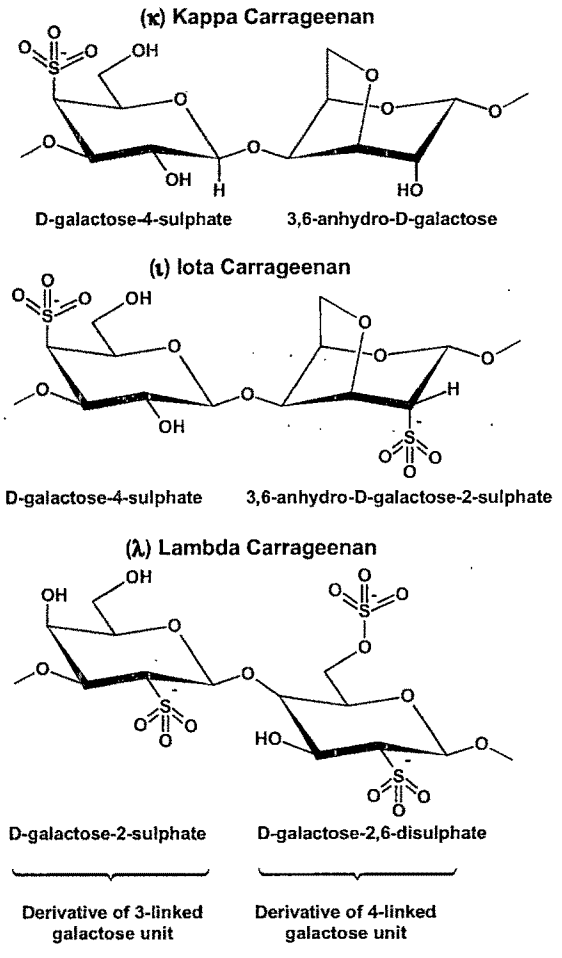


Figure 3.7: Different types of carrageenans.

formation of 3,6-anhydro-D-galactose imparting a higher degree of regularity to the molecule and thus the gelling power is increased. Kappa-type extracts are made from *Eucheuma cottonii* and some *Chondrus* and *Gigartina* species. Iota carrageenan is characterized by having 4-sulfate ester groups on all D-galactose residues and 2-sulfate ester groups on all 3,6-anhydro-D-galactose residues. 2-sulfate ester groups are not removed by alkali treatment. As in the case of kappa carrageenan, iota carrageenan also shows irregularities in the form of 6-sulfate ester groups on some D-galactose residues and through alkali treatment the iota carrageenan becomes more regular. Lambda carrageenan differs from kappa and iota carrageenan by having a disulfated-D-galactose residue and no 4-sulfate in the D-galactose residue. Instead of 4-sulfate ester groups lambda carrageenan contains variable amounts of 2-sulfate ester groups.

Carrageenan has strong, broad absorption bands, typical of all polysaccharides, in the 1000 to 1100 cm^{-1} region. Absorption maxima are 1065 cm^{-1} and 1020 cm^{-1} for gelling and nongelling types, respectively. Other characteristic absorption bands and their intensities relative to the absorbance at 1050 cm^{-1} are as shown in the accompanying table (Table 3.2).

Table 3.2: Characteristic FTIR absorption bands of different types of carrageenans.

Wave number (cm^{-1})	Functional group	Absorbance relative to 1050 cm^{-1}		
		Kappa	Iota	Lambda
1210 -1260	Ester sulfate	0.7 – 1.2	1.2 – 1.6	1.4 – 2.0
1010 -1080	Glycosidic linkage	vs	vs	vs
928 - 933	3,6-anhydro-D-galactose	0.3 – 0.6	0.2 – 0.4	0.0 – 0.2
840 - 850	D-galactose-4-sulfate	0.3 – 0.5	0.2 – 0.4	a
825 - 830	D-galactose-2-sulfate	a	a	0.2 – 0.4
810 - 820	D-galactose-6-sulfate	a	a	0.1 – 0.3
800 - 805	3.6-anhydro-D-galactose-2-sulfate	0.0 – 0.2	0.2 – 0.4	a

vs = very strong s = strong m = medium l = low a = absent

3.2.2.1. Solubility

Carrageenan is water soluble and insoluble in most organic solvents. Water miscible alcohols and ketones, while themselves non-solvents for carrageenan, are tolerated in admixture with carrageenan solutions at levels up to 40%. More highly polar solvents, such as formamide and N,N-dimethylformamide, are tolerated in still higher proportion and alone cause a marked swelling of the polymer. The solubility characteristics (Table 3.3) of carrageenan in water are influenced by a number of factors most important of which are (a) the type of carrageenan, (b) counter ions present, (c) other solutes, (d) temperature, and (e) pH.

Table 3.3: Solubility of different types of carrageenan in different medium.

Medium	Kappa	Iota	Lambda
Hot water	Soluble above 60°C (140°F)	Soluble above 60°C (140°F)	Soluble
Cold water	Sodium salt soluble. Potassium and calcium salt, insoluble	Sodium salt soluble Calcium salts gives thixotropic dispersions	Soluble
Hot milk	Soluble	Soluble	Soluble
Cold milk	Sodium salt, calcium salt and potassium salt insoluble, but swells markedly	Insoluble	Soluble

3.2.2.2. Stability in Solution

Acid and oxidizing agents may hydrolyze carrageenan in solution leading to loss of physical properties through cleavage of glycosidic bonds. Acid hydrolysis depends on pH, temperature and time. The acid hydrolysis takes place only when the carrageenan is dissolved, and the hydrolysis is accelerated as the processing temperature and/or the processing time is increased. However, when the carrageenan is in its gelled state the acid hydrolysis no longer takes place.

Table 3.4: Stability of different types of carrageenan solutions.

Stability	Kappa	Iota	Lambda
At the neutral and alkaline pH	Stable	Stable	Stable
At acid pH	Hydrolyzed in solution when heated. Stable in gelled form.	Hydrolyzed in solution. Stable in gelled form.	Hydrolyzed

3.2.2.3. Reaction with other Electrically Charged Hydrocolloids

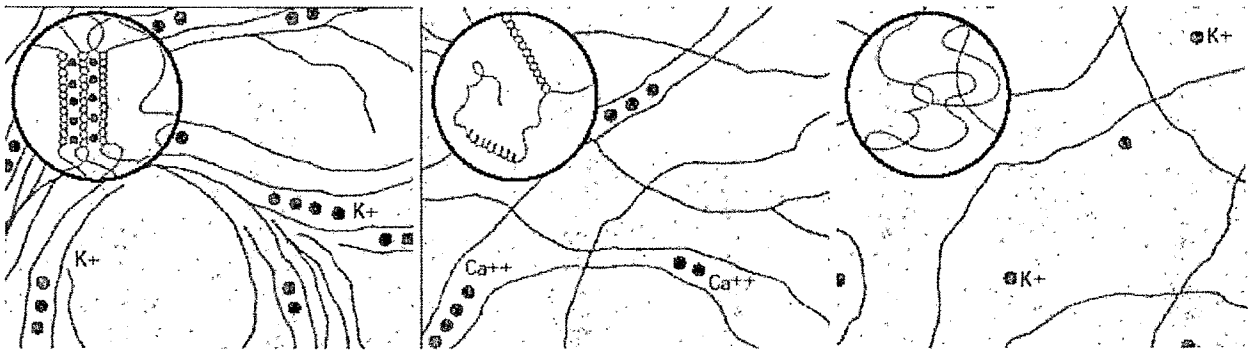
Carrageenan may interact with other charged macromolecules, e.g. proteins, to give various effects such as viscosity increase, gel formation, stabilization or precipitation. The result of the carrageenan-protein interaction is highly dependent on pH of the system and the isoelectric pH of the protein.

3.2.3. GELLING MECHANISM & RHEOLOGY

When a polyelectrolyte (like carrageenan) is combined with a uni/multivalent ion of the opposite charge, it may form a physical hydrogel known as an 'ionotropic' hydrogel. Ionotropic hydrogel, which may degrade and eventually disintegrate and dissolve, are held together by molecular entanglements, and/or secondary forces including ionic, H-bonding or hydrophobic forces (Prestwich et al., 1998). All of these interactions are reversible, and can be disrupted by changes in physical conditions such as ionic strength, pH, temperature, application of stress, or addition of specific solutes that compete with the polymeric ligand for the affinity site on the protein. The structure of kappa and iota carrageenan allows segments of the two molecules to form so called double helices which bind the chain molecules in the three dimensional network, a gel. Lambda carrageenan has a structure that does not allow such double helix formation.

