UTILIZATION OF CARRAGEENAN FROM PHILIPPINE *EUCHEUMA* SPECIES IN GENE/PROTEIN DELIVERY SYSTEM AND ANTI-BACTERIAL SURFACE COATING

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CHAPTER 1

General Introduction and Thesis Outline

Chapter 1

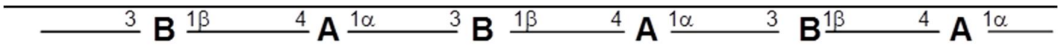
1. Background of the problem

Carrageenan is an extract of red seaweeds found in various parts of the world. These are primarily sulfated polysaccharides of varying ester content that give the three basic types of carrageenan (κ, *kappa*; ι, *iota*; λ, *lambda*) their interesting and unique properties [1]. It is used extensively as a suspending, stabilizing and emulsifying agent in food products [2] as well as pro-inflammatory agent for screening potential anti-inflammatory agents [3]. Carrageenan is the only hydro-colloid that is produced in the Philippines in commercial scale. The Philippines is the world's leading supplier of *Eucheuma* seaweeds, accounting for 80% of world supply and the largest supplier of Philippine Natural Grade (PNG) carrageenan, a product of Filipino-developed technology [4]. Hence, the Philippine carrageenan industry is one of the top dollar earners among other industries in the country. It also ranks among the top 10 as world supplier of refined carrageenan. It played a major role in boosting up the Philippine economy in terms of dollar income and job opportunities to hundreds of Filipinos. Due to increasing demand for use of polysaccharides in gene/drug delivery system as well as for antibacterial surface coating, carrageenan being a hydrocolloid and a polysaccharide find its way to other applications aside from being utilized as food additive.

Carrageenan is a sulphated linear polysaccharide of D-galactose and 3,6-anhydro-D-galactose (3,6-AG) [5]. Due to these half-ester sulphate groups, carrageenan is considered an anionic polysaccharide. All carrageenans are made up of alternating 1,3 - linked β - D - galactopyranosyl and 1,4 - linked

α-D-galactopyranosyl units (Figure 1). The 1,3-linked units occur as the 2- and 4-sulfates, or occasionally unsulfated [5] while the 1,4-linked units occur as the 2- and 6-sulfates, the 2,6-disulfate, the 3,6-anhydride, and the 3,6-anhydride-2-sulfate [6]. Sulfation at C_3 apparently never occurs [5].

In *Kappa*-carrageenan (Figure 2), the 1,3- and 1,4 linked units are respectively D-galactose-4-sulfate and 3,6-anhydro-D-galactose [5]. *Iota*-carrageenan (Figure 2) is of the alternating 1,3-linked β-D-galactose-4-sulfate and 1,4-linked 3,6-anhydro-α-d-galactose-2-sulfate residues [5]. *Lambda*-carrageenan consists of alternating 1,3-linked β-D-galactose having about 70% of the C_2's



B Units:	Found in:
D-Galactose	λ, θ
D-Galactose 2-sulfate	λ, ε, θ
D-Galactose 4-sulfate	μ, ν, κ, ι
A Units:	
D-Galactose 2-sulfate	ε
D-Galactose 6-sulfate	μ
D-Galactose 2,6-disulfate	λ, ν
3,6 – Anhydro-D-Galactose	κ
3,6 – Anhydro-D-Galactose 2 – sulfate	ι, θ

Figure 1. Repeating structure of carrageenan [5]

sulfated and 1,4-linked α-D-galactose-2,6-disulfate [5]. *Lambda*-carrageenan is non gelling in water.

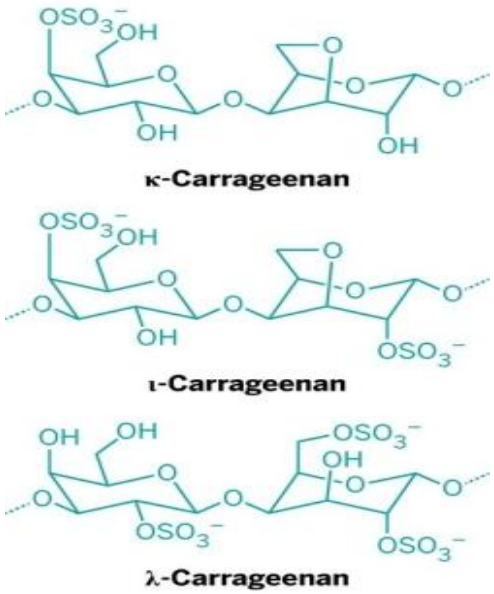


Figure 2. Chemical Structures of Carrageenan [5]

Kappa-carrageenan has an ester sulfate content of about 25 to 30% and a 3,6AG content of about 28 to 35%. *Iota*-carrageenan has an ester sulfate content of about 28 to 30% and a 3,6-AG content of about 25 to 30%. *Lambda*-carrageenan has an ester sulfate content of about 32 to 39% and no contentof 3,6-AG [6]. The chemical reactivity of carrageenans is primarilydue to their half-ester sulfate groups which are strongly anionic, being comparable to inorganic sulfate in this respect. The free acid is unstable, and commercial carrageenans are available as stable sodium potassium and calcium salts or, most commonly, as a mixture of these. The associated cations together with the conformation of the sugar units in the polymer chain determine the physical properties of the carrageenans. Carrageenan has no nutritional value and is used in food preparation for its gelling, thickening, and

emulsifying properties [7] and in pharmaceutical applications [7]. In experimental medicine this substance is often used for the testing of anti-inflammatory agents [8].

The study therefore will focus on the evaluation and assessment of Philippine carrageenan for potential applications in gene delivery, drug/protein delivery as well as antibacterial surface coating. It is supported by the assumption that carrageenan being a polysaccharide and a hydrocolloid can be used in the said applications and can be interacted electrostatically with other polymers like polyethylenimine (PEI) and chitosan.

Carrageenan is an anionic polymer that can bind to cationic polymers through electrostatic interaction in order to form a complex or polyelectrolytes (9-10).

2. STATEMENT OF THE PROBLEM

The application of carrageenan from Philippine *Eucheuma* species has been limited to emulsifier, thickener, binder and filler. Local market of carrageenan in the Philippines are mostly food industries and manufacturers of toothpaste. The general problem is to find other applications of Philippine carrageenan in the field of drug/gene delivery and as anti-bacterial surface coating.

3. OBJECTIVES OF THE STUDY

The study intends to utilize carrageenan in drug/gene delivery system and as a anti-bacterial surface coating. It also aims to interact carrageenan with other polymers like Polyethylenimine (PEI) and Chitosan and evaluate its ability to form complexes or polyelectrolytes through electrostatic interaction.

4. SCOPE AND LIMITATION OF THE STUDY

The study is limited to the utilization of Philippine carrageenan (*Kappa, Iota, Lambda* types) interacted with PEI and Chitosan to form polyelectrolytes through electrostatic interaction for possible application in gene and drug delivery system as well as anti-bacterial coating.

5. STRUCTURE OF THE THESIS

The following is an annotated thesis organization:

CHAPTER 1: ***Introduction***

This chapter presents the general introduction of the study that includes the background of the study, the statement of the problem, purpose of the study, objectives, scope and limitations of the study.

CHAPTER 2: ***Literature review***

This chapter provides the general overview of gene delivery and drug delivery system. It also includes the overview of anti-bacterial surface coating as well as a brief note on the properties of carrageenan, polyethylenimine and chitosan.

CHAPTER 3: ***Evaluation of pDNA-Polyethylenimine Complex Coated with Iota Carrageenan for In Vitro Gene Delivery***

This chapter demonstrates the addition of iota-carrageenan to pDNA-PEI complex which resulted to the formation of a ternary polyion complex that showed potential use and its viability in gene delivery. The carrageenan was attached to the cationic surface of the pDNA/PEI complex. It increased the stability of the pDNA/PEI complex, decreased its cytotoxic effects and protected the DNA from DNase degradation and other gly**c**osaminoglycans without losing its efficiency for gene

expression in CHO-K1, HUH-7 and COS-7 cells. The use of carrageenan as a protective coating for pDNA/PEI complex in gene delivery is promising.

CHAPTER 4: ***Encapsulation of glucose oxidase (GOD) in polyelectrolyte complexes of chitosan–carrageenan***

This chapter shows the potential ability of carrageenan (κ, ι, λ) and chitosan to form a controlled-release system for glucose oxidase (GOD). GOD was encapsulated in chitosan/carrageenan complexes at charge ratios (+/-) of 3 and 5 in mildly acidic solution (pH 6.0). The simple preparation of chitosan/carrageenan complexes and their ability to protect protein integrity under acidic conditions make them a promising drug delivery system for the oral administration of peptides and proteins.

CHAPTER 5: ***Ability of chitosan/carrageenan complex to encapsulate bovine serum albumin (BSA) for potential use in protein delivery***

This chapter focuses on examining the capability of chitosan/carrageenan complex formed by electrostatic interaction as a carrier system for protein delivery. Chitosan/carrageenan complex was formulated at charge ratios (+/-) of 1,3,5,7 while bovine serum albumin (BSA) was the model protein. The ease of preparation and the ability of protecting protein integrity, make chitosan/carrageenan complex a promising drug delivery system for oral administration of peptides and proteins.

Chapter 1

CHAPTER 6: ***Structural Studies on ι-Carrageenan Derived Oligosaccharides and Its Application***

This chapter discusses the two oligosaccharides isolated from ι-carrageenan by mild acid hydrolysis and its application for protein delivery. The oligosaccharides were characterized in terms of chemical structure and sugar units. It was also evaluated for its efficiency to encapsulate bovine serum albumin (BSA).

CHAPTER 7: Formation of Polyethylenimine/Carrageenan Bi-layer as Monitored by Atomic Force Microscopy and Biomolecular Interaction Analysis

This chapter describes the forming of the bi-layer of polyethylenimine and carrageenan monitored by atomic force microscopy and biomolecular binding analysis.

CHAPTER 8: ***Antibacterial activity of polyethylenimine/carrageenan multi-layer against pathogenic bacteria***

This chapter describes the formation of multi-layers of polyethylenimine (PEI) and carrageenan (κ, ι, λ) by layer-by-layer assembly and its antibacterial activity against Enterobacter cloaceae, Staphylococcus aureus and Enterococcus faecalis 29505 for potential use as coating on biomaterial surface. All the multi-layers exhibited growth inhibition.

CHAPTER 9: ***Summary of the thesis***

6. REFERENCES

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CHAPTER 2

Literature Review

Chapter 2

LITERATURE REVIEW

1. Gene delivery/therapy

The underlying principle of gene therapy is based on the introduction of genetic material into living cells in order to achieve a therapeutic biological effect [1]. Generally, this involves introducing DNA encoding a gene for a therapeutic protein. The therapeutic benefits of gene therapy can be expanded to a wide range of diseases that are not strictly hereditary, such as cancer (2) and cardiovascular disease (3). A gene delivery system (GDS) or vector consists of a polynucleotide, encoding the therapeutic gene, and a carrier. According to Thomas et al. 2013 [4], the carrier has several important properties (Fig.1). First, it condenses the polynucleotide, protecting it from mechanical stress and enzymatic attack. Second, the carrier should facilitate transport of the therapeutic gene from the extracellular space into the nuclear compartment, where transcription can take place [4]. Living organisms are generally well protected by intra- and extracellular barriers against invasion of foreign genetic material. This is required to ensure the genetic stability of the species. Depending on the method of administration, the vector needs to circumvent host defense mechanisms, i.e. extracellular barriers. For example, when injected into the bloodstream, circulating antibodies and complement factors may inactivate the vector [5-7]. Vectors delivered to the airways face a spectrum of host defense mechanisms: the vector can be entrapped in the mucus layer and subsequently removed by cilliary clearance. Scavenging macrophages and dendritic cells can degrade the vector by phagocytosis and induce an inflammation response.

In addition, many potential receptors for GDSs are located on the basolateral membrane of airway epithelial cells, safely shielded from the milieu exterieure by tight junctions thus the receptors are inaccessible for GDSs [8]. Gene delivery systems can be can be divided into two classes. These are viral and non-viral (based on synthetic self-assembling systems). The viral gene delivery systems can be based on various viruses. These include adenoviral [9], adeno-associated viral (AAV) [10], lentiviral vectors [11-12]. In addition, viral vectors based on poxvirus [13-14], sendai [15] and herpes virus [16] are under investigation for gene transfer. Viral capsids and envelopes of these known viruses are well adapted to perform gene delivery. When applied *in vivo*, the main advantage of viral vectors is the relatively high transduction efficiency, which is either defined as the number of transgene expressing target cells per transduced genome, or as the percentage of transduced target cells. In addition, the use of integrating vectors such as AAV and HIV derived vectors results in stable transduction of a population of mitotically active target cells [1], which can not be achieved with the commonly used plasmid derived synthetic vectors. On the down side, viral-based gene therapy poses serious safety concerns. Administration of viral vector evokes a host defense response to the viral vector [17-18] and transduced cells, containing viral components. This response can range from a relatively mild acute inflammation to a cytotoxic lymphocyte response. Indeed, one fatality was reported due to an escalated immune response to an administrated adenoviral vector [19].

The viral genome of the vector can recombine with or be activated by wild type viral genome, giving rise to potentially hazardous agents.

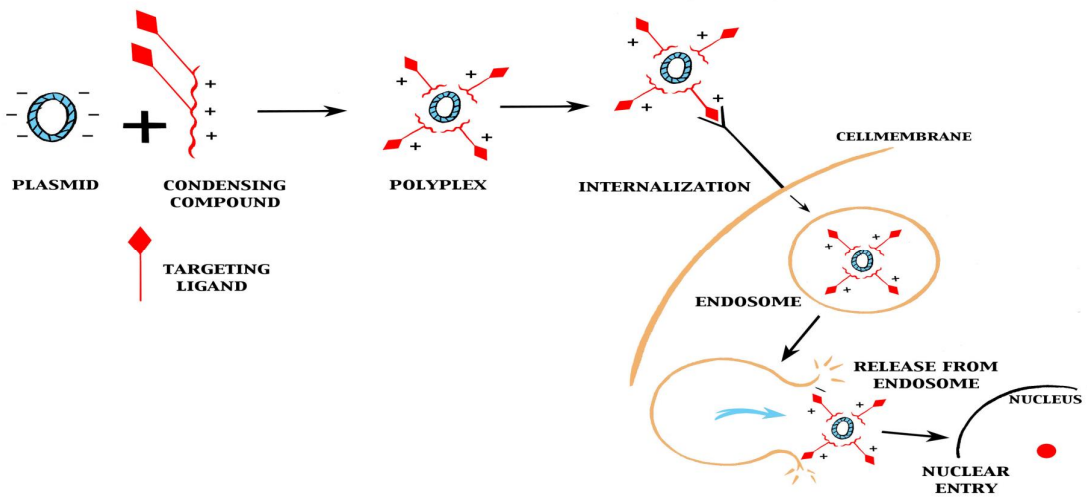

Figure 1. Schematic diagram in non-viral gene transfer [20].

When integrating vectors are used, there is a risk of insertional mutagenesis, as randomly integrating genes can alter the host genome. As recently reported, two patients treated with bone marrow cells transfected with a retroviral vector were diagnosed with leukemia caused by an insertional modification of a proto-oncogene by the therapeutic gene containing vector [21-22]. In summary, viral vectors are highly efficient for gene transfer *in vivo,* but their clinical applications may be restricted due to their safety concerns. The restrictions imposed on viral vectors have led to the development of synthetic gene delivery systems such as cationic lipids and polymers. These vectors may overcome the current problems in safety, immunogenicity and mutagenesis associated with viral vectors [23]. In addition, the size of the therapeutic gene is not limited for self-assembling synthetic vectors and cost-effective large-scale production is feasible. The major challenge is that non-viral

vectors lack the high *in vivo* transduction efficiency demonstrated by viral vectors. Viral based vectors have addressed the complexity of the gene delivery process with specialized mechanisms for intracellular transport and nuclear delivery [24] and non-viral vectors need to be designed to do so either.

In gene transfer, viruses are parasitic non-cellular organisms that require cells for their viability and growth. They have evolved over time to deliver their genome to the host cell nucleus with high efficiency. In many different shapes and molecular designs, viruses consist of a polynucleotide genome packaged in a protein complex called a capsid. The capsid proteins are well adapted to allow efficient binding to target cells, penetration of the plasma- or endosomal membrane, facilitation of intracellular transport, and subsequent import of the genome into the nucleus [1]. Viruses have developed many different mechanisms to approach this trafficking problem. What they have in common is the use of innate cellular transport systems, through interactions of their capsids with intracellular transport systems of the host. The latest generation of non-viral vectors is designed to mimic the properties of viral capsids with target proteins.

Viral vectors are generated by replacing part of the viral genome (either DNA or RNA) with a therapeutic gene. Most viral genes are removed from the genomic backbone of the virus with the exception of the genetic components involved in the genome packaging in the capsid and replication. The genes removed are required for capsid assembly and genome replication and these gene products are provided by packaging cells. Packaging cells transfected with the therapeutic viral vector can

then produce infectious therapeutic virus. The size of the vector sequence and thus of the therapeutic gene insert is limited by the properties of the viral capsid [25].

Non-viral vectors generally comprise cationic polymers (Fig 1) or lipids that are able to condense the negatively charged DNA. For example, poly-L-lysine (PLL) is a widely used component of transfection agents. The primary amino group on the side chain of the monomer L-lysine allows for protonation and thus a positive charge. At physiological pH nearly all N-atoms of the polymer are protonated. Electrostatic interaction with the negatively charged phosphate groups of the DNA results in compact, ordered particles varying from 20 to 200 nm diameter in size [26]. The packaged genetic material, usually plasmid DNA isolated from bacteria, is protected from mechanic stress and enzymatic degradation [27].

1.1 How the vectors target cell binding and entry

In gene delivery system (GDS), it has to target a specific cell to achieve its therapeutic goal and minimize unwanted side effects. Prior to cell entry, non-viral and viral GDSs attach to the surface of target cells. Subsequently the attached vehicle is endocytosed (Fig.1). Viral vectors generally target specific receptors on the plasma membrane and can enter the cell by fusion with the plasma membrane (HIV, influenza), pore formation (polio virus) or by acid induced membrane disruption after receptor-mediated endocytosis (adenovirus) [28].

Adenovirus and adenoviral vectors bind to the cell following two sequential receptor interactions. Firstly, the capsid fiber binds to a glycoprotein of the immunoglobulin family called CAR (Coxsackie, Adenovirus Receptor), which is expressed in many human cell types but is located on the basolateral membrane of

airway epithelial cells [29]. Secondly, the fibronectin-binding integrin (integrin _v_5) binds to the penton base protein, an adenoviral capsid protein [30]. The binding is followed by receptor-mediated endocytosis on the basolateral membrane of airway epithelial cells for cell entry [31]. A receptor (heparan sulfate) for adeno associated virus derived vectors has been located on the basolateral membrane of airway epithelial cells [32] and it has been demonstrated that the apical membrane contains an abundant high affinity receptor for serotypes AAV5 and AAV4 [33-34]. Vesicular stomatitis virus G protein (VSV-G) pseudotyped retro- and lentiviral vectors are not able to infect airway epithelia efficiently when administrated to the apical surface of polarized airway epithelial cells due to the lack of an apical receptor. To overcome this problem retroviral vectors were pseudotyped with apical membrane-binding envelope proteins [35], leading to the development of a filovirus-pseudotyped HIV vector that efficiently infects airway epithelia *in vivo* [36]. Also, a feline immunodeficiency virus (FIV)-based lentiviral vector was pseudotyped with the glycoproteins from Ross River Virus, resulting in a vector that displayed an increased tropism for the liver [37].

Non-viral gene delivery systems generally are completely dependent on endocytosis for cell entry. Polycations complexed to DNA result into positively charged particles named polyplexes. They interact electrostatically with negatively charged proteoglycans of the cell membrane, which leads to endocytosis [38-39]. This non-specific mode of cell entry can be altered by the addition of a targeting ligand to the surface of the DNA delivery vehicle. The first targeting ligand used for non-viral gene delivery was asialorosomucoid which is specific for hepatocytes [40].

Numerous ligands are under investigation and are reviewed. They include transferrin, folate, specific monoclonal antibodies [24, 41], invasin [42] and several carbohydrates [43]. While such formulations were effective *in vitro,* specific cell targeted gene delivery *in vivo* is still problematic. The positive surface charge of the synthetic vector, causes non-specific electrostatic interaction with non-target cells [44]. This unwanted interaction of synthetic vectors with biomolecules of non-target cells can be reduced by addition of hydrophilic segments such as poly(ethylene glycol) (PEG). A PEGylated GDS has a hydrophilic shell at the exterior that masks the positive surface charge and prevents interaction by steric repulsion [45] resulting in less aggregation and reduced transfection of non-target cells.

After endocytosis, the normal endosomal transition into lysosomal vesicles results in acidification, which provides a milieu for the activity of hydrolytic enzymes. In order to avoid the degradation of the therapeutic DNA, a GDS must escape from endosomes [46]. To alleviate this degradation problem viral vectors take advantage of pathways that were selected during the evolution of natural viruses. Adenovirus, a non-enveloped virus, uses the acidification of endosomes to activate three capsid proteins, which then lyse the endosomal membrane [47-48]. The enveloped influenza virus uses an amphiphilic protein, the haemagglutinin protein (HA) to escape from the endosome. When the pH in the endosome drops, the geometric structure of HA changes rapidly, leading to a fusion of the viral and endosomal membrane and so endosomal escape of the virus particle [49].

Non-viral vectors must also have mechanisms to circumvent lysosomal degradation: some polycationic polymers such as polyethylenimine [50] and

pDMAEMA [51] are able to escape the endosome due to intrinsic properties of the polymer. First, the high buffer capacity of the compounds results in swelling of the endosomes as the lysosomal proton pump attempts to reduce the luminal pH. Further, cationic polymers in high concentrations tend to destabilize membranes. However, since the bulk of the DNA delivered this way does not escape hydrolysis, the DNA-containing particles are largely retained in perinuclear endosomes/lysosomes [52]. This phenomena remains one of the bottlenecks in delivery by synthetic GDS.

Pharmacological agents such as chloroquine can be used to disrupt the normal internal routing of the GDS from the endosomes to the lysosomes [53]. Chloroquine is a weak base (pKa 8.1 and 10.2) that accumulates in the endosome when the luminal pH is lowered. It is thought this accumulation results in osmotic swelling, followed by the destabilization of the endosomal membrane and subsequent release of the contents into the cytosol [47]. The presence of chloroquine enhanced transfection efficiency of lactosylated PLL considerably [54], which is generally low unless endosomolytic or lysosomaltropic agents are present [55]

Another strategy to promote endosomal release is the addition of endosome-disrupting peptides such as N-terminal amphilic anionic peptide of HA2 [56], derived from the influenza virus HA [57]. Also the synthetic amphipathic peptides GALA (glutamatic acid-alanine-leucine-alanine) [58] has been shown to enhance the transfection efficiency of liposomes [59]. LALA (lysine-alanine-leucine-alanine) is a cationic amphipatic peptide and was designed to both condense DNA and

destabilize the endosomal membrane. When compared to gene transfer using PLL, KALA was more efficient and yielded a 100-fold greater reporter gene expression [60]. Glycerol is another agent that promotes endosomal escape. It was reported that PLL had endosomal membrane disrupting properties in the presence of glycerol [61]. Poly-L-histidine (PLH) has been used in studies of membrane fusion and has served as a pH-responsive fusogen [62]. PLH was used in combination with PLL-based GDS for pH responsive escape [63]. Also partial substitution of the ε-amino groups of PLL with histidine resulted in a gene transfer agent that was able to transfect HepG2 cells without the addition of helper agents such as chloroquine, glycerol and fusogenic peptide [64-65].

A relatively new approach to facilitate endosomal release of the gene delivery vehicle is photochemical internalization (PCI), based on light-induced photochemical reactions [66]. A number of photosensitizers such as tetra(4-sulfonatophenyl)porphine (TPPS4) and meso-tetraphenylporphine (TPPS2a) localize primarily to endosomes and lysosomes [67]. When exposed to light, these photosensitizers induce the formation of reactive oxygen species. These are short-lived and have a short range of action (10-20 nm) [68]. Therefore, once light-activated, photosensitizers located in endo- or lysosomes may destroy the membrane but leave the organelle contents intact [69]. PCI enhanced the transfection efficiency of PLL by 10-fold and of adenovirus-mediated gene transfer by 6-fold [70].

1.2 The intracellular transport of vector DNA

The vector DNA must reach the nucleus to be effective once released from the endosomes. Extremely large molecular structures such as viral capsids and non-viral vector complexes do not diffuse easily through the viscous and crowded cytoplasm. Viral vectors exploit the intracellular transport system of the host cell, including the microtubule/motors or actin filaments for intracellular movement [71]. Baculovirus is thought to transport itself in mammalian cells *via* actin filaments [72]. Vaccinia virus is also reported to use actin filaments for transport. Herpes viruses employ microtubulebased transport to move within the host cell towards the nucleus, presumably mediated by the attachment of viral capsid to the motor protein dynein [71]. It is generally thought that viral-based vectors employ the same strategies as their wild-type virus counterparts.

Little is known about how synthetic gene delivery vehicles move within the host cell. They may diffuse freely through the cell or “piggy back ride” with the host cell’s own intracellular carriers. An attractive option to enhance intracellular transport of synthetic vectors is the addition of peptides, derived from viral proteins responsible for intracellular transport.

Once in the perinuclear region, the foreign DNA must enter the nucleus in order to be trasnscribed. However, the nucleoplasm is separated from the cytosol by a hydrophobic barrier: the nuclear envelope that consists of an outer nuclear membrane and an inner nuclear membrane. These membranes are connected to each other by the nuclear pore complex (NPC), a protein structure [73]. NPCs form aqueous channels through the double membrane of the nucleus [74] and allow for

transport of cargo. Small molecules (up to 9 nm in diameter, or proteins up to 50 kDa) can passively diffuse, but larger cargo (up to 25 nm in diameter, or ~1000 kDa) is transported in an active signal-mediated manner. Nuclear localization signals (NLS) on cargo, binding to cytoplasmatic proteins named a-importins, which in turn bind to b-importin. The b-Importin is responsible for docking the import cargo on the NPC. The translocation into and across the NPC involves a key regulatory molecule, GTPase Ran [75].

In principle, there are two approaches for viral and non-viral vectors to deliver the genetic material into the nucleus. First, the vector resides in the cytosol until the nuclear envelope is disassembled during mitosis. The foreign genome can enter a newly assembling nucleus. Alternatively, the genomic material can be delivered through the envelope of the interase nucleus *via* the NPC [76].

Many viruses that are used as GDS are able to transport their genome into the cell nucleus. Retroviruses, with the exception of lentiviruses, cannot deliver their genome into an intact nucleus and can therefore only infect dividing cells. Most viruses have developed a mechanism to deliver their genome through the NPC. For example, influenza virus transports its genome through the NPC in small RNA segments packaged into viral ribonucleoprotein complexes [77]. Adenoviral DNA has a linear structure and is transported to the nucleus stored in a protective capsid. The capsid docks to the nucleus via nucleoporins, the building stones of the NPC. Subsequently, the viral DNA is transported through the NPC [78]. Baculovirus has a cigar-shaped plasmid that is able to penetrate the NPC of mammalian cells [72]. The nature of the interactions between capsid and NPC is yet unclear.

Lentiviruses such as HIV-1 have developed mechanisms to transport their genome into cell nucleus of non-dividing cells [79]. After the reverse transcription of genomic HIV-1 RNA into linear double stranded DNA, the DNA remains part of a nucleoprotein complex called the pre-integration complex. Proteins involved in the nuclear import are matrix protein, Vpr and integrase. Their function remains unclear but they may act as a NLS. In addition, Vpr may facilitate nuclear docking of the complex and induce transient herniations of the nuclear envelope allowing nuclear translocation of the complex [76]. HIV-1 nuclear import has been reported to also depend on signals present in viral DNA: the reverse-transcribed HIV-1 genome contains a short triple-stranded overlap, the central DNA flap. When the central DNA flap is mutated, the virus cannot enter the nucleus in an integration-competent form [80].

Non-viral GDS do not have such sophisticated mechanisms as their viral counterparts and it is still unknown how DNA of therapeutically relevant sizes (up to 10 kbp) can pass through the NPC [81]. It was postulated that nuclear uptake occurs preferentially in those cells entering mitosis, consequent to break down of the nuclear membrane [56, 82]. In contrast, there are also reports that suggest a nuclear import mechanism through the NPC. Since transferrin/polylysine complexes were found to gave high transgene expression levels in growth arrested fibroblasts [83]. Moreover, Pollard *et al* [84] reported that some polycations facilitate the nuclear uptake of DNA complexes. The size-restrictions for NPC-mediated import were also challenged by reports that plasmid DNA up to 14.4 kbp was found to be localized in the nucleus in the absence of cell division. The nuclear import required energy,

cytoplasmic factors and was blocked by wheat germ agglutinin an inhibitor of NPC function. Furthermore plasmid DNA is thought to form a complex with NPC proteins prior to transit and translocation [85]. Likewise it has been shown that single-stranded DNA/protein complexes were efficiently imported in mammalian nuclei following the classical importin-dependent nuclear import pathway [86]. Polycations modified by the addition of a NLS have been reported to increase gene transfer [87].

However, none of these studies appear completely conclusive. In experiments with supposedly 'non-mitotic cells', actual controls of mitotic activity after addition of the vector are often missing. It should be noted that non-viral GDS are generally cytotoxic. Application of such GDS can easily result in a burst of transient mitotic activity. With regards to transport studies using fluorescent-labelled DNA, fragmentation of the DNA prior to nuclear transport cannot be excluded. It is well known that small DNA fragments (<500 bp) readily accumulate in the nucleus, as opposed to larger molecules that are required for a therapeutic gene [88].

Well-documented studies have shown that viruses have efficient strategies for nuclear import. How and if non-viral vectors can enter an intact nucleus still remains unclear despite reports suggestive for NPC-mediated nuclear import. Indisputable evidence of transport of synthetic vectors through the NPC has not yet been presented.

1.3. Transgene expression.

After entry in the nucleus, transcription of the therapeutic gene should take place. A prerequisite is the availability of the DNA for the host cell transcriptional machinery. Transgene expression is generally transient [89]. Viral and non-viral GDS

must address this problem. Lentiviral vectors and recombinant AAV address the problem of poor persistence [90] by integration into the host DNA. However, the major drawback of integration is the possible risk of mutagenesis [22]. Several strategies are developed to resolve the problem of transient reporter gene expression in non-viral gene transfer [89]. So far, expression vectors are generally of relatively simple design, comprising a core promoter/enhancer, therapeutic cDNA, and a simple intron structure if any. Clearly, this does not match the intricate mosaic of regulatory and structural elements found in chromosomal genes, which is required for stable and cell-specific expression. The construction of minichromosomes that have full telomere and centromer functions, offers the possibility to introduce a large genomic element, complete with distal regulatory elements. [91]. Transfer of such a therapeutic mini chromosome to a target cell population should lead to stable and perfectly controlled expression of the therapeutic gene. However, successful transfer of such a large construct into a sufficient number of relevant target cells remains a challenge [92-93]. Another approach to extend reporter gene expression is the design of tissue specific self-replicating plasmid. A replicating episomal plasmid mediated under the human beta locus control region sustained reporter gene expression in culture up to 60 generations [94]. Development of integrating vectors based on the Sleeping Beauty transposon system resulting in sustainable transgene expression have been reported [95-97]. In addition, in non-viral gene transfer using plasmid DNA, absence of the methylated sequences may contribute to prolonged longevity of transgene expression of bacterial plasmid DNA in the human host cell [98].

1.4 Non-viral vectors: DNA condensing components and their glycosylated derivatives.

As described above a GDS must be well designed to a) condense DNA, b) bind to a specific cell surface and promote uptake, c) escape from the endosomal compartment, and d) provide for nuclear translocation either by nuclear import or by entry during mitosis. While vectors based on viruses take advantage of the strategies that were developed by their natural counterparts during evolution, non-viral GDSs will have to be constructed *de novo* to meet the high demands of efficient gene transfer. Essentially, a synthetic GDS is an artificial virus, consisting of DNA, usually in the shape of a plasmid and a carrier. The carrier therefore needs to be equipped with tools that can fulfill the task of the viral capsid. In order to condense DNA, the carrier has to be positively charged. Carriers can be roughly divided in two groups: cationic lipids and cationic polymers. Both can be modified to mimic the actions and functions of the viral capsid. One specific modification is glycosylation. The presence of carbohydrates on the DNA condensing compound allows for interaction with lectins which are proteins that bind to specific carbohydrate structures [99].

1.5 Glycosylation to target cellular lectins

The natural ligands for most lectins are typically complex glycoconjugates that carry clustered arrays of the cognate carbohydrate, thus cooperating with clustered lectin-binding sites to generate high-avidity binding [100]. Some membrane-bound lectins are internalized upon ligand binding, followed by delivery to internal acidic compartments [101]. The covalent linkage of carbohydrates to non-viral GDS may enhance cell specific binding by targeting membrane-bound lectins and triggering

receptor-mediated endocytosis. There are two well-known lectins that are carbohydrate-binding receptors. The mannose receptor recognizes glycoproteins with mannose, glucose, fucose and *N*-acetylglucosamine residues in exposed positions [102] and is located on a variety of macrophage subtypes [103]. Macrophages are targets for gene therapy of diseases as Gaucher's disease [104] and HIV infection [105] but may also be a target cell for expressing an exogenous gene with therapeutical effects [106]. The asialoglycoprotein receptor is located on the hepatocyte membrane and specifically recognizes terminally linked b-galactose or Gal*NAc* (*N*-acetyl galactosamine) residues on circulating glycoproteins and cells [107]. A variety of DNA carriers are in development using galactose/lactose as a tageting ligand for the hepatocyte asialoglycoprotein receptor, taking advantage of receptor-mediated endocytosis to both enhance efficiency and specificity of gene transfer [43].