Table 3.5: Gelling mechanism of different types of carrageens.

	Kappa	Iota	Lambda
Effect of cations	Gels most strongly with potassium ions	Gels most strongly with calcium ions	Non-gelling
Type of gel	Strong and brittle with syneresis	Elastic and cohesive without syneresis	Non-gelling
Synergistic effect with locust bean gum	High	High	None
Freeze/thaw stability	None	Stable	None



Kappa Iota lambda

Figure 3.8: Schematic presentation of gelling mechanism of different types of carrageenans.

When dissolved by heating, followed by cooling below certain temperatures, kappa and iota carrageenans form thermoreversible water gels in a concentration as low as 0.5%, provided gelling cations are present. Kappa carrageenan gels in the presence of potassium ions, the rigidity of the gel increasing with increasing potassium ion concentration. Potassium ions also have the effect of increasing the melting and gelling temperature.

Table 3.6: Effect of different metallic ions on ionic and hydrated radium of kappa-carrageenan gels.

	Li+	Na+	K+	Rb+	Cs+
Ionic radius, Å	0.60	0.95	1.33	1.48	1.69
Hydrated radius, Å	3.40	2.76	2.32	2.28	2.28
Helix radius, Å			1.9 – 1.4		

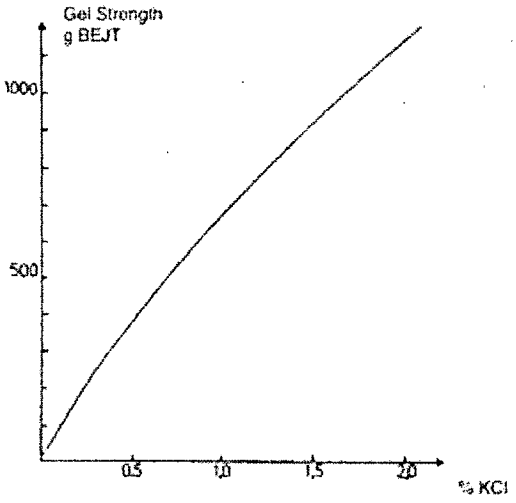


Figure 3.9: Effect of potassium chloride on gel strength of kappa carrageenan gel 1.50% kappa carrageenan.

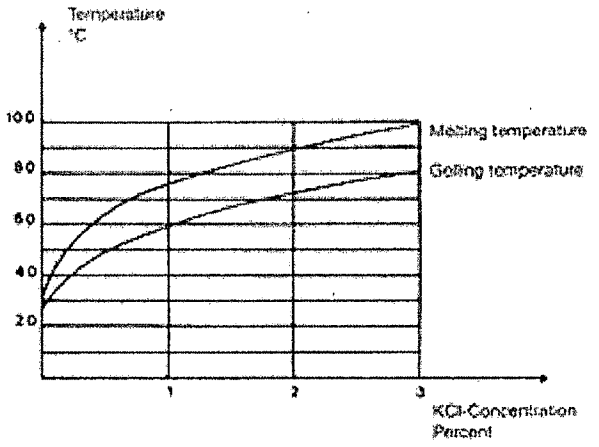


Figure 3.10: Effect of potassium chloride on gelling and melting temperature of carrageenan.

Calcium ions and barium ions increase the rigidity of a carrageenan gel, the effect being most pronounced when potassium ions are added as well.

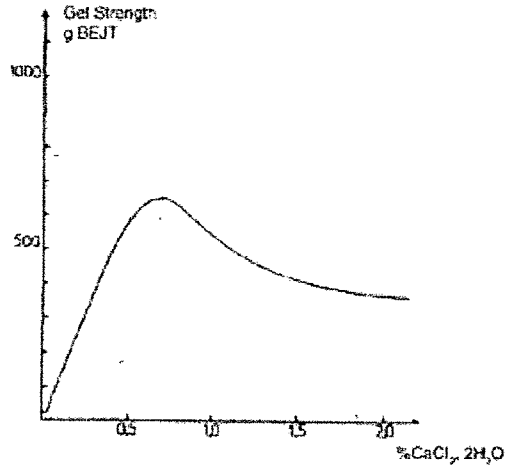


Figure 3.11: Effect of calcium chloride on gel strength of 1.50% kappa carrageenan gel.

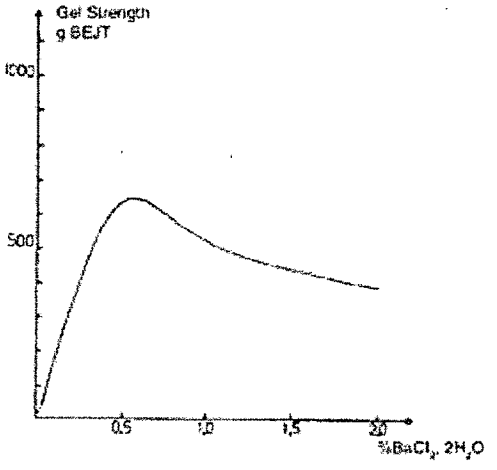


Figure 3.12: Effect of barium chloride on gel strength of 1.50% kappa carrageenan gel.

It is believed that calcium and barium ions form bridges between adjacent double helices through an electrostatic binding to two adjacent sulfate groups, thus stabilizing and strengthening the network. When removing cations which cause gelation of carrageenan

from the medium as well as from the carrageenan, a solution of carrageenan is obtained which does not form a gel irrespective of the temperature. Thus, the gelling temperature of a carrageenan solution is a function of the concentration of gelling cations present in the system. Kappa carrageenan forms strong and brittle gels which exude water (syneresis) and in many applications such textural properties are disadvantageous.

3.2.3.1. Rheology

Carrageenan solutions show pseudoplastic flow behavior as do most hydrocolloids: With increasing shear rate the viscosity decreases whereas the viscosity instantly increases as the shear rate is decreased. Solutions of carrageenan have low viscosity and are thus easy to handle. A kappa carrageenan water gel is irreversibly destroyed when subjected to shear. Kappa carrageenan water gels are thus not thixotropic.

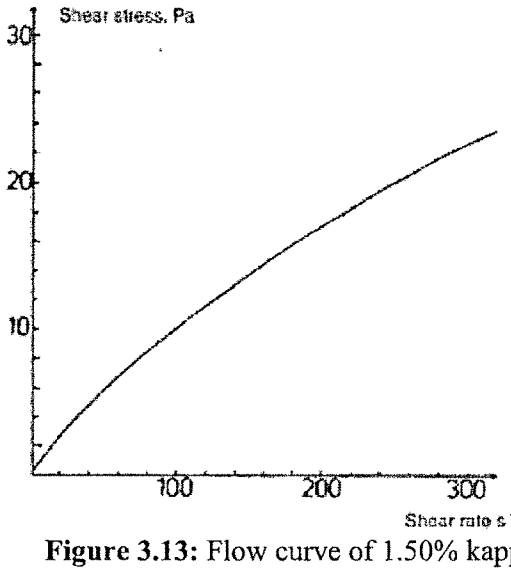


Figure 3.13: Flow curve of 1.50% kappa carrageenan water solution.

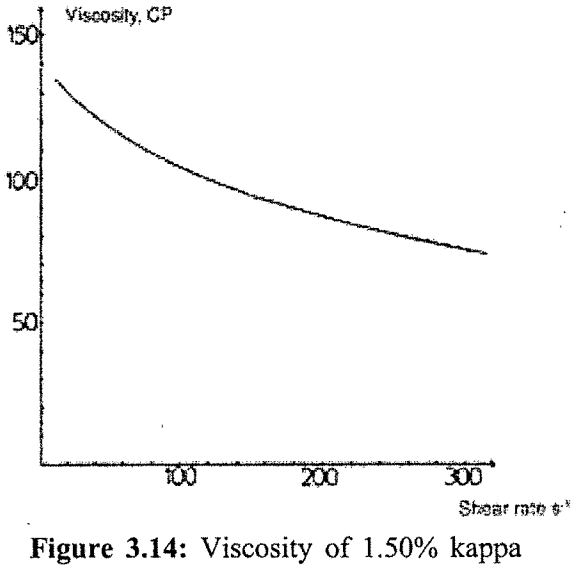


Figure 3.14: Viscosity of 1.50% kappa carrageenan water solution.