1.6. Glycosylation of cationic lipids

Cationic-lipid based gene delivery systems are composed of lipid:DNA complexes (liposomes). Such complexes have been successfully delivered to e.g. airway epithelium *in vivo* [108], although at generally less efficiency rates than viral vector gene delivery [109]. Initial clinical trials have indicated that liposomes have a relatively low toxicity profile when administrated to nasal epithelium [110-113]. However, liposome administration to human lungs caused mild influenza-like symptoms which may be related to the DNA component of the complex [114].

The composition of lipids in the liposome based gene delivery vehicles varies and is known to be critical to vector targeting and efficiency. Additional components

such as carbohydrates can both increase efficiency and promote selective targeting [115].

Indeed, when the biodistribution of mannosylated liposomes in mice was studied, it was found that intravenously administered mannosylated liposomes were taken up mainly by non-parenchymal cells in the liver [116]. Moreover, mouse peritoneal macrophages were efficiently transfected *in vitro* [117]. However, *in vivo* expression of the reporter gene was detected in the non-parenchymal cells of the liver. This was reduced by pre-dosing with mannosylated bovine serum albumin and was enhanced by the incorporation of PEI into the liposome complex [118]. Correspondently, galactosylated liposome/DNA complex transfected the mouse liver through asialoglycoprotein receptor-mediated endocytosis [119]. The liver is reported to express two types of galactose receptors, one specific for parenchymal cells and one for Kupffer cells. The lactosyl residue of galactose provides the targeting ligand. Indeed, when bound to low density lipoprotein the lactosyl residue can target both Kupffer cells and parenchymal cells of the liver. The specificity depends on the degree of lactosylation. At high substitution of lactose, lactosylated low-density lipoprotein is mainly taken up by Kupffer cells. At low substitution of lactose, parenchymal cells are the main site of uptake of lactosylated low-density lipoprotein [120].

1.7. Cationic polymers for DNA condensing

Polymers with primary and secondary amino groups that are protonated at physiological pH have the potential to interact with the negatively charged DNA through their positive charges. The electrostatic interaction results in the formation of

a transfection complex [24] and that packages the DNA and protects it from degradation [27]. Numerous natural and synthetic polyations are under investigation for use in gene transfer. Natural polycations include histones [121] and chitosan, an aminopolysaccharide [122]. Among synthetic vectors are peptides such as poly-lysine and poly-L-ornithine, as well as polyamines such as polyethylenimine [50]. Also synthetically manufactured are poly(ß-aminoesters) both linear [123] and dendritic [124] and metacrylate-based polymers such as poly(2-dimethylamino) ethyl methacrylate (pDMAEMA) [125]. Currently most synthetic vectors are being modified so as to optimize important steps in the transfection process. The addition of a targeting ligand, e.g. carbohydrate, can enhance cell specificity. Also, adding a nuclear localization signal appears to improve nuclear delivery [126]. Despite all efforts, a satisfactory gene delivery system for clinical applications has not been developed.

The most popular cationic polymer as non-viral vector is PEI. It forms a complex with pDNA and showed high gene expressions in both in vitro and in vivo gene delivery [127-128]. The complex binds non-specifically to negatively charged proteoglycans on cell membranes and agglutinates with blood components, such as erythrocytes and serum albumins. This often leads to its rapid elimination and adverse effects, such as embolism and inflammatory reaction [129-132]. To solve these problems, studies were made to reduce the cytotoxicity and agglutination with blood components. These involve preparation and development of several novel polymers of PEI covalently bind to hydrophilic polymers like: polyethylene glycol

(PEG), polyhydroxypropylmethacrylamide (pHPMA), and polyvinylpyrrolidine to modify the complex surface [130,132-133]. Aside from hydrophilic polymers, studies were also done on the use of anionic polymers. Studies showed that the introduction of a protective polyanion coating of pDNA/polycation binary complex using polyacrylic acid derivatives resulted in high level gene expression in lung after injection into mouse tail vein wherein the surface of the resulting ternary complex was recharged to negative one [134]. Anionic polymers can bind to the cationic complex electrostatically to modify the complex surface [134-135]. These polyanions do not decompose the pDNA/polycation complex but attached to the cationic surface of the complex forming a ternary polyion (pDNA/polycation/polyanion) complex that retains its transfection efficiency [136].

Polysaccharides have been used for quite some time as controlled release coatings, matrices, macromolecular carriers, and biodegradable carriers [137]. These consist mostly of chains of monosaccharide or disaccharide units linked together by O-glycosidic linkages with a large number of derivatizable groups, a wide range of molecular weights, varying chemical compositions, and for the most part, a low toxicity and biodegradability, yet a high stability [137].

The group of Kawakami [138] reported that mannosylated, fucosylated, and galactosylated liposomes showed high accumulation in the liver via each specific receptor. An example of this, is hyaluronic acid which is taken by a specific receptor-mediated endocytosis and used for targeted drug delivery systems [139-140]. Hyaluronic acid also forms a ternary complex with polycation and DNA with high gene expression and stability in serum-containing medium [135].

Kurosaki et al, 2009 [141], has developed a gene vector electrostatically assembled with a polysaccharide capsule. λ-Carrageenan was one of the polysaccharides used in the coating of pDNA-PEI complex. They reported that the ternary complex coated with λ-carrageenan and other polysaccharides except for chitosan showed little uptake and gene expression. In this study, ι-carrageenan was used as polyanion coating for pDNA-PEI complex through electrostatic interaction. The resulting ternary complexes were then evaluated according to its transfection efficiency, physical properties and morphology. The ratios of the ternary complexes were also modified using lower N/P (PEI/DNA) ratios of 3.3 and 3.5 than the study of Kurosaki et al [141] which used an N/P ratio of 8 with varying ratios of carrageenan. In their study, transfection was carried out in reduced serum medium using B16-F10 cells. In our study, we used CHO-K1, COS-7 and HUH-7 cells for the evaluation of transfection efficiency in the presence of 10% FBS for four hours.

2. Drug delivery system

A drug delivery system can be defined as a mechanism to introduce therapeutic agents into the body. An optimal drug delivery system ensures that the active drug is available at the site of action for the correct time and duration. Significant advances have been made in drug delivery technologies throughout the past 3 decades, and drug delivery at a desired release rate is now possible [142]. Even highly sophisticated drug delivery technologies, however, often fail to produce marketable oral controlled-release dosage forms, as a result of the physiological limitations of the gastrointestinal (GI) tract and/or the utilization of non-feasible pharmaceutical components [142].

Chapter 2

In oral drug delivery, there are many scientific challenges that could be studied for years to come, and breakthrough technologies are required to generate novel dosage forms raising drug delivery to higher level [142].

A controlled release system utilized polymer matrix or pump as a rate-controlling device to deliver the drug in a fixed, pre-determined pattern for a desired time period [143]. The system offered several advantages [144] namely: (1) the possible maintenance of plasma drug levels to a therapeutically desirable range, (2) possible elimination or reduction of harmful side effects from systemic administration (3) improvement and facilitation of underprivileged areas where good medical supervision is unavailable (4) facilitation in the administration of drugs with a short *in vivo* half-life (5) continuous small amounts of drug maybe less painful than several large doses (6) improvement of patient compliance (7) less expensive product and less waste of drug. Since the pioneering work in controlled drug delivery, it was demonstrated that when a pharmaceutical agent is encapsulated within, or attached to, a polymer or lipid, drug safety and efficacy may be greatly improved and new therapies are possible (145). This concept prompted active and intensive investigations for the design of degradable materials, intelligent delivery systems, and approaches for delivery through different portals in the body. Recent efforts have led to development of a new approach in the field of controlled drug delivery with the creation of responsive polymeric drug delivery systems [146]. There are different approaches for controlled drug delivery. These are: Localized Drug Delivery; Targeted Drug Delivery; Sustained Drug Delivery (Zero Order Release Profile); Modulated Drug Delivery (Nonzero – Order Release Profile);

Feedback Controlled Drug Delivery; Implantable Controlled Drug Delivery Devices [144].

Despite extensive research in drug delivery, the advances in oral applications have been relatively slow [142]. While the oral route is the most convenient method of drug administration, advances in oral drug delivery technologies have been limited. One of the reasons for this is the limitations imposed by the unique GI physiology illustrated in Figure 2. In these areas, even small improvements in drug delivery technology can make significant differences in enhancing patient compliance and drug bioavailability [147].

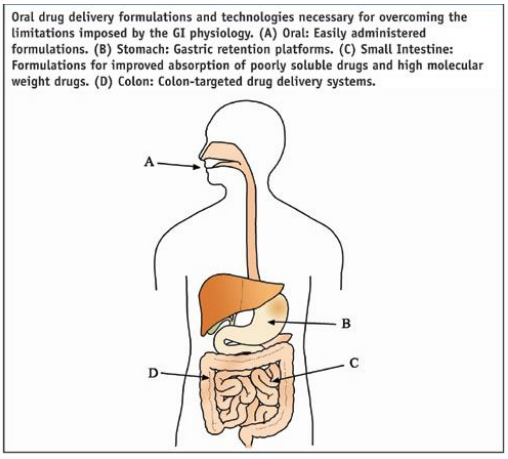

Figure 2. Illustration of GI Physiology [142]

One of the "holy grails" in oral drug delivery is to develop gastric retention platforms for long-term (ranging from 6 to 24 hours) delivery of drugs by oral administration. Currently, there are a number of drugs that can be delivered using once-daily formulations. This is possible only for drugs that are well absorbed through out the GI tract or typically have long terminal elimination half-lives [142].

There are a large number of drugs that are not absorbed to the same extent once they pass the upper small intestine, and thus, once-daily formulations are elusive for these drugs. One method to overcome the fast GI transit is to maintain the dosage form in the stomach for extended periods of time, and therefore, research efforts have been focused on development of gastric retention platforms [142].

2.1 Oral delivery of proteins

The development of peptides and proteins as therapeutic agents has been greatly accelerated by advances in the fields of biotechnology. A class of peptides receiving much attention are the various growth factors, many of which have been synthesized by genetic engineering or isolated from natural sources [148].

Currently, the clinical administration of these drugs requires repeated intramuscular or subcutaneous injections because oral administration is not suitable due to degradation caused by proteolytic enzymes in the gastrointestinal tract [149]. Clearly, there is a need for novel drug delivery systems capable of prolonged release in order to minimize dosing frequency and improve patient compliance [150-153].

The major problem encountered when using orally administered peptides and proteins is the interaction with the proteolytic enzymes such as peptidases [154]. It has recently been estimated that an orally administered peptide might encounter in excess of 40 different peptidases during the passage through the GI tract [155]. Peptidases are enzymes that hydrolyze the peptide bond formed between two amino acids [154]. They have been traditionally divided into two major classes: the endopeptidases, which hydrolyze peptide bonds interior to the terminal bonds of the

peptide chain, and the exopeptidases, which hydrolyze the bond linking the N-terminal or C-terminal amino acid to the peptide chain [154].

2.2 Route of drugs in the GI tract

In Figure 2, the sites of GI tract are shown where the orally administered peptides and proteins will pass. In each of these sites, the lumen and the cell membranes constitute the major barriers prior to any absorption. The stomach constitutes an extremely hostile environment for peptides and proteins [154]. The stomach lumen is very acidic (pH 1-2) and the endopeptidase pepsin is secreted into the stomach lumen via the gastric pits [154]. The small intestine is the most favored site for the absorption of orally administered drugs, due to the very large surface area of brush border membrane, which in humans is estimated to be between 200 and 500 m^2 [154]. The small intestine has a mucosa, which has a rapid cell turnover. Cells migrate from the crypts at the base of the villi and gradually work their way up the villi. Finally, the cells are extruded or sloughed off from the top of the villi into the lumen of the intestine. In a human the entire process takes from 3 to 6 days [154].

In recent years, the colon has been considered as a potential site for the delivery of therapeutic peptides and proteins since it represents less of an enzymatic barrier to proteins. There is also a development of delivery systems able to target the colon [156-158].

2.3 Mechanisms of uptake of proteins by the intestinal epithelium

There are four routes of transport of proteins across the cell [154]: The first one is pinocytosic transport (*Figure 3a*), which is one of endocytic processes. In this process, a region of the plasma membrane engulfs external solutes to form an

intracellular vesicle having less than 0.1 μm in diameter. This vesicle is transported through the cell and the engulfed solutes are secreted to the other side. The second is a receptor-mediated endocytosis transport (*Figure 3b*). This is a similar process with the pinocytosis but a specific receptor on the surface of membrane recognizes an extracellular macromolecule (the ligand), and binds to it then the plasma membrane region containing the receptor-ligand complex forms a vesicle. The third mechanism is phagocytosis transport (*Figure 3c*). In this process, the target particle is bound to the cell surface then the plasma membrane expands along the surface of the particle and eventually engulfs it. Vesicles formed by phagocytosis are typically 1-2 μm or greater in diameter, much larger than those formed by endocytosis. The fourth and most interesting in this study is paracellular transport (*Figure 3d*). The intestinal epithelia are aggregates of individual cells and they are bound together by tight junctions. In certain conditions such as removal of Ca2+ from extracellular media, the tight junctions are open and the protein can diffuse to other side [159].

2.4 Polymer-based drug-delivery systems

Polymers have been used extensively in controlled drug delivery systems. These can be classified as (1) nondegradable polymeric reservoirs and matrices, and (2) biodegradable polymeric devices [160]. The two most commonly used nondegradable polymers are silicone and poly(ethylene-covinyl acetate) (EVAc) [161]. In nondegradable polymeric systems, these are prepared by homogeneous dispersement of drug particles throughout the matrix [161]. Drug release occurs by diffusion through the polymer matrix or by leaching or a combination of both [162].

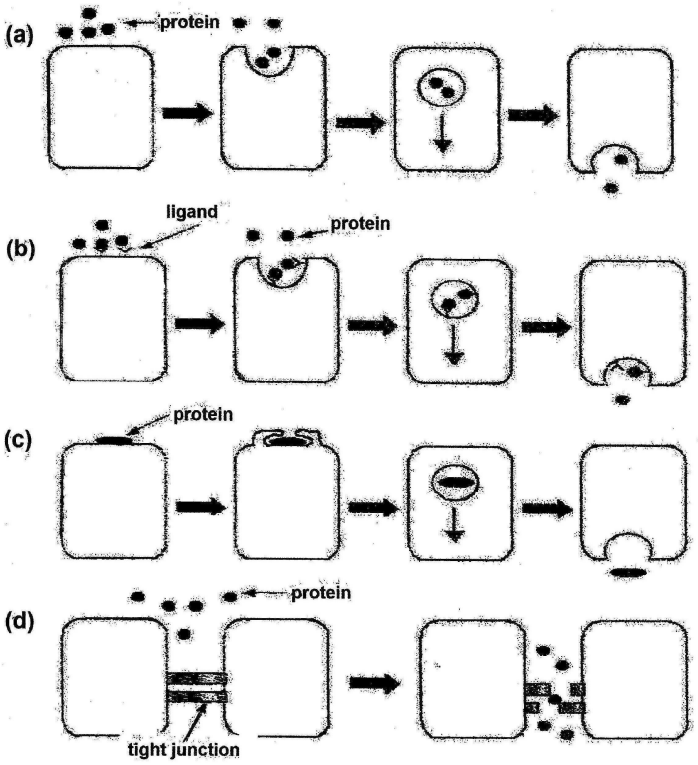

Figure 3- Possible transport mechanisms of orally administered protein across intestinal epithelium: (a) pinocytosis transport, (b) receptor mediated endocytosis transport, (c) phagocytosis transport, and (d) paracellular transport [154].

The matrix may be composed of either a lipophilic or hydrophilic polymer depending on the properties of the drug and the rate of release desired. However, it is difficult to achieve constant rates of drug release with nondegrad able matrix systems, for example, the rate of release of carmustine from an EVAc matrix device drops continuously during incubation in buffered water [163]. Biodegradable polymeric devices are formed by physically entrapping drug molecules into matrices or microspheres. These polymers dissolve when implanted (injected) and release drugs.

Examples of biodegradable polymers are poly(lactide-co-glycolide) (PLGA), and poly (p – carboxy phenoxy propane – co - sebacic acid) (PCPP - SA) [164]. Some of the commercially available polymeric devices are Decapeptyl, Lupron Depot (microspheres), and Zoladex (cylindrical implants) for prostate cancer and Gliadel for recurrent malignant glioma. The half-life of therapeutics administered by microspheres is much longer than free drug injection. Polymers are also being investigated for treating brain tumors [165], and delivery of proteins and other macromolecules [166].

The use of natural biodegradable polymers to deliver drugs continues to be an area of active research despite the advent of synthetic biodegradable polymers (167-174). The desirable characteristics of polymer systems used for drug delivery, whether natural or synthetic, are minimal effect on biological systems after introduction into the body; in vivo degradation at a well-defined rate to nontoxic and readily excreted degradation products; absence of toxic endogenous impurities or residual chemicals used in their preparation, e.g., crosslinking agents [148]. Natural polymers remain attractive primarily because they are natural products of living organisms, readily available, relatively inexpensive, and capable of a multitude of chemical modifications [148].

A majority of investigations of natural polymers as matrices in drug delivery systems have centered on proteins (e.g., collagen, gelatin, and albumin) and polysaccharides (e. g., starch, dextran, inulin, cellulose, and hyaluronic acid) [175]. There are three general mechanisms by which drugs are delivered from polymer or lipid systems: (1) diffusion of the drug species from or through the system; (2) a

chemical or enzymatic reaction leading to degradation of the system, or cleavage of the drug from the system; and (3) solvent activation, either through osmosis or swelling of the system [176]. Advantages and disadvantages of these systems have been discussed previously [177]. A combination of mechanisms is possible. Polymer-based systems have had an enormous impact on drug therapies (Fig. 4). In one approach, the drug is physically entrapped inside a solid polymer that can then be injected or implanted in the body. Early forms of these systems involved non-degradable polymers (membrane - controlled diffusion) (Fig. 4a) such as silicone rubber, which could release low molecular mass lipophilic drugs for extremely long times [178]. This type of approach led to the development of Norplant, small silicone capsules containing contraceptives that are slowly released by diffusion through the polymer for 5 years. However, this approach does not permit the slow delivery of either ionic species or molecules with a relative molecular mass (*M*r) over about 400 because they are not able to diffuse through such polymers. To address this problem, drugs were physically embedded in polymers at concentrations high enough to create a series of interconnecting pores through which the drug could subsequently slowly diffuse [179] (a type of matrix system, Fig. 4b). Early studies utilized ethylene–vinyl acetate copolymer or various hydrogels as model polymers. Subsequently, to develop biodegradable systems with such properties, lactic/glycolic acid copolymers were utilized. In this case, the combination of diffusion through pores as well as polymer matrix degradation allows control of release rates. This approach has provided the basis for injectable delivery systems lasting for 1 to 4 months for normally short-lived polypeptide hormones such as luteinizing hormone releasing

hormone analogues [180] now used by about 300,000 patients annually for treating advanced prostate cancer, endometriosis or precocious puberty.

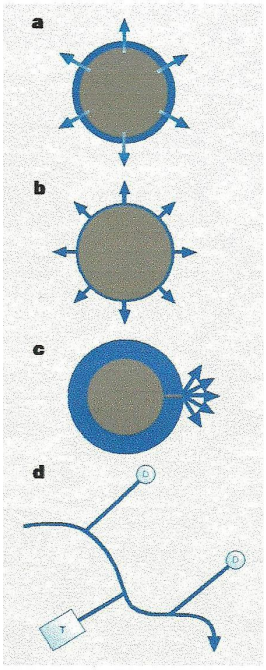

Figure 4. Examples of polymer-based delivery systems. In a-c, small dots represent drug, and arrows show the direction in which drug is released. a, Reservoir system in which drug diffuses through a polymer membrane (blue rim). This is the design for Norplant, Ocusert and a number of other systems. b, Matrix system in which the drug is evenly distributed through a polymer system. The drug can be released by diffusion through the polymer. Alternatively, drug release can occur by a combination of drug diffusion and polymer erosion. c, Osmotic system in which drug is pumped out through a laser-drilled hole. Water is attracted through the blue semi permeable membrane (the membrane is permeable to water but impermeable to drug) by osmosis due to the drug or a coencapsulated salt in the system. d, Polymeric drug conjugates. The curved line represents polymer. The bonds connecting drug (D) and polymer are cleavable inside the body. The targeting moiety, T, is optional [181].

The major scientific challenges in the development of gastric retention devices is to overcome strong gastric contractions that occur every few hours, particularly in the fasted state [181]. If the gastric retention technology is based on swelling or expansion of the system, this then must be sufficient to maintain the delivery system in the stomach in the fasted condition. The extensive swelling or expansion, however, tends to result in mechanically weak properties, which may not withstand the compression exerted by the housekeeper waves. For this reason, fast swelling hydrogels with mechanically strong and elastic polymers have been developed [182].

2.5 Hydrogels for drug delivery applications

Hydrogels are hydrophilic polymer networks capable of absorbing large amounts of water, yet insoluble because of the presence of cross-links, crystalline

regions, or entanglements [142]. Hydrogels are either neutral or ionic, depending on the nature of the side groups. They can be classified based on the network morphology as amorphous, semicrystalline, hydrogen-bonded structures, supermolecular structures, and hydrocolloidal aggregates. Additionally, in terms of their network structures, hydrogels can be classified as macroporous, microporous, or nanoporous [183-184]. Since the development of poly(2-hydroxy ethyl methacrylate) gels, hydrogels have been considered for use in a wide range of biomedical and pharmaceutical applications, mainly due to their high water content and rubbery nature. Because of these properties, hydrogel materials resemble natural living tissue more than any other class of synthetic biomaterials. Furthermore, the high water content allows these materials to exhibit excellent biocompatibility. Some applications of hydrogels include contact lenses, biosensors, artificial organs, dental materials, and drug delivery systems [185-190].

2.6 pH-Sensitive complexation of hydrogels

Depending on the nature of the side groups along the polymer chains, hydrogels have the ability to respond to their environmental changes such as pH, ionic strength, temperature, or specific chemical compounds [191-192]. This environmentally sensitive behavior has led to the extensive use of hydrogels in controlled drug delivery systems, where they can respond to changes in the environment thus, regulate drug release [193-198]. One of the hydrogels that exhibit pH-responsive behavior is complexation hydrogels. Osada studied complex formation in PMAA hydrogels [199]. In acidic media, the PMAA membranes collapsed in the linear PEG chains due to the formation of interpolymer complexes

between the PMAA and PEG. The gels swelled when placed in neutral or basic media. The permeability of these membranes was strongly dependent on the environmental pH and PEG concentration [200]. Similar results were observed with hydrogels of PAA and linear PEG [201]. Interpenetrating polymer networks of PVA and PAA that exhibit pH-sensitive behavior due to complexation between the polymers were prepared and the release behavior of indomethacin was studied [202-203]. Peppas *et al.* [204-208] have developed a class of graft copolymer hydrogels of PMAA with grafted PEG (P(MAA-g-EG)). These gels exhibited pH-dependent swelling behavior due to the presence of acidic dependent groups and the formation of interpolymer complexes between the ether groups on the graft chains and protonated pendent groups. Klier *et al.* [206] studied the responsive abilities of the P(MAA-g-PEG) materials to their environment. The materials exhibited a dynamic volume change depending on the pH of their environment. Klier *et al.* [206] also studied the ability of the material to act as a controlled release drug delivery device. The materials exhibited a release of drugs dependant on their environment possessing a controlled release at high pH values, while hindering the release at low pH.

In 1997, Lowman and Peppas first discussed the use of P(MAAg- PEG) as a carrier for oral protein delivery systems [209]. Their work showed that the hydrogels possessed the ability to regulate the release of drugs depending on the surrounding environmental pH. Thus, in low pH environments the network would stay collapsed and the mesh of the network would keep the drug inside of the polymer. Whereas in neutral or high pH environments, the network would swell open and the meshwork of

the polymer chains would open up. This would allow the drug to diffuse out of the polymer and into the surrounding environment. These studies were conducted with microspheres of the material made via an emulsion polymerization that used silica oil and water as the phases. This technique yielded particles of a specific size but used an organic solvent. The solvent had to later be removed from the materials to avoid causing problems with other studies [155].

Madsen and Peppas [210] conducted studies into the ability of P(MAA-g-PEG) hydrogels to inhibit proteolytic enzymes. It was found that the calcium binding properties of the copolymer inhibited the enzymatic activities of model digestive enzymes. This study is important because it shows that not only is the material able to provide a stable carrier that is sensitive to pH and can release the insulin into the targeted upper small intestine, but also that the material is able to provide protection to the insulin inside by deactivating some of the digestive enzymes in the stomach. By protecting its contents the amount of active insulin that reaches the upper small intestine increases.

2.7 Polymer complexes in stimuli-sensitive behavior

Polymer complexes are insoluble, macromolecular structures formed by the non-covalent association of polymers with an affinity for one another. The complexes form due to association of repeating units on different chains (interpolymer complexes) or on separate regions of the same chain (intrapolymer complexes). Polymer complexes are classified by the nature of the association. The major classes of polymer complexes are stereocomplexes, polyelectrolyte (polyionic) complexes, and hydrogen-bonded complexes [211-213]. The non-ionic association

of stereoisomers caused by van der Waals interactions forms stereocomplexes. One example of this type of complex is van der Waals complexes between poly(methyl methacrylate)PMMA and poly(methacrylic acid) (PMAA) [211].

Polyelectrolyte complexes are formed between macromolecular acids and bases or their salts. These complexes are stabilized by ionic bonds. Some of the polymers that exhibit polyelectrolyte complexation include poly(acrylic acid) (PAA) and PMAA with poly(ethyleneimine) [214-216], and PAA with chitosan [217].

Polyelectrolytes have been identified for use as desalination and ultrafiltration membranes, dialysis membranes, and peptide stabilizers [218]. Interpolymer complexes stabilized by hydrogen bonds are formed between polymers containing electron-donating protons, typically poly(carboxylic acids), and polymers containing electron-donating groups such as poly(ethylene glycol) (PEG), polypyrrolidones, or alcohols. Some examples of polymer systems that form complexes due to hydrogen bonding include PAA and polyacrylamide [216], PAA and poly(vinyl alcohol) [204, 219-220], PAA and PEG [221-223], PMAA and poly(vinyl pyrrolidone) [224-226], and PMAA and PEG [208-209, 221, 227-228].

Hydrogen-bonded complexes are generally formed in aqueous media within a narrow range of solvent composition, pH, and ionic strength. In addition, the complexes are stabilized by the composition and structure of the copolymer, as well as by hydrophobic interactions. The stability of hydrogen-bonded complexes is also dependent on many environmental factors such as temperature, nature of the solvent, pH, or ionic strength. Hydrogen-bonded complexes involving polyacids are only formed when the pH if the solution is sufficiently low for substantial protonation

of the acidic groups. Additionally, this type of interpolymer complexation process is reversible in nature. Because of their nature, hydrogen-bonded complexation polymer systems have the ability to function in a wide variety of applications. Of various applications, the application for drug delivery has received significant interest. In particular, complexation copolymers of PMAA/PEG have been studied great detailed for use as drug delivery systems [206-207, 229]. In these copolymers, interpolymer complexes form in acidic media due to hydrogen bonding between protonated carboxylic groups and the PEG ether groups. These complexes dissociate in neutral or basic media due to ionization of the acid groups (Figure 5).

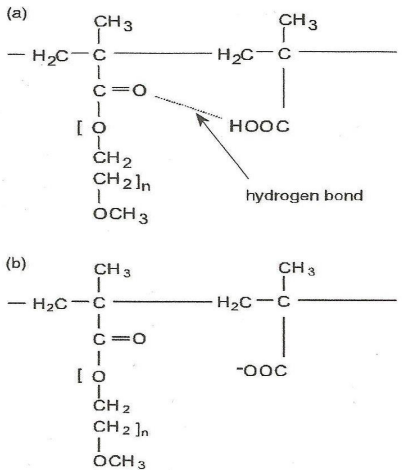

Figure 5 - Complexation/decomplexation of P(MAA-g-EG) networks: (a) in acidic media when the carboxylic acid group is protonated, and (b) is in neutral or basic media when the carboxylic acid group is ionized [154].

Polyelectrolyte complexes (PECs) [230], formed by mixing polysaccharides of opposite charge, have recently attracted considerable attention because of their

potential for use in drug delivery systems as well as in various biotechnological applications [231-234]. The multitude of reactions in biology that involve polyelectrolyte-polyelectrolyte interaction increases the interest in investigating this area. The characteristic properties of the polyelectrolytes, e.g. charge density, chain conformation and the response of PECs to variation in pH, ionic strength and temperature, offer a wide set of variables with a potential to be employed in pharmaceutical formulations. Interaction between a completely dissociated polyanion and a polycation, both of comparatively high charge densities, may be considered primarily to be of an electrostatic nature since such interactions are strong as well as of relatively long-range order. The possible influence of, for example, hydrophobic interactions and Van der Waals forces on the formation of PECs must also be considered, especially when the charge-charge interaction is screened by a supporting electrolyte of high ionic strength. Not only is the intermolecular interaction governed by the nature and structure of the polyelectrolytes involved but it can indeed be significantly affected by the prevailing solute – solvent and solvent-solvent conditions.

2.8 Protein and water diffusion in hydrogels

When a hydrophilic polymer network is brought into contact with water), the latter enters the polymer network resulting in a swollen gel phase. If a solute (protein or drug) is incorporated in the glassy polymer network, the solute will be released [235-237]. There have been many efforts to make use of this process of water penetration and subsequent protein release in polymer materials for controlled protein delivery systems. For many applications, the equilibrium swelling of the hydrogels may not be

the most important characteristic of the gel. For some applications, it is vital that the gel be able to exhibit fully reversible swelling and de-swelling in response to pH changes or ionic strength. The rate at which the gel swells or collapses in response to changes in the environmental pH are vital in determining whether or not a particular gel is suitable for a given application. The early work of Katchalsky [238-240] in the area of polyelectrolyte gels usually used in stimuli-sensitive devices established the fact that the collapse and expansion of poly(methacrylic acid) gels occurred reversibly by simply adjusting the pH of the fluid. The group of Tanaka [241] performed significant work in this area as well and confirmed the reversible behavior of polyelectrolyte networks. Ohmine and Tanaka have also observed the sudden collapse of ionic networks in response to sudden changes in the ionic strength of the swelling medium. Studies by Khare and Peppas [242] examined the swelling kinetics of poly(methacrylic acid) or poly(acrylic acid) with poly(hydroxylethyl methacrylate). They observed pH and ionic strength dependent swelling kinetics in these gels.

3. Polyethylenimine

Polyethylenimine (PEI) is a weakly basic, aliphatic, non toxic synthetic polymer which is polycationic owing to the presence of primary, secondary, and tertiary amino groups [243]. The most popular cationic polymer as non-viral vector is PEI. It forms a complex with pDNA and showed high gene expressions in both in vitro and in vivo gene delivery [244-245]. PEI is a highly water soluble, positively charged, synthetic polymer in which every third atom is a nitrogen that can be protonated as well as provide a potential branching point [246]. Approximately 20%

of the nitrogen of PEI are protonated under physiological conditions [247-248]. As a result, the polymer can change its ionization state over a broad pH range [246]. PEI is a highly efficient vector for delivering oligonucleotides and plasmids both *in vitro* and *in vivo* [249]. It is an organic macromolecule with the highest cationic-charge-density potential [249]. PEI has been used previously to coat surfaces to promote the attachment of neurons in primary cell cultures [250-253]. Polyethylenimine is an effective attachment factor for weakly anchoring cell lines and primary cells [254]. Its use in lipofection protocols makes the procedures more reliable and increases the yield of expressed products with commonly used cell lines such as PC-12 and HEK-293 cells [254]. Since 1995, PEI has been found to be a versatile polymeric vector for gene delivery that tightly condenses plasmid DNA and is able to promote transgene delivery to the nucleus of mammalian cells [244,249,253-255]. PEI has a strong permeabilizing effect but no bactericidal effect on gram-negative bacteria [255]. PEI prepared in nanoparticles were incorporated in dental provisional cements and found to had antibacterial effect against *Streptococcus mutans* [256]. Insoluble cross-linked quaternary ammonium polyethylenimine (PEI) nanoparticles incorporated at 1 or 2% w/w in a resin composite also has antimicrobial effect against *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Pseudomonas aeruginosa* and *Escherichia coli* [257].

4. Chitosan

Chitosan (β-(1→4)-2-amino-2-deoxy-D-glucan) is prepared by the alkaline deacetylation of chitin (β-(1→4)-2-acetamido-2-deoxy-D-glucan) which is at the second place in the nature among the renewable organic resources after cellulose

[258–260]. Chitosan is the only cationic polyelectrolyte among the natural polysaccharides [261-262]. The presence of positively charged amino groups in its molecule causes this polysaccharide to bind to metal ions via chelation and to be adsorbed at various interfaces, in most cases negatively charged. Chitosan is widely used in the industry as a flocculant for wastewater purification and a thickener for aqueous media. It is also used to prepare fibers, films, membranes, coatings, and microcapsules for manufacturing paper, fabrics, photographic materials, food packages, and drug deliverysystems [258-260, 263-264]. Chitosan is absolutely nontoxic and exhibits excellent biocompatibility, as well as immunostimulating, wound healing, antiseptic, and high sorption effects. All these properties explain a drastically increasing chitosan application as a component of drugs for the external use; an enterosorbent added to preparations for weight loss and health improvement; and a component of other compositions used in biological medicine [259-260, 263-267]. Because chitosan is a cationic polyelectrolyte, it is widely used to form PECs with various anionic polysaccharides and synthetic polymers [258, 268–274]. Its mixtures with carrageenans were studied in a number of works. The insoluble complexes of chitosan with k-carrageenan were used in [275] for the preparation of microcapsule shells, and in [276] these complexes were applied for chitosan separation from wastewater of food industry enterprises. Chitosan–k-carrageenan gels were obtained in quite concentrated solutions (4%) [277-278]. The interactions between chitosans deacetylated to different degrees and carrageenans of the three types (κ, ι, λ) were studied in detail by a group of Swedish researchers [279]. Chitosan, the deacetylated form of chitin is non-toxic and easily

bioabsorbable [122] with gel forming ability at low pH. Moreover, chitosan has antacid and anti-ulcer activities that prevent or weaken drug irritation in the stomach [122]. Also, chitosan matrix formulations appear to float and gradually swell in acid medium. All these interesting properties of chitosan made this polymer an ideal candidate for controlled drug release formulations [123]. Chitosan is also used in drug delivery systems in nanoparticles, hydrogels, microcapsules and transdermal devices [1].