Because of the above characteristics, κ-carrageenan is used as an entrapment matrix for cells and enzymes as well as for pharmaceutical and food adjuvant. In the past, conventional crosslinked potassium-κ-carrageenan beads have been investigated for the development of a multiple unit drug delivery system. However, not even a single reference in the literature could be cited to date for improvisation of shelf-life of the entrapped enzyme in κ-carrageenan beads, hence was studied here.

3.2.4. CHARACTERIZATION

3.2.4.1. Fourier Transform Infra-Red Spectroscopy (FTIR)

. FTIR spectrum of κ-carrageenan powder (Figure 3.15) showed various distinct peaks: very broad band spreading 3150-3600 cm^{-1} (strong; s) due to polyhydroxy $(-OH)_n$ group; 2968 cm^{-1} (s), 2920 cm^{-1} (s), and 2850 cm^{-1} (medium; m) due to C-H stretch; 1425 cm^{-1} (s) and 1375 cm^{-1} (s) due to C-H deformation; 1225 cm^{-1} (s) due to S=O stretch of sulfate ester salt; 1070 cm^{-1} due to C-O stretch of cyclic ethers; 925 cm^{-1} (s) due to C-O stretch of polyhydroxy groups attached to carbons; etc.

3.2.4.2. Differential Scanning Calorimetry (DSC)

DSC thermogram of κ-carrageenan was obtained using an automatic thermal analyzer system (DSC-60, Shimadzu, Japan) and is shown in Figure 3.16. Broad endothermic peak at 85°C in the thermogram of κ-carrageenan was observed due to the presence of water molecules. Two minor peaks at 259° and 266°C and major exothermic peak at 344°C were due to the degradation exotherm κ-carrageenan.

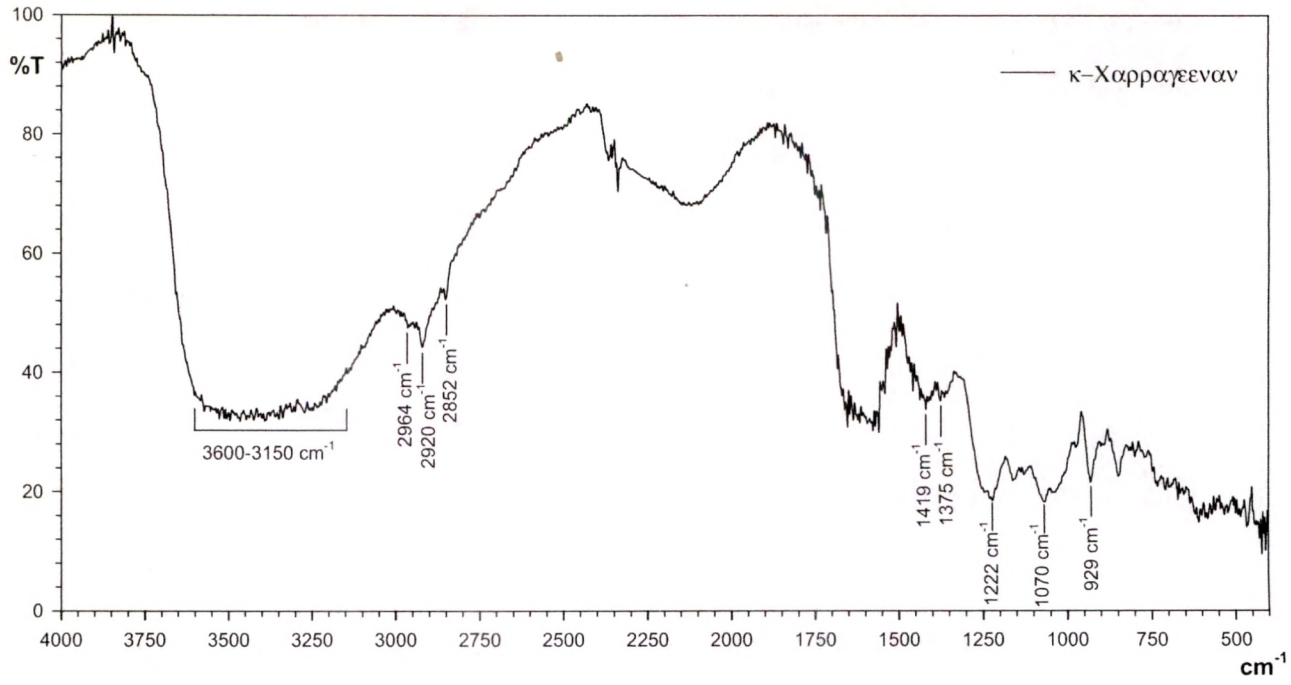


Figure 3.15: FTIR spectrum of sodium κ-carrageenan.

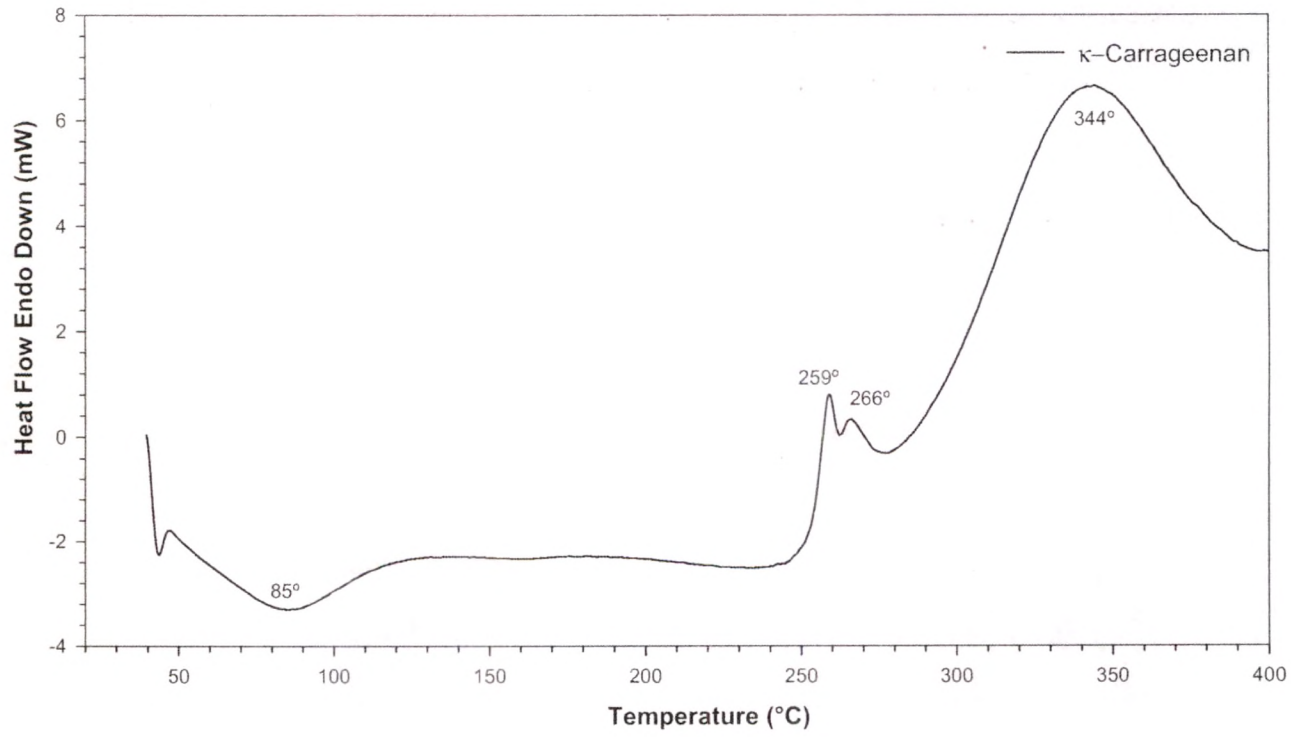


Figure 3.16: DSC thermogram of κ-carrageenan.

3.3. Chitosan

Chitin (β(1-4)-*N*-acetyl-D-glucosoamine), the second most abundant naturally occurring biopolymer after cellulose, is the major structural component of the invertebrate exoskeleton and the fungal cell wall (Ravi Kumar et al., 2004). Seafood processing waste, mainly from crab, shellfish, lobster, and shrimp, is an abundant chitin source. Chitosan, obtained by partial alkaline deacetylation of chitin, is a poly-cationic polysaccharide comprising of β-[1→4]-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc; A-unit) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN; D-unit) (Muzzarelli, 1973; Casal et al., 2005). Chitosan and chitin are commercially interesting compounds because of their high nitrogen content (6.89%, the repeating unit contains $-NH_2$ group on the C–2 positon) (Charlot et al., 2003) compared to synthetically substituted cellulose (1.25%). This makes chitosan a useful chelating agent (Rabea et al., 2003).