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CHAPTER 3

Evaluation of pDNA-Polyethylenimine Complex Coated with *Iota* Carrageenan for In Vitro Gene Delivery

Chapter 3

ABSTRACT

In the present study, we evaluated pDNA/PEI complex coated with *iota* carrageenan via electrostatic interaction at various N/P ratios for its efficiency in transfecting CHO-K1, COS-7 and HuH-7 cells in the presence of 10% (w/v) fetal bovine serum (FBS) with four hours exposure. Results showed that at N/P ratio (PEI/pDNA) of 3.3 coated with iota carrageenan showed higher transfection efficiency in CHO-K1 cells as compared to N/P ratios of 5 and 7. The ratio of 1:3.3:0.3 (pDNA/PEI/*Iota*-carrageenan) exceeded the transfection efficiency of pDNA/PEI complex by 72.0%. Comparing the transfection efficiency in HuH-7 cells, we found out that pDNA/PEI/*Iota*-carrageenan is less effective. Adjusting the N/P ratio of PEI/pDNA from 3.3 to 3.5, transfection efficiency is more effective in COS-7 cells and HuH-7 cells. Using N/P ratio of 3.5 in CHO-K1 cells, transfection efficiency is more effective at ratio of 1:3.5:0.5 (pDNA/PEI/*Iota*-carrageenan). It exceeded the efficiency of pDNA/PEI complex by 89.7%. The prepared complexes showed resistance to DNase I degradation and showed no significant cytotoxicity to COS-7 and CHO-K1 cells. It is stable after exposure to increasing concentration of bovine serum albumin, dextran sodium sulfate and chondroitin sulfate solutions. Atomic force microscopy showed that the coated complexes exhibited mostly spherical shape in nanoparticle size with negative to positive zeta potential. The addition of *iota*-carrageenan to pDNA-PEI complex resulted in the formation of a ternary polyion complex that shows potential use in gene delivery.

Chapter 3

INTRODUCTION

The most popular cationic polymer as non-viral vector is PEI. It forms a complex with pDNA and showed high gene expressions in both in vitro and in vivo gene delivery [1,2]. The complex binds non-specifically to negatively charged proteoglycans on cell membranes and agglutinates with blood components, such as erythrocytes and serum albumins. This often leads to its rapid elimination and adverse effects, such as embolism and inflammatory reaction [3-6]. To solve these problems, studies were made to reduce the cytotoxicity and agglutination with blood components. These involve preparation and development of several novel polymers of PEI covalently bind to hydrophilic polymers like: polyethyleneglycol (PEG), polyhydroxypropylmethacrylamide (pHPMA), and polyvinylpyrrolidine to modify the complex surface [4,6,7]. Aside from hydrophilic polymers, studies were also done on the use of anionic polymers. Studies showed that the introduction of a protective polyanion coating of pDNA/polycation binary complex using polyacrylic acid derivatives resulted in high level gene expression in lung after injection into mouse tail vein wherein the surface of the resulting ternary complex was recharged to negative one [8]. Anionic polymers can bind to the cationic complex electrostatically to modify the complex surface [8,9]. These polyanions do not decompose the pDNA/polycation complex but attached to the cationic surface of the complex forming a ternary polyion (pDNA/polycation/polyanion) complex that retains its transfection efficiency [10]. Polysaccharides have been used for quite some time as controlled release coatings, matrices, macromolecular carriers, and biodegradable carriers [11]. These consist mostly of chains of monosaccharide or disaccharide

units linked together by O-glycosidic linkages with a large number of derivatizable groups, a wide range of molecular weights, varying chemical compositions, and for the most part, a low toxicity and biodegradability, yet a high stability [11].

The group of Kawakami [12] reported that mannosylated, fucosylated, and galactosylated liposomes showed high accumulation in the liver via each specific receptor. An example of this, is hyaluronic acid which is taken by a specific receptor-mediated endocytosis and used for targeted drug delivery systems [13,14]. Hyaluronic acid also forms a ternary complex with polycation and DNA with high gene expression and stability in serum-containing medium [9]. Kurosaki et al, 2009 [15], has developed a gene vector electrostatically assembled with a polysaccharide capsule. Lambda type carrageenan was one of the polysaccharides used in the coating of pDNA-PEI complex. They reported that the ternary complex coated with lambda type carrageenan and other polysaccharides except for chitosan showed little uptake and gene expression. In this study, iota-carrageenan was used as polyanion coating for pDNA-PEI complex through electrostatic interaction. The resulting ternary complexes were then evaluated according to its transfection efficiency, physical properties and morphology. The ratios of the ternary complexes were also modified using lower N/P (PEI/DNA) ratios of 3.3 and 3.5 than the study of Kurosaki et al [15] which used an N/P ratio of 8 with varying ratios of carrageenan. In their study, transfection was carried out in reduced serum medium using B16-F10 cells. In our study, we used CHO-K1, COS-7 and HUH-7 cells for the evaluation of transfection efficiency in the presence of 10% FBS for four hours.

RESULTS AND DISCUSSION

Complex formation of pDNA/PEI/Carrageenan

The formation of ternary complex of pDNA-PEI-carrageenan was determined by gel retardation assay. Release of DNA was not observed in the agarose gel chromatogram (Figure 1), hence a ternary complex was formed after addition of carrageenan to pDNA/PEI complex (N/P = 3.3) prepared at various charge ratios (+/-). All images obtained from atomic force microscope (AFM) in Figure 2, showed no unbound DNA confirming a good complex formation. Discrete particles with spherical or toroidal shapes were observed. In Figure 3 (N/P ratio of 3.5), the electron micrographs showed complexes with spherical shapes in most charge ratios. The size of the carrageenan coated complexes was also determined by dynamic laser light scattering (DLS). Both mean size and dispersity depended on the charge ratios (Figure 3). The complex with charge ratio of 1:3.5:2.5 (Figure 3D) has the largest particle size compared to the other ratios which is in conformity with the AFM micrograph. The shape of the complex is circular and is the biggest among the other complexes. The charge ratio of 1:3.5:6.0 has the smallest particle size even smaller than pDNA/PEI complex (N/P = 3.5). Therefore charge ratios greatly influence the particle size of the complex.

Transfection efficiency

As observed in Figure 4, the results of transfection efficiency showed that the complex of PEI/pDNA at N/P ratio of 3.3 coated with iota carrageenan showed higher transfection efficiency in CHO-K1 cells as compared to N/P ratios of 5 and 7. The ratio of 1:3.3:0.3 (pDNA/PEI/*Iota*-carrageenan) exceeded the transfection

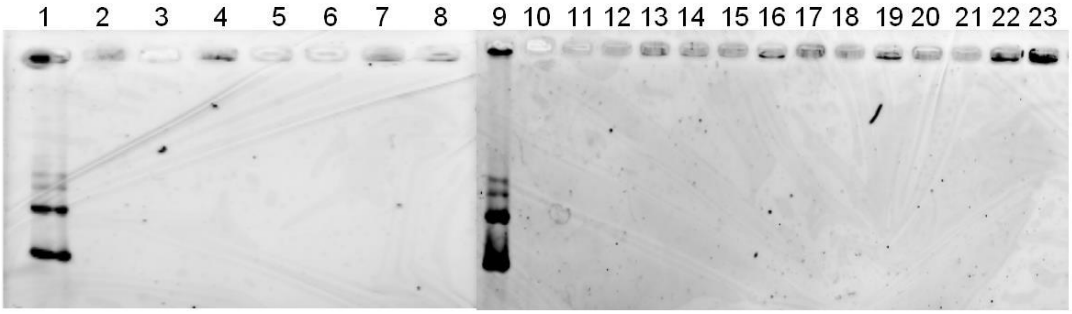

Figure 1. Effect of carrageenan on electrophoretic migration of pDNA through an agarose gel. Each complex was loaded onto agarose gel, and electrophoresis was carried out. Retardation of pDNA was visualized using ethidium bromide. Lanes 1 & 9: pDNA (0.1 μg); lane 2: pDNA/PEI (N/P=3.3); lane 3: pDNA/PEI/*Iota*-carrageenan (1:3.3:8.3); lane 4: pDNA/PEI/*Iota*-carrageenan (1:3.3:3.3); lane 5: pDNA/PEI/*Iota*-carrageenan (1:3.3:2.3); lane 6: pDNA/PEI/*Iota*-carrageenan (1:3.3:1.3); lane 7: pDNA/PEI/*Iota* carrageenan (1:3.3:0.3); lane 8: pDNA/PEI/*Iota*-carrageenan (1:5.0:10.0); lane 10: pDNA/PEI (N/P=5.0); lane 11: pDNA/PEI/*Iota*-carrageenan (1:5.0:5.0); lane 12: pDNA/PEI/*Iota*-carrageenan (1:5.0:4.0); lane 13: pDNA/PEI/*Iota*-carrageenan (1:5.0:3.0); lane 14: pDNA/PEI/*Iota*-carrageenan (1:5.0:2.0); lane 15: pDNA/PEI/*Iota*-carrageenan (1:5.0:1.0); lane 16: pDNA/PEI (N/P = 7.0); lane 17: pDNA/PEI/*Iota*-carrageenan (1:7.0:15.0); lane 18: pDNA/PEI/*Iota*-carrageenan (1:7.0:12.0); lane 19: pDNA/PEI/*Iota*-carrageenan (1:7.0:10.0); lane 20: pDNA/PEI/*Iota*-carrageenan (1:7.0:9.0); lane 21: pDNA/PEI/*Iota*-carrageenan (1:7.0:8.0); lane 22: pDNA/PEI/*Iota*-carrageenan (1:7.0:6.0); lane 23: pDNA/PEI/*Iota*-carrageenan (1:7.0:3.0).

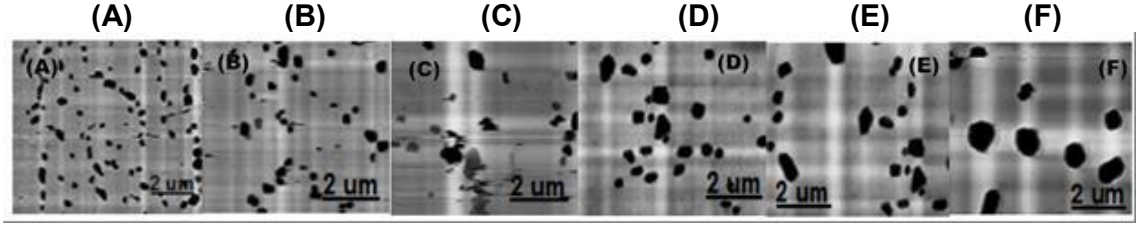

Figure 2. AFM images of pDNA/PEI/Iota carrageenan complexes at 10 μg/mL plasmid concentration. (A). pDNA/PEI (N/P=3.3); (B). pDNA/PEI/*Iota*-carrageenan (1:3.3:8.3); (C). pDNA/PEI/*Iota*-carrageenan(1:3.3:3.3); (D). pDNA/PEI/*Iota*-carrageenan (1:3.3:2.3); (E). pDNA/PEI/*Iota*-carrageenan (1:3.3:1.3); (F). pDNA/PEI/*Iota*-carrageenan (1:3.3:0.3).

efficiency of pDNA/PEI complex by 72.0%. Comparing the transfection efficiency in HuH-7 cells (Figure 5), we found out that pDNA/PEI/*Iota*-carrageenan is less effective. Adjusting the N/P ratio of PEI/pDNA from 3.3 to 3.5, transfection efficiency

is more effective in COS-7 cells and HuH-7 cells as shown in Figures 6 -7. Using N/P ratio of 3.5 in CHO-K1 cells (Figure 8), transfection efficiency is more effective at ratio of 1:3.5:0.5 (pDNA/PEI/*Iota*-carrageenan). It exceeded the efficiency of pDNA/PEI complex by 89.7%. Zeta potential of the complexes was also determined. As shown in the graph (Figure 9), the pDNA/PEI complex at N/P ratio of 3.5 has a positive zeta potential of +19.8mV while the rest of the complexes coated with carrageenan have lower values of zeta potential confirming the complete charge masking of the complex. The zeta potential measurements showed the shielding effect of carrageenan. The complex with a ratio of 1:3.5:0.5 displayed a strongly positive zeta potential and exhibited a good transfection efficiency in CHO-K1 and HuH-7 cells. However it displayed a poor transfection activity in COS-7 cells. The complex with a ratio of 1:3.5:4.0 which displayed a negative zeta potential exhibited good transfection efficiency in COS-7 while the complex with a ratio of 1:3.5:2.5 which has a positive zeta potential also gave high transfection efficiency in COS-7 cells as compared to other ratios. It was reported that cationic pDNA/PEI complex is taken up by the cells through endocytosis according to the electrostatic interaction with cell membrane [9]. Hence the complexes with positive zeta potential showed good transfection efficiency in CHO-K1, HuH-7 and COS-7 cells. Anionic complexes could not be taken by cells in the same manner [15] thus, the poor transfection efficiency of the complexes with negative zeta potential was observed.

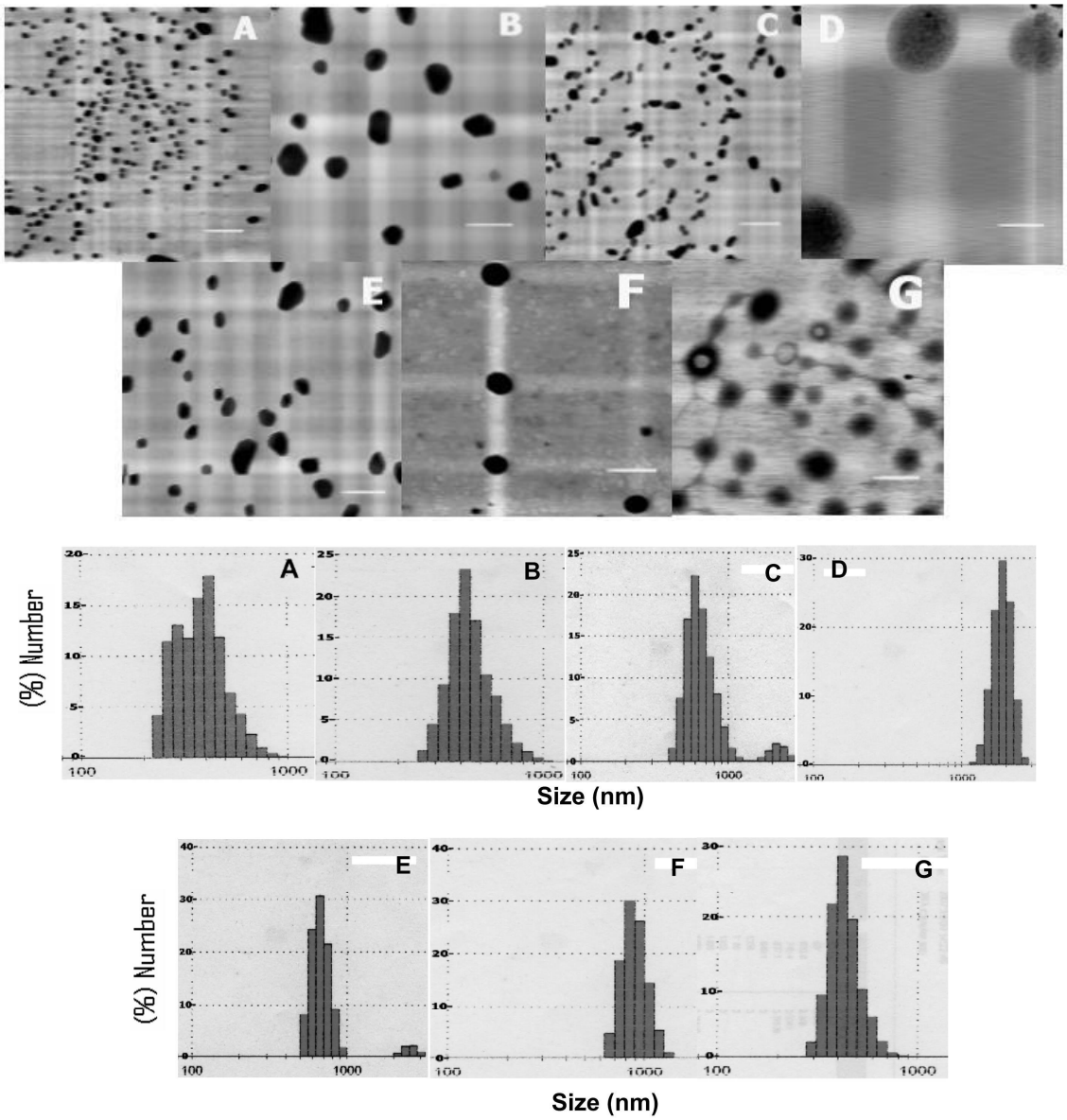

Figure 3. AFM images and size of pDNA/PEI//*iota*-carrageenan complexes with N/P ratio of 3.5 at 10 μg/mL plasmid concentration. Bars represent 1000 nm. A.(1:3.5:6.0); B. (1:3.5:5.0); C. (1:3.5:4.0); D. (1:3.5:2.5); E. (1:3.5:1); F. (1:3.5:0.5). G. pDNA-PEI = 3.5. 10 µL of ternary complexes with DNA (10 µg/mL) was applied in mica disk. A commercial atomic force microscope, SPA-300 system, Seiko Instrument, Inc. was used for imaging with Si_3N_4 tip. Dynamic light scattering shows an increase in particle size for charge ratio of 1:3.5:2.5.

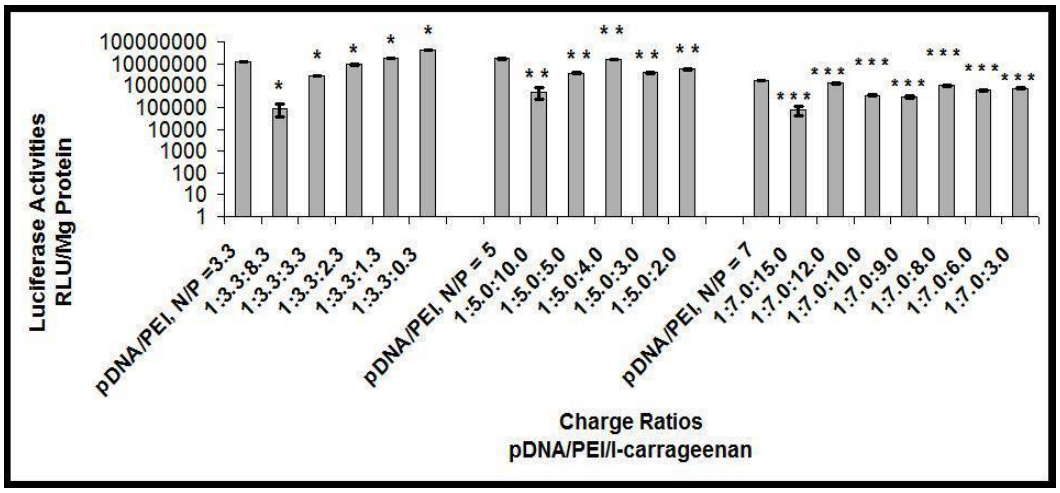

Figure 4. Transgene efficiency of each complex coated with *iota*-carrageenan. CHO-K1 cells were transfected with complex containing pGL3(SV40). Twenty-four hours after transfection the luciferase efficiency was evaluated. Each bar represents the mean ± S.E. of three experiments. * : P = 0.008 vs pDNA/PEI ; ** : P < 0.001 vs pDNA/PEI; *** P = 0.0027 vs pDNA/PEI. Transfection using pDNA-PEI-carrageenan complex was made in the presence of 10% FBS at a plasmid concentration of 5 μg/mL.

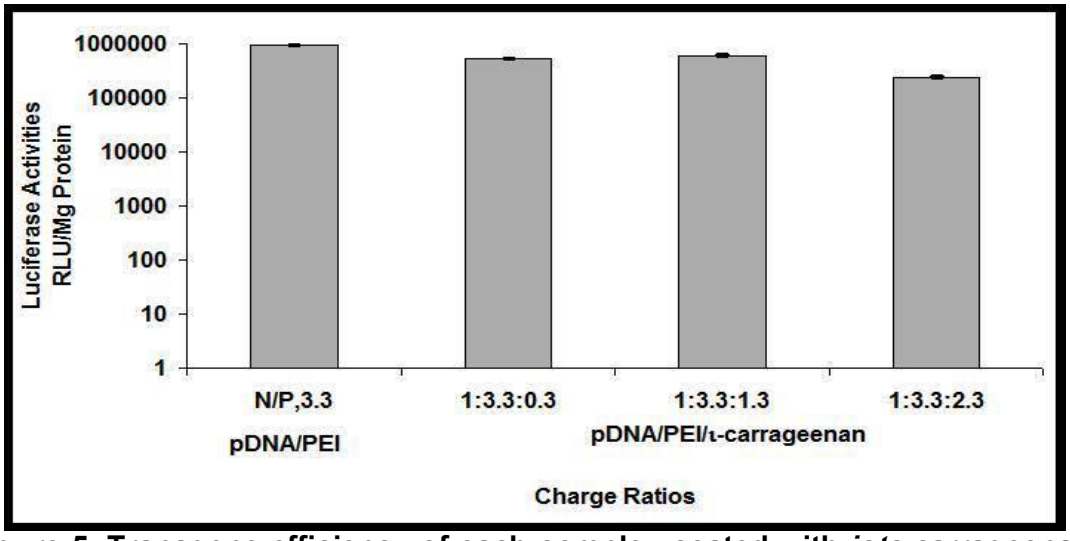

Figure 5. Transgene efficiency of each complex coated with *iota*-carrageenan. HuH-7 cells were transfected with complex containing pGL3(SV40). Twenty-four hours after transfection the luciferase efficiency was evaluated. Each bar represents the mean ± S.E. of three experiments. * : P < 0.001 vs pDNA/PEI. Transfection using pDNA/PEI/*Iota*-carrageenan complex was made in the presence of 10% FBS at a plasmid concentration of 5 μg/mL.

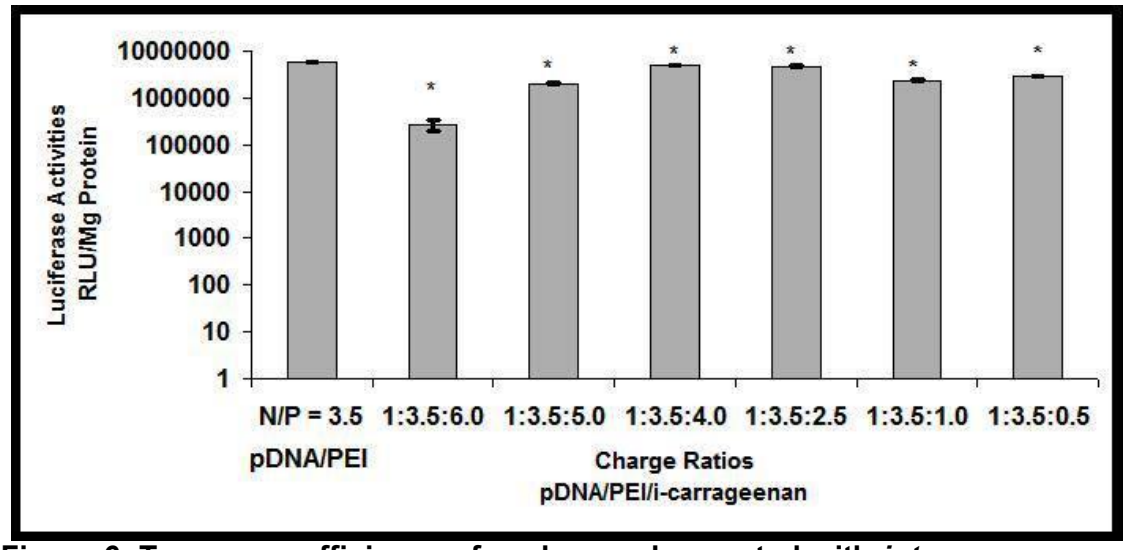

Figure 6. Transgene efficiency of each complex coated with *iota*-carrageenan. COS-7 cells were transfected with complex containing pGL3(SV40). Twenty-four hours after transfection the luciferase efficiency was evaluated. Each bar represents the mean ± S.E. of three experiments. * : P < 0.001 vs pDNA/PEI. Transfection using pDNA/PEI/*Iota*-carrageenan complex was made in the presence of 10% FBS at a plasmid concentration of 5 μg/mL.

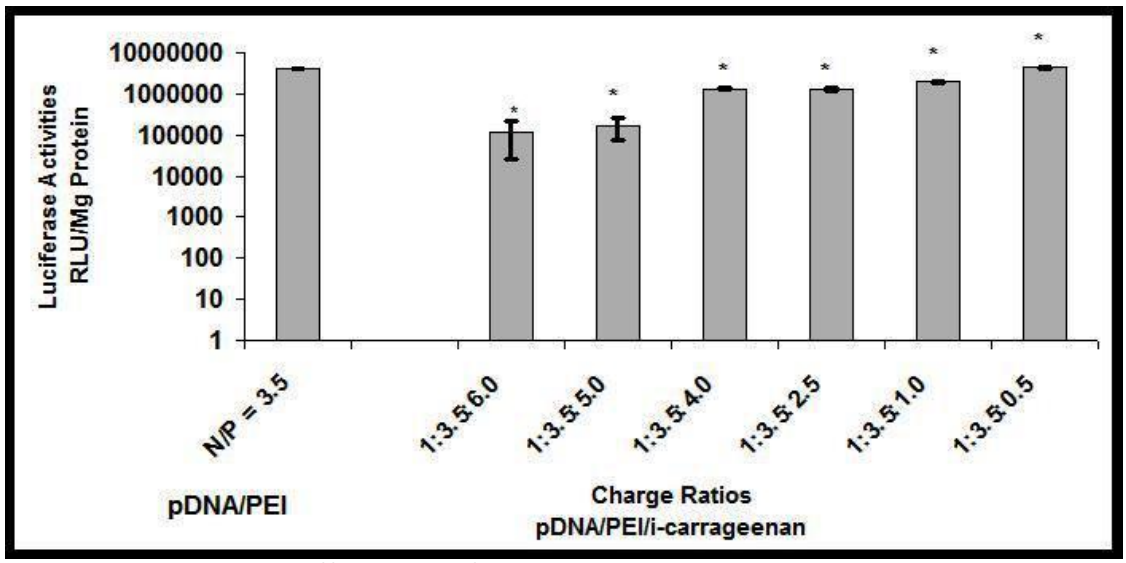

Figure 7. Transgene efficiency of each complex coated with *iota*-carrageenan. HuH-7 cells were transfected with complex containing pGL3(SV40). Twenty-four hours after transfection the luciferase efficiency was evaluated. Each bar represents the mean ± S.E. of three experiments. * : P < 0.001 vs pDNA/PEI. Transfection using pDNA/PEI/*Iota*-carrageenan complex was made in the presence of 10% FBS at a plasmid concentration of 5 μg/mL.

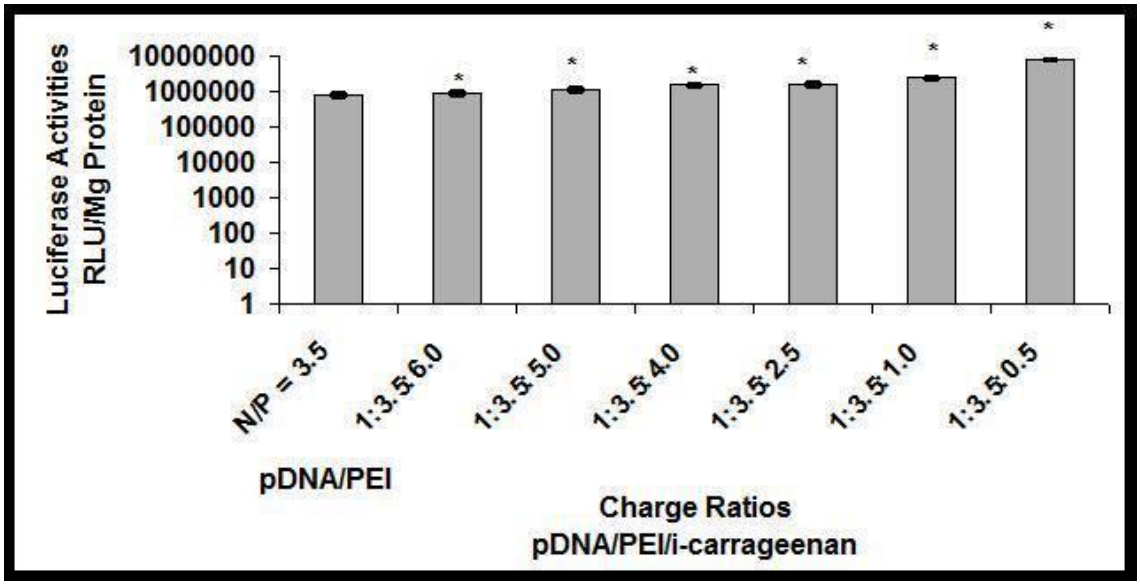

Figure 8. Transgene efficiency of each complex coated with *iota*-carrageenan. CHO-K1 cells were transfected with complex containing pGL3(SV40). Twenty-four hours after transfection the luciferase efficiency was evaluated. Each bar represents the mean ± S.E. of three experiments. * : $P < 0.001$ vs pDNA/PEI. Transfection using pDNA/PEI/*Iota*-carrageenan complex was made in the presence of 10% FBS at a plasmid concentration of 5 μg/mL.

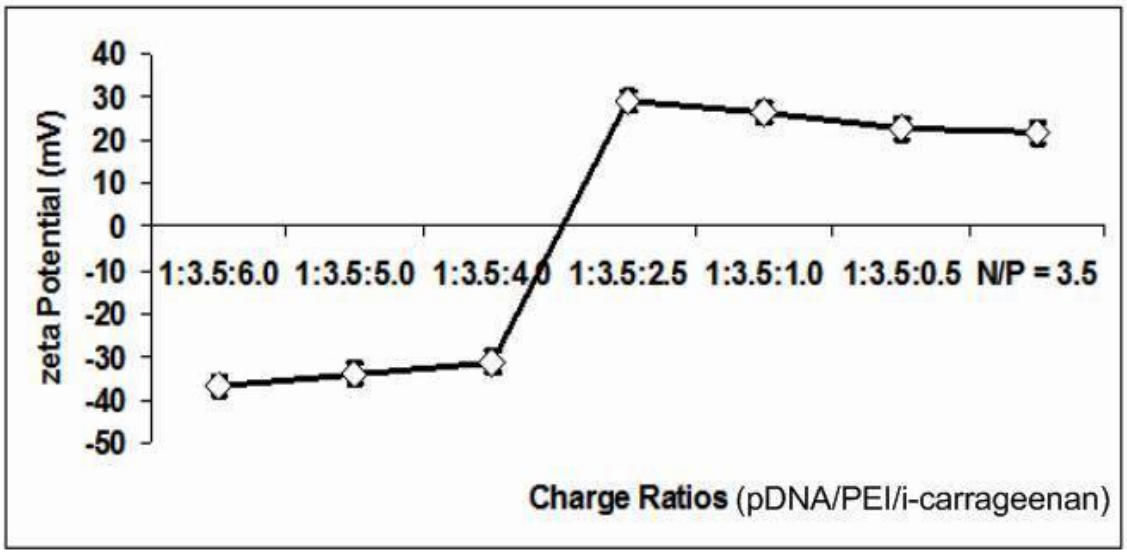

Figure 9. Zeta potential of pDNA/PEI complexes (N/P = 3.5) coated with *iota*-carrageenan. Each bar represents the mean ± S.E. of three experiments. * : $P < 0.001$ vs N/P =3.5 (pDNA/PEI).

Evaluation of Cytotoxicity

As shown in Figure 10, the complex with carrageenan has less toxic effects in the COS-7 cells as compared with pDNA/PEI complex. In previous studies made by Goula *et al.* [20], Bragonzi *et al.* [21] and Kircheis *et al.* [22] branched PEI (25 and 800 kDa) are generally less efficient and often toxic, particularly at high polycation nitrogen to DNA phosphate ratios (N/P) as compared to linear PEI [22]. In this study, the incorporation of carrageenan in the pDNA-PEI complex reduces the cytotoxic effects of PEI in the CHO-K1, HuH-7 and COS-7 cells. This is due to the neutralization effect of carrageenan in PEI. At 1 hour incubation period, viability of cells is above 80% for both cell lines. Viability of CHO-K1 cells slightly decreases after 4 hours incubation with the complex except in COS-7 cells. The complex with charge ratios 1:3.5:2.5; 1:3.5:1.0; 1:3.5:0.5 increases the viability of the COS-7 cells after 4 hours incubation as compared with CHO-K1 cells then decreases for another 4 hours incubation. The pDNA/PEI complex (N/P = 3.5; 3.3) decreases the viability of both CHO-K1, HuH-7 and COS-7 cells all throughout the 48 hours incubation period.