3.3.1. STURCTURE

Chitosan, obtained by partial alkaline deacetylation of chitin, is a poly-cationic polysaccharide comprising of β-[1→4]-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc; A-unit) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN; D-unit) (Figure 3.17) (Muzzarelli, 1973; Casal et al., 2005).

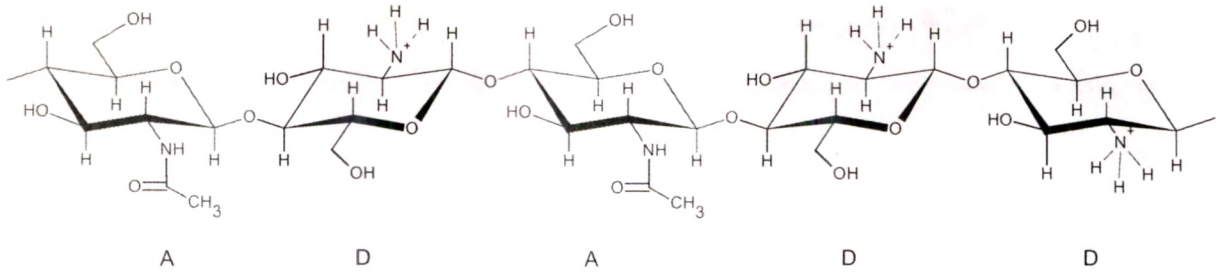


Figure 3.17: Chemical structure of chitosan, comprising of 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc; A-unit) & 2-amino-2-deoxy-β-D-glucopyranose (GlcN; D-unit).

3.3.2. POLYELECTROLYTE COMPLEX (PEC) FORMATION

At a high degree of protonation of the amino groups, the cationic chitosan spontaneously forms macromolecular complexes by reaction with anionic polyelectrolytes (Ravi Kumar et al., 2004). The formation of the polyanion-polycation (polyelectrolyte) complexes is mainly driven by an electrostatic mechanism as represented in Figure 3.18, where the exchange reaction of counterions with different valences is important (Maurstad et al., 2003). Charge neutralization and possible local overcompensation or bridging (such as hydrogen bonding, Coulomb forces, van der Waals forces, and transfer forces) mediated by a multivalent counterion induces attraction between topologically separated segments of the polyelectrolytes (Dumitriu and Chornet, 1998). PEC is characterized by a hydrophilic microenvironment with a high water content and electrical charge density. Besides polycationic and polyanionic biopolymers, PEC requires no auxiliary molecules (i.e. catalysts or initiators) also the reaction is generally performed in aqueous solution, and thus favors biocompatibility and avoids purification before administration. Since chitosan has a rigid, stereo-regular structure containing bulky pyranose rings, the formation of PEC can induce a conformational change of the other polyelectrolyte, if the latter has a non-rigid structure (Yao et al., 1997). PEC can also be reinforced by the

addition of ions inducing the formation of ionically cross-linked systems (i.e. Ca^{2+} can be added with alginate) (Daly and Knorr, 1988). Just as cross-linking density governs the properties of cross-linked hydrogels, the properties of PEC are mainly determined by the degree of interaction between the polymers. This latter depends essentially on their global charge densities and determines their relative proportion in the PEC. Indeed, the lower the charge density of the polymer, the higher is the polymer proportion in the PEC, since more polymeric chains are required to react with the other polymer. Thus, presence of salts, such as NaCl, may weaken the electrostatic attraction between the oppositely charged polyelectrolytes by contributing to the counterion environment and results in 'bulky' PEC (Sakiyama et al., 1999). PEC of different characteristics can be obtained by changing the chemical structure of component polymers, such as molecular weight, flexibility, functional group structure, charge density, hydrophilicity and hydrophobicity balance, stereoregularity and compatibility, as well as reaction conditions: pH, ionic strength, concentration, mixing ratio, and temperature (Dumitriu and Chornet, 1998).

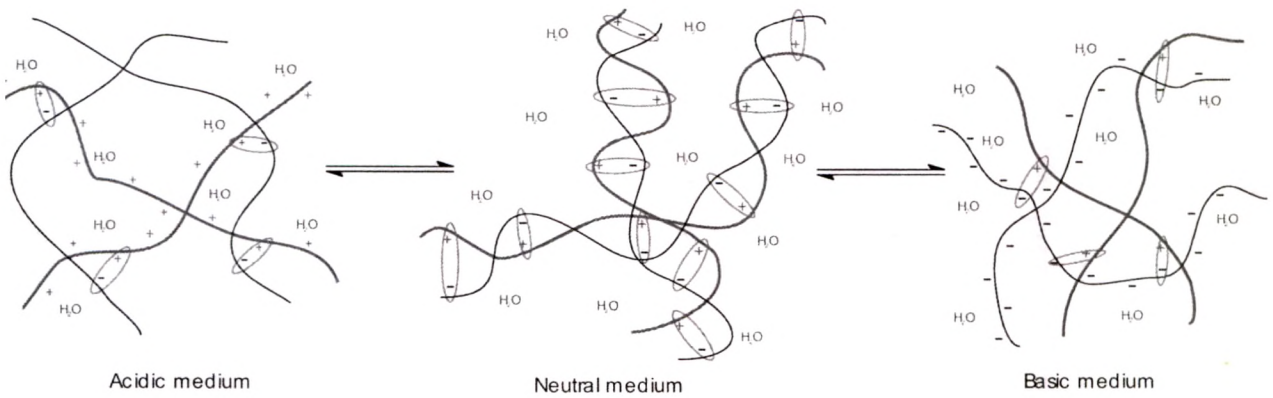


Figure 3.18: Structure of a chitosan-alginate PEC; +, positive charge of chitosan; –, negative charge of the alginate; ○, ionic interaction; + + +, chitosan; – – –, alginate.

Figure 3.19 depicts the schematic representation of the PEC membrane formation which may be divided into three main classes: (i) primary complex formation; (ii) formation process within intracomplexes; (iii) intercomplex aggregation process. Prior mixing

results in randomly arranged primary complex (due to secondary binding sources such as Coulomb forces (very rapid)), which upon further exposure converted to ordered secondary complex (due to formation of new bonds and/or the correction of the distortions of the polymer chain) and contains water molecules. Upon drying, PEC membrane undergoes intercomplex aggregation (because of hydrophobic interactions and/or drying), which constitute a network (Tsuchida, 1994).