Stability in serum albumin

Previous studies showed that cationic complexes bound with serum albumin and formed aggregates [23]. The aggregation of pDNA complexes would induce a rapid clearance from plasma and be a disadvantage for nonviral gene delivery. The interaction of polycations with blood proteins such as albumin can also cause the complex to disassemble exposing DNA to degrading enzymes [23] thus the stability of the complexes with the presence of BSA was examined. Stability of the complex

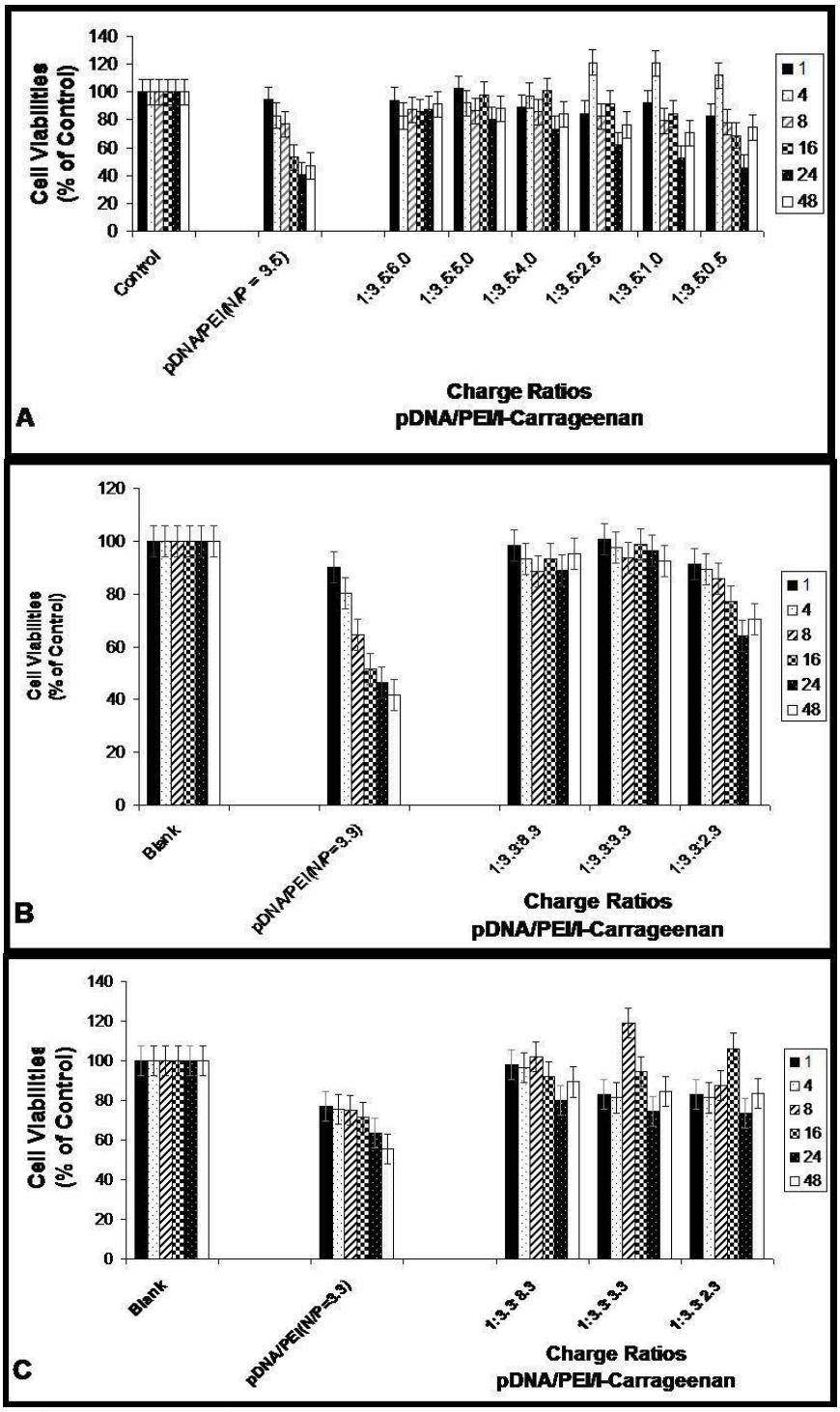

Figure 10. Cytotoxicity of pDNA/PEI/*Iota*-carrageenan (N/P = 3.5) against COS-7 cells (A); HuH-7 cells (B) and CHO-K1 (C), n=4. Each bar represents the mean ± S.E. of three experiments. * : $P < 0.001$ vs Blank.

for one week incubation was determined by turbidity assay at 350 nm. As shown in Figure 11, all of the complexes have lower turbidity reading compared with pDNA/PEI complex. The turbidity reading of pDNA/PEI complex decreased significantly suggesting formation of aggregates while carrageenan coated pDNA/PEI complexes had more stable turbidity readings. Carrageenan coating suppresses BSA-induced aggregation. The addition of carrageenan to the pDNA-PEI complex contributes to the stability of the pDNA complexes in the presence of serum. Carrageenan forms a complex with bovine serum albumin (BSA) through electrostatic interaction. Iota carrageenan gives a stronger electrostatic interaction than kappa type. The strength of complexation is dependent on the charge density on the polysaccharide [24].

Resistance to DNase I digestion

The resistance of ternary complexes to DNA hydrolysis by DNase I was examined. Figure 12 shows the agarose gel chromatogram of the complexes treated with DNase I. All of the ternary complexes showed resistance to DNase I while the free DNA and pDNA-PEI complex were degraded. The assay for DNA digestion showed that the complexes coated with carrageenan at various charge ratios were stable against DNase I degradation. Based on their morphology as observed in the AFM micrographs (Fig. 3), the complexes are mostly in spherical shapes showing a compacted pDNA. The results of the DNA digestion assay are well consistent with the AFM observation.

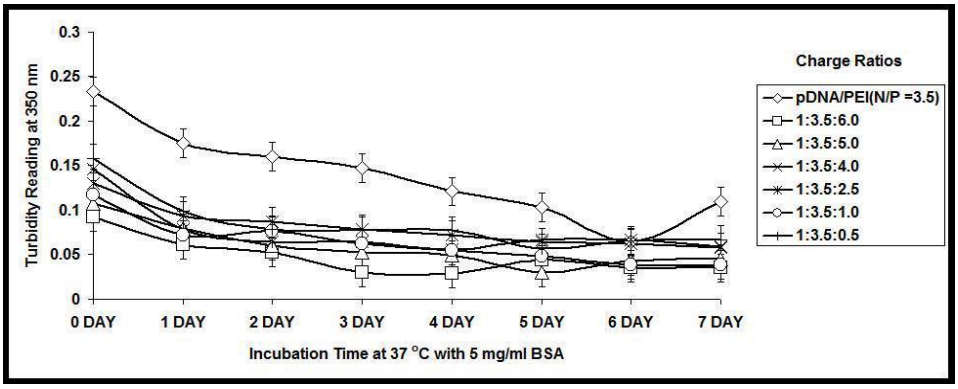

Figure 11. Turbidity of complexes after incubation with BSA (5 mg/mL) at 37 °C with mild agitation for 1 week. Conc. of pDNA = 100 µg/mL. The pDNA/PEI complexes were formed at N/P = 3.5 coated with *iota* carrageenan at various charge ratios. Each bar represents the mean ± S.E. of three experiments. * : P < 0.001 vs pDNA/PEI.

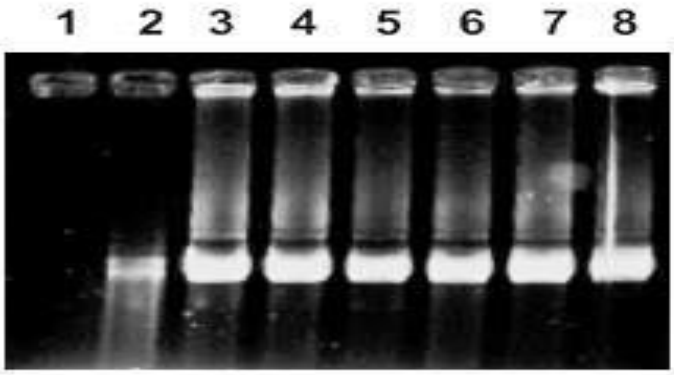

Figure 12. DNase I protection assay. pDNAPEI//*iota*-carrageenan complexes at various charge ratios were separately incubated with DNase I solution (0.5 units) in DNase/Mg^{2+} digestion buffer at 37 ^{0} C for 15 mins. Lane 1: Free DNA (0.1µg,); Lane 2: pDNA/PEI (N/P = 3.5); Lane 3:(1:3.5:6.0); Lanes 4:(1:3.5:5.0); Lane 5: (1:3.5:4.0); Lane 6: (1:3.5:2.5); Lane 7:(1:3.5:1.0); Lane 8: (1:3.5:0.5).

Stability in glycosaminoglycan

Exposure of the complexes with chondroitin sulfate showed no release of DNA. All of the complexes remain intact (Figure 13A). Full retention of the complexes was observed in the well. Release of DNA was not observed since

carrageenan has no binding effect with chondroitin sulfate. Both compounds are all polyanions. Carrageenan shielded the complex from interaction with chondroitin sulfate. In the case of dextran sodium sulfate, release of DNA was observed in ratios 1:3.5:6.0; 1:3.5:5.0; 1:3.5:4.0; 1:3.5:2.5; 1:3.5:1.0; (Figure 13B) but without degradation. pDNA/PEI complex (N/P = 3.5, Lane 2, Fig. 13B) and ternary complex with charge ratio of 1:3.5:0.5 (Lane 8, Fig. 13B) showed resistance in the release of DNA.

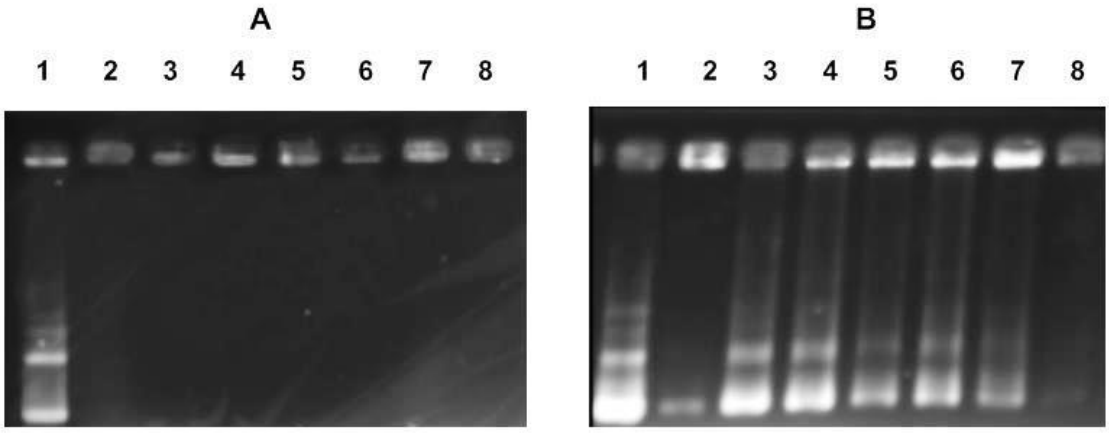

Figure 13. DNA release assay by glycosaminoglycans. pDNA/PEI/*Iota*-carrageenan complex at various +/- ratios were mixed with 2.5 mg/mL chondroitin sulfate and dextran sodium sulfate then incubated at 37 ^{o}C for 20 min. A: Chondroitin sulfate; B. Dextran sodium sulfate. Lane: Free DNA (0.1 ug); Lane 2: pDNA/PEI (N/P = 3.5); Lane 3:(1:3.5:6.0); Lane 4:(1:3.5:5.0); Lane 5: (1:3.5:4.0); Lane 6: (1:3.5:2.5); Lane 7:(1:3.5:1.0); Lane 8: (1:3.5:0.5).

MATERIALS AND METHODS

PEI (average molecular weight, 25 kDa; branched with degree of polymerization, 580) was obtained from Aldrich Company. Iota carrageenan (Benvisco SI-100, average molecular weight, 560 kDa) was obtained from Shemberg Biotech Corporation. Plasmid pGL3(SV40) was amplified in E. coli and isolated with a Qiagen Endotoxin-free Plasmid Giga Kit (Qiagen Inc.) according to the instruction manual. All other chemicals used were of analytical grade.

Preparation of complexes

The PEI-DNA complex was prepared at various molar ratios of PEI nitrogen (N) to DNA phosphate (P) from N/P = 3.3 and N/P = 3.5, by mixing the solution of PEI (which was previously stabilized for 20 minutes) in the DNA solution. The pDNA-PEI complex was stabilized at room temperature for 20 minutes prior to the addition of carrageenan solution. Carrageenan solution was prepared at a concentration of 5.63 mg/mL. All solutions of pDNA/PEI/carrageenan were prepared in phosphate buffer saline (PBS) solution at pH 7.3. Complex formation was verified by electrophoresis on a 1% agarose gel with Tris-acetate (TAE) running buffer, 100V for 30 minutes. DNA was visualized with ethidium bromide (0.2 μg/mL).

Resistance of pDNA-PEI-Carrageenan complex against DNase I degradation

Ternary complexes of pDNA/PEI/Carrageenan, pDNA/PEI complex and pDNA were separately incubated with DNase I solution (130 Units/mL) as previously described [16]. EDTA (0.5M) was used to stop DNase degradation. All samples were then immediately placed in an ice bath. After the addition of 5% heparin, aliquots of the sample were subjected to electrophoresis.

Study of the effect of heparin and other glycosaminoglycan (chondroitin sulfate and dextran sulfate) on the stability of ternary complexes

The effect of heparin at 0.1% concentration on the stability of ternary complexes was assayed by means of the changes in electrophoretic mobility as previously described [16]. The complexes were incubated at 37 ^{o}C for 10 minutes.

The effect of chondroitin sulfate and dextran sulfate on the stability of ternary complexes was conducted by adding 1.0-2.0 mg of chondroitin sulfate and dextran sodium sulfate to the ternary complexes followed by incubation at 37 ^{o}C for 30 minutes as previously described [17]. Release of DNA from the complex was investigated by electrophoresis on a 1% agarose gel.

Atomic force microscopy (AFM) measurement

Solution of ternary complexes (10 μL) with a final DNA concentration of 10 μg/mL at different charge ratios were applied in drop deposition onto the surface of a mica disk as previously described (17,18). Following adsorption for 2 minutes at room temperature, excess fluid was removed by absorption with filter paper. Then 50 μL of water was added to the surface to wash excess salt from the PBS solution. The mica surface was subsequently dried at room temperature prior to imaging. A commercial atomic force microscope (SPA-300 system of Seiko Instruments, Inc. Japan) was used for imaging using a Si3N4 tip on the cantilever (100 nm x 400 nm, SN-AF01-A, Olympus Optical Co.) with tapping mode and non-contact recording under ambient condition.

Transfection of cells

For gene transfer studies, the human hepatoma cell line HuH-7 (JTC-39 established in Japan), Hela, COS-7 and CHO-K1 cells were used in this experiment. Cells were seeded with 1 x 104 cells per cm^2 per well in 24 well plate. These were grown as adherent culture in 1 mL DMEM supplemented with 10%(w/v) fetal bovine serum (FBS) for HuH-7 cells and COS-7 cells and 1 mL F12 + 10 % (w/v) FBS for CHO-K1 cells incubated at 37 ^oC with 5% CO_2 in a humidified chamber. To transfect the cells, 5 μg pGL3(SV40), 3.2825 μg PEI were separately diluted with 50 μL each with phosphate buffer saline (PBS) solution and stabilized for 20 minutes prior to addition of carrageenan solution. 100 μL of the ternary complex solution was added per well. Cells were incubated for 4 hours with the same condition as mentioned before and further incubated to 24 hours after changing the medium to 1 mL. pDNA and pDNA/PEI complexes were used as control.

Measurement of luciferase

After the transfection experiment, the cells were washed with PBS, pH 7.3 and scraped off the dishes in 100 μL of lysis buffer (Promega,USA). The resulting lysate was centrifuged at 12000 X g for 1 minute and 20 μL supernatant was mixed together with 100 μL substrate solution (Promega, USA). A luminometer (Turner Designs) was then used to measure the light units (RLUs) due to luciferase activity. Protein assay was undertaken according to the manufacture's instruction (Bio-Rad Laboratories, Japan).

Measurement of zeta potential and particle size

Surface charges of the ternary complexes of carrageenan was evaluated using a Coulter® DELSA 440SX Zeta Potential Analyzer. Samples of 1.5 mL containing 20 μg/mL of pDNA were used as test solutions. Size measurement was determined by using a High Performance Particle Sizer (Malvern Instrument), UK.

Stability assay

The ternary complexes of carrageenan were mixed with 5.0 mg/mL bovine serum albumin then incubated at 37 ^{o}C with mild agitation. Turbidity measurements at a wavelength 350 nm were undertaken for everyday for one week using a UV/Visible spectrophotometer (Ultrospec 3000) by Pharmacia Biotech.

Evaluation of cytotoxicity

Cellular toxicity of the ternary complexes of carrageenan was carried out using COS-7 and CHO-K1 cells in quadruplicate. Viability of the cells following exposure to the complexes was evaluated using the MTT assay (WST-1/1-methoxy-PMS), by the protocol of Dojindo Laboratories, Japan. Confluent COS-7 and CHO-K1 cells were seeded with 1.5 x 103 cells per cm2 per well in 96 well plate with their corresponding growth medium as mentioned before. These were then exposed to the ternary complexes of carrageenan for 1, 4. 8, 16, 24, 48 hours (incubation time at 37 ^{o}C). After incubation, 10 μL of MTT solution was added followed by incubating again at 37 ^{o}C for 2 hours. Absorbance readings were taken at 450-690 nm using a Lab systems Multiskan MS microplate reader. Cell viability was expressed as percentage of control (untreated cells). MTT solution was prepared by mixing WST-1

in PBS, 16.5 mg/4.5 mL, with 1-methoxy PMS, 0.7 mg/mL at a volume ratio of 1:9, (PMS:WST-1).