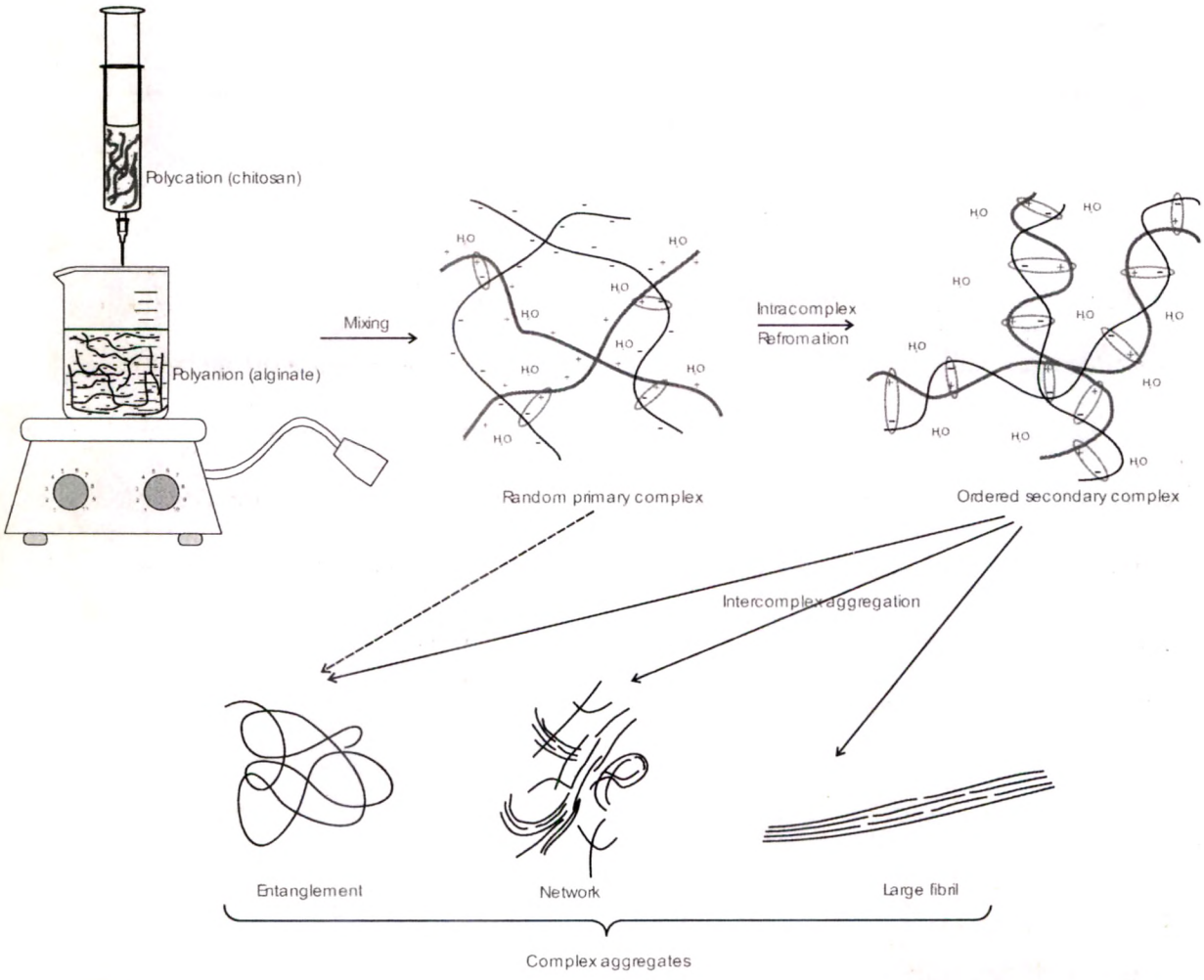


Figure 3.19: Schematic representation of PEC membrane formation.

Beads produced from sodium alginate, with calcium chloride present in the chitosan solution, bind ~100 times more chitosan than capsules produced by dropping the alginate solution into a chitosan solution in the absence of salt and most of the beads, micro-,

macro-capsules, microspheres, are produced by these methods. Reversed chitosan-alginate complex coacervate capsules, formed by the dropwise addition of chitosan solution into alginate solution, were reported to be fragile even following hardening for 3 h (Daly and Knorr, 1988). To avoid the limitation of this reverse coacervation, the present study employs a novel approach, wherein chitosan and alginate were reacted at their completely ionized state in order to increase the counterion charge density of the polymers which increase the overall interaction and hence the mechanical strength of the PEC membrane. This was achieved by maintaining the chitosan and alginate solution pH at 2 and 6.5 respectively. Further, the chitosan solution at pH 2 suppresses the chitolytic activity of the added α-amylase, which is active in the pH range of 4–5.

3.3.3. APPLICATIONS OF CHITOSAN AND CHITOSAN-ALGINATE PEC

Chitosan is currently receiving a great deal of attention for medical and pharmaceutical applications. The main reasons for this increasing interest are undoubtedly due to its appealing intrinsic properties. Indeed, chitosan is known for its use in various medical `applications such as topical ocular application (Alonso and Sanchez, 2003), implantation (Hoemann et al., 2005; Jiang et al., 2005)or injection (Kwak et al., 2005; Lu et al., 2006). In addition, it has been reported that chitosan acts as a penetration enhancer by opening epithelial tight-junctions. This polysaccharide becomes water-soluble in acidic conditions (pH < 6), leading to the preparation of biocompatible and often biodegradable polymer solutions (Khan et al., 2000). Also it has excellent cell adhesive properties (He et al., 1998), promotes wound-healing (Ueno et al., 2001) and has bacteriostatic effects (Liu et al., 2001). Moreover, chitosan is metabolized by certain human enzymes, e.g. lysozyme (Muzzarelli, 1997), α-amylase (Muzzarelli, 1997), hyaluronidase, and can be considered

as biodegradable (Muzzarelli, 1997). Finally, chitosan is abundant in nature, and its production is of low cost and is ecologically interesting (Peter, 1995). Potential fields of application of polyelectrolyte complexes are: as membranes for different end uses (Alexakis et al., 1995; Wang et al., 2002); coatings on films and fibres (Wang et al., 2001); implants for medical use; microcapsules (Haque et al., 2005; Iyer et al., 2005; Zheng et al., 2005); supports for catalysts (Sardar et al., 2003); binding of pharmaceutical products (Ribeiro et al., 1999); isolation and fractionation of proteins (Wibowo et al., 2005).

3.3.4. CHARACTERIZATION

3.3.4.1. Fourier Transform Infra-Red Spectroscopy (FTIR)

FTIR spectrum of chitosan (Figure 3.20) showed, absorption bands at 3500–3100 cm^{-1} ascribed to combined peaks of O–H stretching (polyhydroxy $(–OH)_n$ carbohydrate) and intermolecular hydrogen bonding. The N–H stretching from primary amines are overlapped in the same region (Borges et al., 2005). Also it showed a weak band at 2875 cm^{-1} due to C–H stretching, the bridge oxygen C–O–C (cyclic ether) stretching band at 1151 cm^{-1}, and the C–O stretching bands 1070, 1031, and 893 cm^{-1} (Khor, 2001). The carbonyl (C=O) stretching of secondary amide (amide I band) absorption bands at 1655 cm^{-1}, N–H bending vibration of non-acylated 2-aminoglucose primary amines band at 1570 cm^{-1}, and N–H bending vibrations of (*N*-acetylated residues) amide II band at 1558 cm^{-1} were observed as well (Le-Tien et al., 2004). The peaks at 1419 cm^{-1} and 1321 cm^{-1} belongs to the N–H stretching of the amide and ether bonds and N–H stretching (amide III band) respectively.

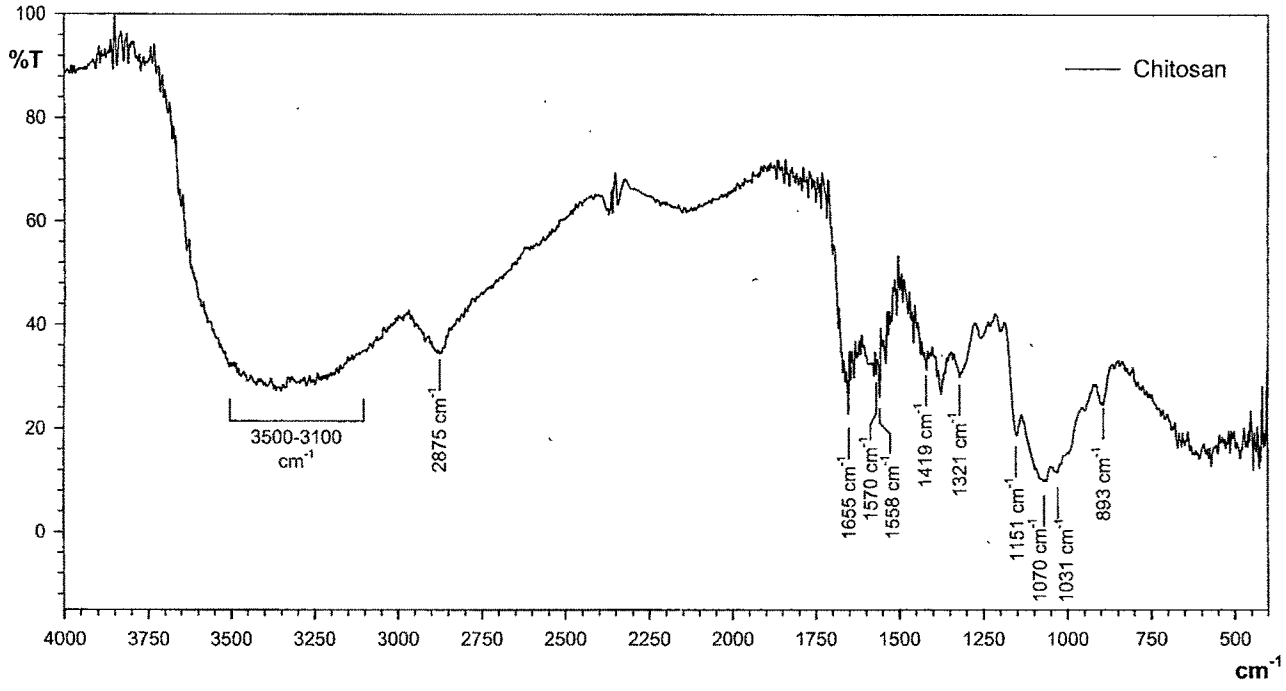


Figure 3.20: FTIR spectrum of sodium chitosan.

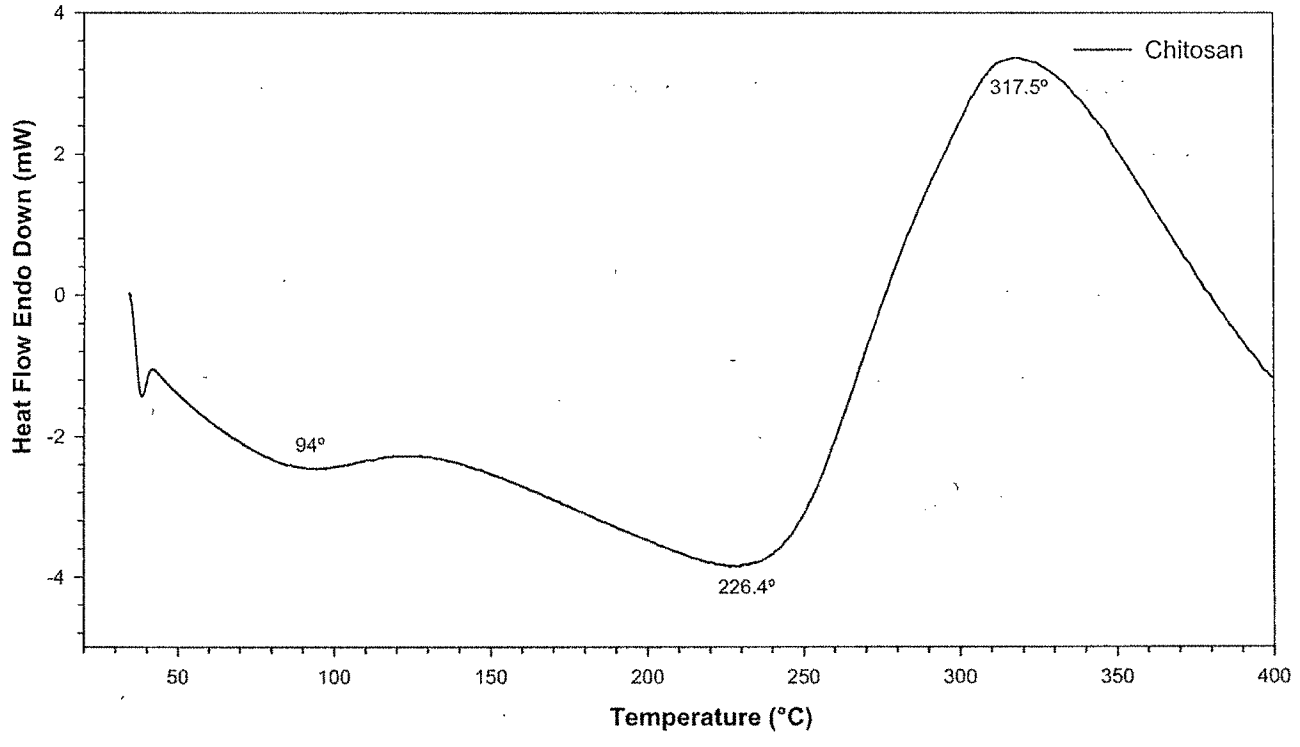


Figure 3.21: DSC thermogram of chitosan.

3.3.4.2. Differential Scanning Calorimetry (DSC)

DSC thermogram of chitosan was obtained using an automatic thermal analyzer system (DSC-60, Shimadzu, Japan) and is shown in Figure 3.21. The thermogram of the chitosan polymer exhibited an endothermic peak at about 94°C that has been attributed to the evaporation of absorbed water. The exothermic baseline deviation beginning around 250°C indicates the onset of chitosan degradation (Khalid et al., 2002; Borges et al., 2005).

3.3.5. CHITOSAN DIGESTED BY PEPSIN

In our study, we used the commercial porcine pepsin as one enzyme and it was observed that, addition of pepsin to the chitosan solution decrease its viscosity drastically and make it difficult to drop in to the alginate solution. This may be due to presence of chitosanase isozymes in the commercial pepsin samples, which digest chitosan rapidly in acidic environment maintained to dissolve the chitosan. Hence this system was not studied further. Yalpani and Pantaleone (Yalpani and Pantaleone, 1994) recently reported that a substantial number of commercially available crude enzyme preparations, including pepsin, lipase, and glucanases, among others, display lytic activity toward chitosan. These investigators, however, did not purify and characterize the chitosanolytic enzyme. On the other hand, Liao et al. (Liao et al., 2000) partially purified chitosanolytic enzyme from a commercial pepsin preparation and demonstrated the presence of chitosanase. Recently Fu et al. (Fu et al., 2003) described further purification and characterization of three chitosanolytic enzymes isolated from a commercial porcine pepsin preparation. All three isozymes hydrolyzed chitosan polymer as well as *N, N', N''*-triacetylchitotriose oligomer.

3.4. References

Kelco Inc., Alginate Products for Scientific Water Control Third Edition, (1987).

ALEXAKIS, T.; BOADI, D. K.; QUONG, D.; GROBOILLOT, A.; O'NEILL, I.; PONCELET, D. and NEUFELD, R. J. (**1995**). Microencapsulation of DNA within alginate microspheres and crosslinked chitosan membranes for in vivo application. *Appl Biochem Biotechnol* 50:*(1)*, 93-106.

ALONSO, M. J. and SANCHEZ, A. (**2003**). The potential of chitosan in ocular drug delivery. *J Pharm Pharmacol* 55:*(11)*, 1451-1463.

ARICA, B.; CALIS, S.; KAS, H. S.; SARGON, M. F. and HINCAL, A.A. (**2002**). 5-Fluorouracil encapsulated alginate beads for the treatment of breast cancer. *Int. J. Pharm.* 242:*(1-2)*, 267-269.

ATKINS, E. D. T.; MACKIE, W.; PARKER, K. D. and SMOLKO, E. E. (**1971**). Crystalline structure of poly-D-mannuronic and poly-L-guluronic acids. *J. Polymer Sci. Part B:Polymer Lett.* 9, 311-316.

BORGES, OLGA; BORCHARD, GERRIT; VERHOEF, J. COOS; SOUSA, ADRIANO DE and JUNGINGER, HANS E. (**2005**). Preparation of coated nanoparticles for a new mucosal vaccine delivery system. *Int. J. Pharm.* 299, 155-166.

BOUHADIR, K. H.; KRUGER, G. M.; LEE, K. Y. and MOONEY, D. J. (**2000**). Sustained and controlled release of daunomycin from cross-linked poly(aldehyde guluronate) hydrogels. *J Pharm Sci* 89:*(7)*, 910-919.

CASAL, E.; CORZO, N.; MORENO, F. J. and OLANO, A. (**2005**). Selective recovery of glycosylated caseinmacropeptide with chitosan. *J. Acric. Food Chem.* 53, 1201-1204.

CHARLOT, AURÉLIA; AUZÉLY-VELTY, RACHEL and RINAUDO, MARGUERITE (**2003**). Specific interactions in model charged polysaccharide systems. *J. Phys. Chem. B* 107, 8248-8254.

DALY, M. M. and KNORR, D. W. (**1988**). Chitosan-alginate complex coacervate capsules: Effects of calcium choloride, plasticizersand polyelectrolytes on mechanical stability. *Biotechnol. Prog.* 4, 76-81.

DUMITRIU, SEVERIAN and CHORNET, ESTEBAN (**1998**). Inclusion and release of proteins from polysaccharide-based polyion complexes. *Adv. Drug Deliv. Rev.* 31, 223-246.

EFENTAKIS, M. and BUCKTON, G. (**2002**). The effect of erosion and swelling on the dissolution of theophylline from low and high viscosity sodium alginate matrices. *Pharm Dev Technol* 7:*(1)*, 69-77.

FU, JU-YUEH; WU, SHENG-MING; CHANG, CHEN-TIEN and SUNG, HSIEN-YI (**2003**). Characterization of threechitosanase isozymes isolated from a commercial crude porcine pepsin preparation. *J. Agric. Food Chem.* 51, 1042-1048.

GARCIA, A. M. and GHALY, E. S. (**1996**). Preliminary spherical agglomerates of water soluble drug using natural polymer and cross-linking technique. *J. Controlled Release* 40, 179-186.

GRANT, G. T.; MORRIS, E. R.; REES, D. A.; SMITH, P. J. C. and THOM, D. (**1973**). Biological Interactions Between Polysaccharides and Divalent Cations: The Egg-Box Model. *FEBS Lett.* 32, 195-198.

GURSOY, A. and CEVIK, S. (**2000**). Sustained release properties of alginate microspheres and tabletted microspheres of diclofenac sodium. *J Microencapsul* 17:*(5)*, 565-575.

HAQUE, T.; CHEN, H.; OUYANG, W.; MARTONI, C.; LAWUYI, B.; URBANSKA, A. M. and PRAKASH, S. (**2005**). In vitro study of alginate-chitosan microcapsules: an alternative to liver cell transplants for the treatment of liver failure. *Biotechnol Lett* 27:*(5)*, 317-322.

HAUG, A. (**1964**). Composition and properties of alginates. *Rep. Norw. Inst. Seaweed Res.* 30, 25-45.

HE, P.; DAVIS, S. S. and ILLUM, L. (**1998**). In vitro evaluation of the mucoadhesive properties of chitosan microspheres. *Int. J. Pharm.* 166, 75-88.

HE, ZHIMIN and WANG, KANG (**2002**). Alginate-konjac glucomannan-chitosan beads as controlled release matrix. *Int. J. Pharm.* 244, 117-126.

HOEMANN, C. D.; HURTIG, M.; ROSSOMACHA, E.; SUN, J.; CHEVRIER, A.; SHIVE, M. S. and BUSCHMANN, M. D. (**2005**). Chitosan-glycerol phosphate/blood implants improve hyaline cartilage repair in ovine microfracture defects. *J Bone Joint Surg Am* 87:*(12)*, 2671-2686.

HUA, L.; DACHUAN, X.; LEI, Y.; SHAOHU, X.; XIAOJING, L.; TAO, H. and SHIZHENG, Z. (**2003**). Microencapsulation and culture in vitro of rat pinealocytes. *Int J Artif Organs* 26:*(10)*, 958-964.

IYER, C.; PHILLIPS, M. and KAILASAPATHY, K. (**2005**). Release studies of Lactobacillus casei strain Shirota from chitosan-coated alginate-starch microcapsules in ex vivo porcine gastrointestinal contents. *Lett Appl Microbiol* 41:*(6)*, 493-497.

JIANG, H.; WANG, Y.; HUANG, Q.; LI, Y.; XU, C.; ZHU, K. and CHEN, W. (**2005**). Biodegradable hyaluronic acid/n-carboxyethyl chitosan/protein ternary complexes as implantable carriers for controlled protein release. *Macromol Biosci* 5:*(12)*, 1226-1233.

KHALID, M. N.; AGNELY, F.; YAGOUI, N.; GROSSIORD, J. L. and COUARRAZE, G. (**2002**). Water sate characterization, swelling behavior, thermal and mechanical properties of chitosan based networks. *Eur. J. Pharm. Sci.* 15, 425-432.

KHAN, T. A.; PEH, K. K. and CHING, H. S. (**2000**). Mechanical, bioadhesive strength and biological evaluations of chitosan films for wound dressing. *J. Pharm. Pharm. Sci.* 3, 303-311.

KHOR, E. (**2001**). The structural properties of chitin as it is known today. *Chitin: Fulfilling a Biomaterials Promise*, Elsevier, New York, pp. 73-82.

KWAK, C.; HONG, S. K.; SEONG, S. K.; RYU, J. M.; PARK, M. S. and LEE, S. E. (**2005**). Effective local control of prostate cancer by intratumoral injection of (166)Ho-chitosan complex (DW-166HC) in rats. *Eur J Nucl Med Mol Imaging* 32:*(12)*, 1400-1405.

LEE, B.; MIN, G. and KIM, T. (**1996**). Preparation and in vitro release of melatonin loaded multivalent cationic alginate beads. *Arch. Pharm. Res.* 19, 280-285.

LE-TIEN, CANH; MILLETTE, MATHIEU; MATEESCU, MIRCEA-ALEXANDRU and LACROIX, MONIQUE (**2004**). Modified alginate and chitosan for lactic acid bacteria immobilization. *Biotechnol. Appl. Biochem.* 39, 347-354.

LIAO, I. C.; WAN, A. C.; YIM, E. K. and LEONG, K. W. (**2005**). Controlled release from fibers of polyelectrolyte complexes. *J Control Release* 104:*(2)*, 347-358.

LIAO, Y. M.; SU, J. L.; CHIANG, C. L. and CHANG, C. T. (**2000**). Studies on the degradation of chitosan by crude proteolytic enzymes from hog stomach mucosa and *Bacillus subtilis*. *Taiwanese J. Agric. Chem. Food Sci.* 38, 239-247.

LIN, S. S.; UENG, S. W.; LEE, S. S.; CHAN, E. C.; CHEN, K. T.; YANG, C. Y.; CHEN, C. Y. and CHAN, Y. S. (**1999**). In vitro elution of antibiotic from antibiotic-impregnated biodegradable calcium alginate wound dressing. *J Trauma* 47:*(1)*, 136-141.

LIU, X. F.; GUAN, Y. L.; YAND, D. Z.; LI, Z. and YAO, K. D. (**2001**). Antibacterial actins of chitosan and carboxymethylated chitosan. *J. Apppl. Polym. Sci.* 79, 1324-1335.

LU, B.; XIONG, S. B.; YANG, H.; YIN, X. D. and ZHAO, R. B. (**2006**). Mitoxantrone-loaded BSA nanospheres and chitosan nanospheres for local injection against breast cancer and its lymph node metastases II: Tissue distribution and pharmacodynamics. *Int J Pharm* 307:*(2)*, 175-181.

MARTINSEN, A.; SKJAK-BRAEK, G. and SMIDSROD, O. (**1989**). Alginate as immobilization material: I. Correlation between chemical and physical properties of alginate gel beads. *Biotechnol. Bioeng.* 33, 79-89.

MARTINSEN, A.; STORRO, I. and SKJAK-BRAEK, G. (**1992**). Alginate as immobilization material: III. Diffusional properties. *Biotechnol. Bioeng.* 39, 186-194.

MAURSTAD, GJERTRUD; DANIELSEN, SIGNE and STOKKE, BJØRN (**2003**). Analysis of compacted semiflexible polyanions visualized by atomic force microscopy: Influence of chain stiffness on the morphologies of polyelectrolyte complexes. *J. Phys. Chem. B* 107, 8172-8180.

MITREVEJ, A.; SINCHAIPANID, N.; RUNGVEJHAVUTTIVITTAYA, Y. and KOSITCHAIYONG, V. (**2001**). Multiunit controlled-release diclofenac sodium capsules using complex of chitosan with sodium alginate or pectin. *Pharm Dev Technol* 6:*(3)*, 385-392.

MORRIS, E. R.; REES, D. A. and THOM, D. (**1973**). Characterization of polysaccharide structure and interactions by circular dichroism: Order disorder transition in the calcium alginate system. *Chem. Comm.*, 245-246.

MURATA, Y.; JINNO, D.; KOFUJI, K. and KAWASHIMA, S. (**2004**). Properties of calcium-induced gel beads prepared with alginate and hydrolysates. *Chem Pharm Bull (Tokyo)* 52:*(5)*, 605-607.

MUZZARELLI, R. A. A. (**1973**). Chitosan. in: MUZZARELLI, R. A. A. (Ed.) *Natural Chelating Polymers*, Pergamon Press, Oxford, pp. 144-176.

MUZZARELLI, R. A. A. (**1997**). Human enzymatic activities related to the therapeutic administration of chitin derivatives. *Cell Mol. Life Sci.* 53, 131-140.

NOKHODCHI, A. and TAILOR, A. (**2004**). In situ cross-linking of sodium alginate with calcium and aluminum ions to sustain the release of theophylline from polymeric matrices. *Farmaco* 59:*(12)*, 999-1004.

OH, S. H.; KIM, J. K.; SONG, K. S.; NOH, S. M.; GHIL, S. H.; YUK, S. H. and LEE, J. H. (**2005**). Prevention of postsurgical tissue adhesion by anti-inflammatory drug-loaded pluronic mixtures with sol-gel transition behavior. *J Biomed Mater Res A* 72:*(3)*, 306-316.

OHTA, M.; SUZUKI, Y.; CHOU, H.; ISHIKAWA, N.; SUZUKI, S.; TANIHARA, M.; MIZUSHIMA, Y.; DEZAWA, M. and IDE, C. (**2004**). Novel heparin/alginate gel combined with basic fibroblast growth factor promotes nerve regeneration in rat sciatic nerve. *J Biomed Mater Res A* 71:*(4)*, 661-668.

PARK, P.G.; SHALABY, W.S.W. and PARK, H. (**1993a**). Ch. 5. *Biodegradable Hydrogels for Drug Delivery*, Technomic, Lancaster, pp. 99-140.

PARK, P.G.; SHALABY, W.S.W. and PARK, H. (**1993b**). Physical Gels. *Biodegradable Hydrogels for Drug Delivery*, Technomic Publishing Compony, Inc. (now CRC Press), Lancaster, PA, pp. 99-140.

PETER, M. G. (**1995**). Applications and environmental aspects of chitin and chitosan. *J. Macromol. Sci.* A32, 629-640.

PICKER, KATHARINA M. (**1999**). Matrix tablets of carrageenans. II. Release behavior and effect of added cations. *Drug Dev. Ind. Pharm.* 25:*(3)*, 339-346.

PILLAY, V.; DANCKWERTS, M. P.; MUHIDINOV, Z. and FASSIHI, R. (**2005**). Novel modulation of drug delivery using binary zinc-alginate-pectinate polyspheres for zero-order kinetics over several days: experimental design strategy to elucidate the crosslinking mechanism. *Drug Dev Ind Pharm* 31:*(2)*, 191-207.

PILLAY, V.; DANGOR, C.M.; GOVENDER, T.; MOOPANAR, K.R. and HURBANS, N. (**1998**). Drug release modulation from crosslinked calcium alginate microdiscs. 1. Evaluation of the concentration dependency of sodium alginate on drug entrapment capacity, morphology and dissolution rate. *Drug Deliv.* 5, 25-34.

PILLAY, V. and FASSIHI, R. (**1999**). In vitro release modulation from crosslinked pellets for site-specific drug delivery to the gastrointestinal tract. I. Comparison of pH-responsive drug release and associated kinetics. *J Control Release* 59:*(2)*, 229-242.

PRESTWICH, G. D.; MARECAK, D. M.; MARECAK, J. F.; VERCRUYSSE, K. P. and ZIEBELL, M. R. (**1998**). Controlled chemical modification of hyaluronic acid. *J. Control. Rel.* 53, 93-103.

RABEA, ENTSAR I.; BADAWY, MOHAMED E. -T.; STEVENS, CHRISTIAN V.; SMAGGHE, GUY and STEURBAUT, WALTER (**2003**). Chitosan as antimicrobial agent: Applications and mode of action. *Biomaterials* 4:*(6)*, 1457-1465.

RASTELLO DE BOISSESON, M.; LEONARD, M.; HUBERT, P.; MARCHAL, P.; STEQUERT, A.; CASTEL, C.; FAVRE, E. and DELLACHERIE, E. (**2004**). Physical alginate hydrogels based on hydrophobic or dual hydrophobic/ionic interactions: bead formation, structure, and stability. *J Colloid Interface Sci* 273:*(1)*, 131-139.

RAVI KUMAR, M. N. V.; MUZZARELLI, R. A. A.; MUZZARELLI, C.; SASHIWA, H. and DOMB, A. J. (**2004**). Chitosan chemistry and pharmaceutical perspectives. *Chem. Rev.* 104, 6017-6084.

REES, D.A. (**1969**). Structure Conformation and Mechanism in the Formation of Polysaccharide Gels and Networks. in: WOLFROM, M. L. and TIPSON, R. S. (Eds.), *Advances in Carbohydrate Chemistry and Biochemistry*, Vol. 24, Academic Press, New York, pp. 267-332.

RIBEIRO, A. J.; NEUFELD, R. J.; ARNAUD, P. and CHAUMEIL, J. C. (**1999**). Microencapsulation of lipophilic drugs in chitosan-coated alginate microspheres. *Int J Pharm* 187:*(1)*, 115-123.

SAKIYAMA, T.; TAKATA, H.; KIKUCHI, M. and NAKANISHI, K. (**1999**). Polyelectrolyte complex gel with high pH-sensitivity prepared from dextran sulfate and chitosan. *J. Apppl. Polym. Sci.* 73, 2227-2233.

SARDAR, M.; ROY, I. and GUPTA, M. N. (**2003**). A smart bioconjugate of alginate and pectinase with unusual biological activity toward chitosan. *Biotechnol Prog* 19:*(6)*, 1654-1658.

SHI, X. W.; DU, Y. M.; SUN, L. P.; YANG, J. H.; WANG, X. H. and SU, X. L. (**2005**). Ionically crosslinked alginate/carboxymethyl chitin beads for oral delivery of protein drugs. *Macromol Biosci* 5:*(9)*, 881-889.

SIPAHIGIL, O. and DORTUNC, B. (**2001**). Preparation and in vitro evaluation of verapamil HCl and ibuprofen containing carrageenan beads. *Int. J. Pharm.* 228, 119-128.

SMIDSROD, O. and HAUG, A. (**1972**). Properties of poly(1,4-hexuronates) in gel state. II. Comparison of gels of different chemical composition. *Acta. Chem. Scand.* 26, 79-88.

SUGAWARA, S.; IMAI, T. and OTAGIRI, M. (**1994**). The controlled release of prednisolone using alginate gel. *Pharm Res* 11:*(2)*, 272-277.

SUZUKI, S. and LIM, J. K. (**1994**). Microencapsulation with carrageenan-locust bean gum in a multiphase emulsification technique for sustained drug release. *J. Microencapsul.* 11, 197-203.

TALUKDER, R. and FASSIHI, R. (**2004**). Gastroretentive delivery systems: hollow beads. *Drug Dev Ind Pharm* 30:*(4)*, 405-412.

TANAKA, H.; MATSUMURA, M. and VELIKY, I. A. (**1984**). Diffusion characteristics of substrates in Ca-alginate gel beads. *Biotechnol. Bioeng.* 26, 53-58.

TSUCHIDA, E. (**1994**). Formation of polyelectrolyte complexes and their structures. *J. Macromol. Sci. Pure Appl. Chem.* A31, 1-15.

UENG, S. W.; YUAN, L. J.; LEE, N.; LIN, S. S.; CHAN, E. C. and WENG, J. H. (**2004**). In vivo study of biodegradable alginate antibiotic beads in rabbits. *J Orthop Res* 22:*(3)*, 592-599.

UENO, H.; MORI, T. and FUJINAGA, T. (**2001**). Topical formulations and wound healing applicatins of chitosan. *Adv. Drug Deliv. Rev.* 52, 105-115.

WANG, L.; KHOR, E. and LIM, L. Y. (**2001**). Chitosan-alginate-$CaCl_2$ system for membrane coat application. *J Pharm Sci* 90:*(8)*, 1134-1142.

WANG, L.; KHOR, E.; WEE, A. and LIM, L. Y. (**2002**). Chitosan-alginate PEC membrane as a wound dressing: Assessment of incisional wound healing. *J Biomed Mater Res* 63:*(5)*, 610-618.

WIBOWO, S.; VELAZQUEZ, G.; SAVANT, V. and TORRES, J. A. (**2005**). Surimi wash water treatment for protein recovery: effect of chitosan-alginate complex concentration and treatment time on protein adsorption. *Bioresour Technol* 96:*(6)*, 665-671.

YALPANI, M. and PANTALEONE, O. (**1994**). An examination of the unusual susceptibilities of aminoglycans to enzymatic hydrolysis. *Carbohydr. Res.* 256, 159-175.

YAO, K. D.; TU, H.; CHENG, F.; ZHANG, J. W. and LIU, J. (**1997**). pH-sensetivity of the swelling of a chitosan-pectin polyelectrolyte complex. *Angew Makromol. Chem.* 245, 63-72.

YOTSUYANAGI, T.; OHKUBO, T.; OHHASHI, T. and IKEDA, K. (**1987**). Calcium-induced gelation of alginic acid and pH-sensitive reswelling of dried gels. *Chem. Pharm. Bull.* 35, 1555-1563.

ZEKORN, T. B. C. and BRETZEL, R. G. (**1999**). Immunoprotection of Islets of Langerhans by Microencapsulation in Barium Alginate Beads. in: KUHTREIBER, W. M.; LANZA, R. P. and CHICK, W. L. (Eds.), *Cell Encapsulation Technology and Therapeutics*, Birkhauser, Boston, pp. 90-96.

ZHENG, C. H.; LIANG, W. Q.; LI, F.; ZHANG, Y. P. and FANG, W. J. (**2005**). Optimization and characterization of chitosan-coated alginate microcapsules containing albumin. *Pharmazie* 60:*(6)*, 434-438.