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CHAPTER 4

Encapsulation of glucose oxidase (GOD) in polyelectrolyte complexes of chitosan–carrageenan

Chapter 4

ABSTRACT

The purpose of this study was to investigate the potential ability of carrageenan (κ-, ι-, λ-) and chitosan to form a controlled-release system for glucose oxidase (GOD). GOD was encapsulated in chitosan/carrageenan complexes at charge ratios (+/-) of 3 and 5 in mildly acidic solution. The encapsulation efficiency and activity of the loaded GOD were investigated. Among the different complexes prepared, chitosan/κ-carrageenan complex showed high encapsulation efficiencies of 79% and 62.5% at charge ratios of 3 and 5, respectively. The order of encapsulation efficiency decreases toward chitosan/λ-carrageenan complex (κ > ι > λ). After treatment with chitosanase and pepsin solutions, the activity of encapsulated glucose oxidase (GOD) was preserved for all complexes. The chitosan/κ-carrageenan complex was able to preserve 80.2% of GOD activity in pH 1.2 solution, 73.3% in chitosanase solution and 66.4% in pepsin solution. Controlled release of GOD was observed when the complexes were treated with different physiological and enzyme solutions; the complex of chitosan/κ-carrageenan had the lowest release rate of GOD. The simple preparation of chitosan/carrageenan complexes and their ability to protect protein integrity under acidic conditions make them a promising drug delivery system for the oral administration of peptides and proteins.

Chapter 4

INTRODUCTION

Oral delivery of peptide and protein drugs has been a highly active research area [1]. Although many highly sophisticated drug delivery technologies are available, these often fail to produce marketable controlled-release dosage forms because of the physiological limitations of gastrointestinal (GI) tract and the utilization of non-feasible pharmaceutical components [1].

For effective peptide and protein delivery via the gastrointestinal tract, a carrier system should overcome significant enzymatic and diffusion barriers. Various protein formulations have been reported [2] to deal with these limitations. These include hydrogels [2], nano/microspheres [3–7] and lipid-based systems such as liposomes [8], solid lipid nanoparticles and water-in-oil (W/O) emulsions [9–11]. Of these methods, the one that is the most widely used for protein formulation technology is encapsulation using polymers which aims to maintain the activity of protein-based drugs and prolong the their circulation time [2]. Proteins immobilized in polymeric microparticles have the advantages of improved biological and thermal stabilities, pH suitability, extended therapeutic effect and the possibility for targeted delivery [2]. The entrapment efficiency and retention of protein activity in polymeric protein delivery systems are largely dependent on the polymer and reagents used, the type of immobilization technique, and other process variables [9,12]. As reported in previous studies [13,14], the presence of a water/organic solvent interface in the case of poly(D/L-lactic-coglycolic acid) (PLGA) microspheres was believed to be responsible for the low encapsulation efficiency, inactivation and aggregation of proteins hence, hydrogel systems are more desirable for protein delivery [15].

Hydrogel systems have relatively high water content with a soft consistency similar to natural tissue and are more compatible with proteins [15].

Among the hydrogels used in protein delivery systems, alginate and chitosan are the most widely-used materials. Alginate has been studied for the sustained release or immobilization of bioactive agents such as enzymes [16], vaccines [17], insulin [18] and cytokines [19-20]. Chitosan has also been extensively studied as a promising protein carrier [21–25].

There have been no studies reported yet on the use of a chitosan–carrageenan polyelectrolyte complex for controlled release of glucose oxidase. Hence, the objective of this study is to evaluate the possibility of using polyelectrolyte complexes from chitosan and different types of carrageenan (κ, ι, λ) to encapsulate glucose oxidase and control or prolong its release. In this study, a simple coacervation process was used to prepare a chitosan–carrageenan polyelectrolyte complex. This method is a mild process occurring in a pure aqueous environment and is ideal for the in process stability of proteins and peptides [26]. Because chitosan is water soluble, the encapsulation of glucose oxidase was carried out at pH 6.0 as compared to the study of Liu et al. [2] which used Ph 4.0 to encapsulate GOD. The use of chitosan–carrageenan complex to encapsulate produced nanospheres in contrast with the alginate/chitosan microspheres. The most suitable type of carrageenan to form a polyelectrolyte complex with chitosan was also determined in this study.

Published studies on the use of the polyelectolyte complex of chitosan–carrageenan bhave focused on the prolonged release of diltiazem bchlorhydrate [27]

and the controlled release of sodium diclofenac [28] and theophylline [29] in tablet and capsule forms. It was also used for efficient b-galactosidase immobilization [30].

Carrageenan has also been used in many published studies for controlled release of drugs. These include: diltiazem HCl, bupropion HCl, metoprolol tartrate, and tramadol HCl [31]; acetaminophen [32]; verapimil HCl and ibuprofen [33]; mefenamic acid [34]; ketoprofen [35]; chlorpheniramine maleate [36]; and theophylline [37]. It has also been cross-linked with polyacrylic acid-co-poly- 2-acrylamido-2-methylpropanesulfonic acid (PAA-co-PAMPS) to form a biodegradable hydrogel as a candidate for a drug delivery system [38]. Carrageenan is classified as a food additive [39] and shows potential use for a new drug delivery system providing more control over the release rate of drugs.

Studies done on the encapsulation and immobilization of glucose oxidase were based on alginate/chitosan microspheres [2]; alginate microspheres [3]; sodium alginate–cellulose sulfate–poly(methylene-co-guanidine) capsules [4]; chitosan matrix cross-linked with glyoxal [5]; layer-by-layer films of chitosan [7]; alginate microspheres with photoreactive diazoresin nanofilm coatings [6]; and copolymers of N-pyrrolyl terminated polydimethylsiloxane/polypyrrole (PDMS/PPy) matrices via electrochemical polymerization [40].

RESULTS AND DISCUSSION

Encapsulation efficiency

Chitosan is known to form stable complexes with carrageenans [42]. The maximum yield of complex formation was observed for the molar mixing ratio of 1:1 which suggests a stoichiometric anion–cation interaction [42]. The complexation of chitosan and carrageenan was due to the opposite charges between the positively charged amine groups (NH_3) in chitosan and the negatively charged sulfate groups (SO_4) in carrageenan. In this study, chitosan/carrageenan complexes were prepared at charge ratios (+/-) of 3 and 5 at 1 mg/mL. GOD (isolectric point of 4.2 [43]) was negatively charged when the pH > 4.2 and could form a complex with chitosan at pH 6.0 through electrostatic interactions. The resulting cationic GOD-loaded chitosan was then combined with carrageenan to form a complex via electrostatic interaction. As shown in Fig. 1A, higher encapsulation efficiency of GOD in chitosan/carrageenan complexes was observed as compared to single polymer (Fig. 1B). The efficiency was doubled in polymer blends, which exhibited greater ability to encapsulate protein compared to a single polymer. At a charge ratio of ±3, chitosan/κ-carrageenan complexes showed the highest encapsulation efficiency of 79%, followed by chitosan/ι-carrageenan at 54% and chitosan/λ-carrageenan at 40.5%. The obtained results were compared to the encapsulation efficiency of chitosan/dextran sodium sulfate as the positive control, which showed an efficiency of 75%. In the study by Liu et al. [2], using a 1 mg/mL concentration of GOD, the chitosan/alginate microspheres had an encapsulation efficiency of 79.8%, which is slightly higher than that of the chitosan/carrageenan complex. At a charge ratio of

5 (Fig. 1A), lower efficiency was observed (compared with charge ratio of 3) but higher than that of the single blends. The observed efficiencies are as follows: 62.5% for chitosan/κ-carrageenan, 43.5% for chitosan/ι-carrageenan and 33.8% for chitosan/λ-carrageenan. An increase in the charge ratio indicates an increase in the amount of chitosan, which may affect the encapsulation efficiency. Chitosan concentration had a significant effect on GOD loading, encapsulation efficiency and the loaded GOD activity according to Liu et al. [2]. They determined that GOD loading and encapsulation decreased with increasing chitosan concentration due to desorption of GOD as a result of competition between the chitosan and GOD for ionic binding sites [2]. Although their study [2] involved chitosan/alginate microspheres for the encapsulation of GOD, this behavior also applies in chitosan/carrageenan complex because carrageenan and alginate are both polyanions. The chemical structure and molecular weight of carrageenan used in this experiment may also influence the encapsulation efficiency. λ-Carrageenan had the highest molecular weight of 750 kDa and was the most anionic due to its three sulfate groups of the alternating 1,3-linked β-D-galactopyranosyl and 1,4-linked β-D-galactopyranosyl sugar units. ι-Carrageenan, on the other hand, has two sulfate groups attached to its alternating sugar units [44]. The presence of these excess sulfate groups may cause competitive interaction between carrageenan and glucose oxidase, thus lowering the encapsulation efficiency of the complex. κ-Carrageenan, however, has only one sulfate group attached to its alternating sugar units thus, competitive interaction was weaker [44].

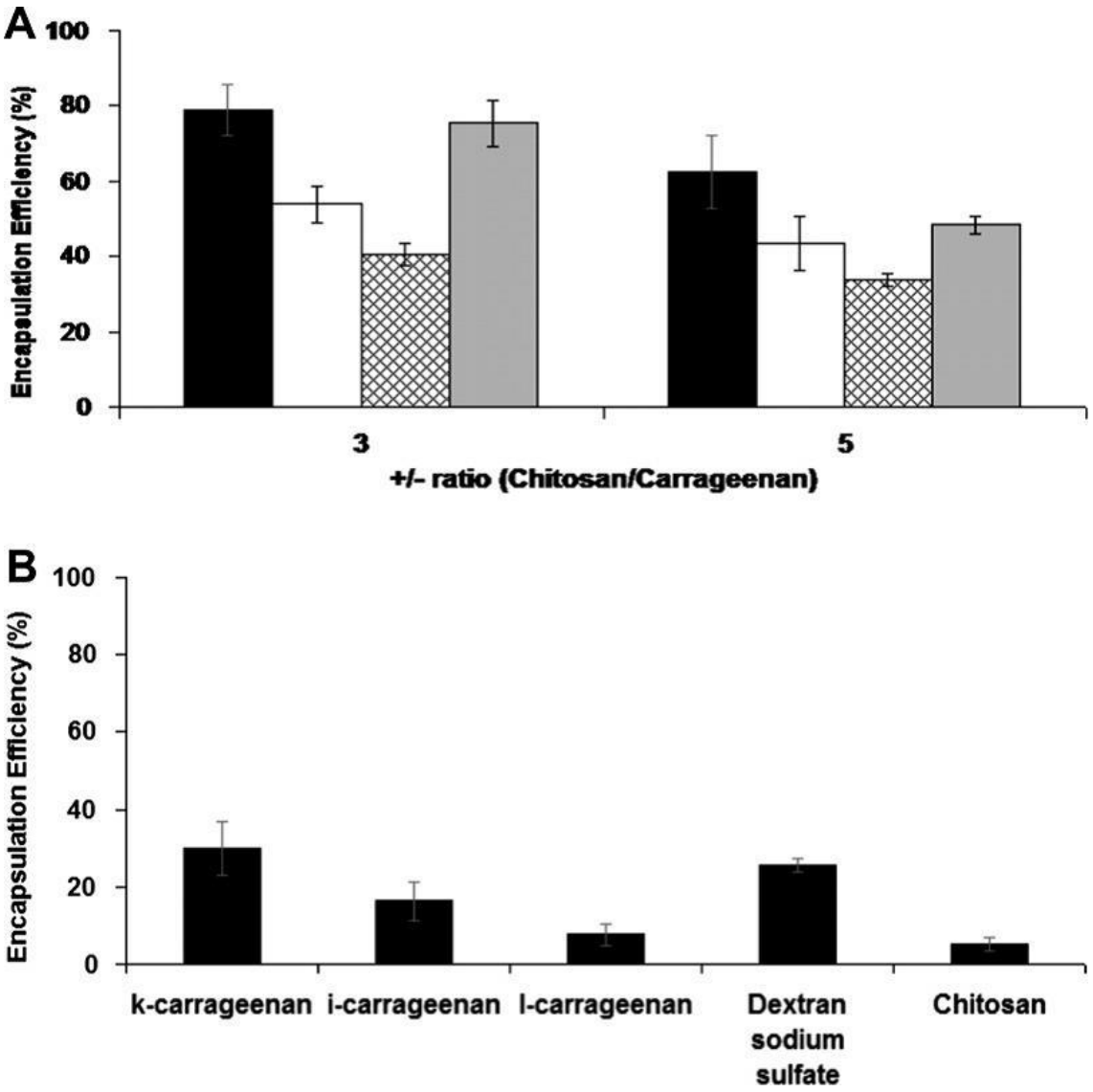


Fig. 1. Efficiency of chitosan/carrageenan complex (A) and single polymers (B) to encapsulate GOD(1 mg/mL). A. ■ : chitosan/κ-carrageenan; □ : chitosan/ι-carrageenan; ■ :chitosan/λ-carrageenan; ▩: chitosan/dextran sodium sulphate. The values represent the mean ± SD, *n* = 4. GOD indicates glucose oxidase.

Morphology of the chitosan/carrageenan complex loaded with GOD

AFM images of the polymers (carrageenan, chitosan) and GOD are shown in Fig. 2J–N. The complexation of chitosan with carrageenan through electrostatic interaction forms a good complex with GOD (Fig. 2A–H). It shows compact, almost uniform circular structure confirming complex formation. The complexes of chitosan/ κ-carrageenan (Fig. 2A and B) and chitosan/dextran sodium sulfate (Fig. 2D and H) show good complex formation with GOD, compared to ι-, λ-carrageenan and chitosan (Fig. 2B and F, C and G, I), where some GOD could be seen looping out of the complex. These findings conform to the results of the encapsulation efficiency (Fig. 1) of the polyion complex. κ-Carrageenan (Fig. 2J) forms an aggregated network, in conformity with the results of McIntire and Brant [45], who used water to dilute the carrageenan samples and prepared by aerosol spray deposition. There was no difference noted in this study, which used PBS solution adjusted to pH 6.5 to dilute the carrageenan. The presence of single stranded chains was also confirmed in the micrograph (Fig. 2J). ι-Carrageenan (Fig. 2K) displays a peculiar structure that is quite different from that previously reported by McIntire and Brant in 1999 [45]. The molecular structure of ι-carrageenan has ‘‘kinks” that increase its flexibility and reduce the space occupied by the molecule as described by Kirby et al. [46]. This might explain the irregularities of the structure. In λ-carrageenan (Fig. 2L), the structure exhibits a network of typical chains for the polymeric molecule. It has the same morphology as described by Oliva *et al.* [47] in their studies. A dendritic

topography was observed in the AFM image of chitosan (Fig. 2M). According to Xiaoqing et al. [48], based on AFM images, chitosan forms a prominent and delicate dendritic structures, which are fully-simulated and grow in clusters composed of nanoparticles with varying sizes. The findings of Xiaoqu ing et al. [48] were also observed in this study. Fig. 2N shows an image of GOD. Most of the nanospheres were of similar size, while some show a dimeric structure. This observation corresponds to the folded form of two identical polypeptide chains of the GOD molecule [49].

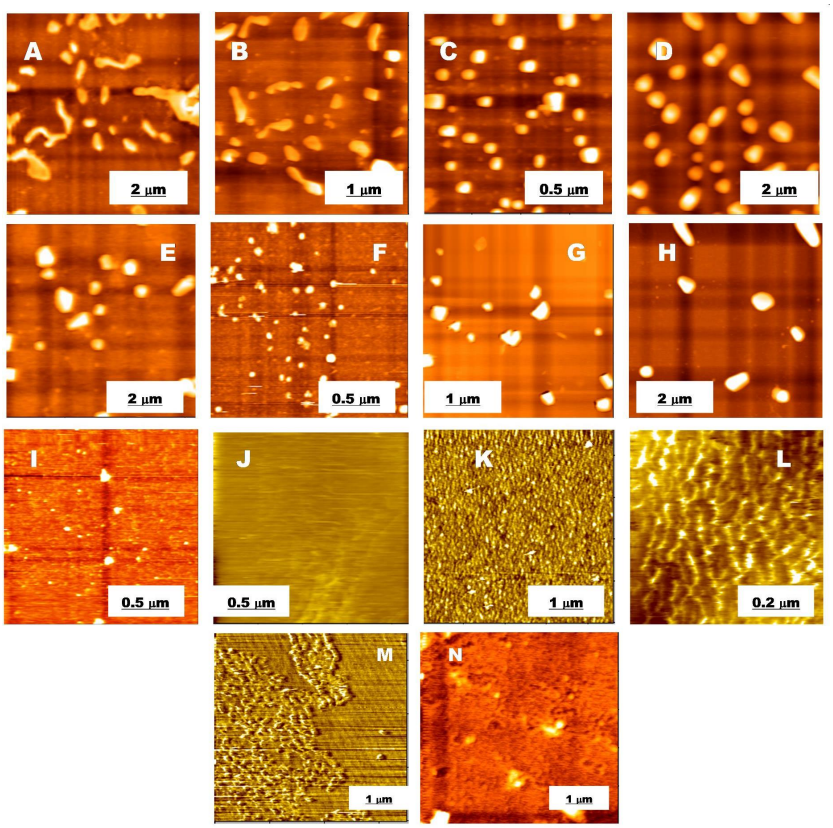


Fig. 2. AFM images of GOD loaded chitosan/carrageenan complex. A-D: +/- ratio = 3; E-H: +/- ratio = 5; A & E: chitosan/κ-carrageenan; B & F: chitosan/ι– carrageenan; C & G: chitosan/λcarrageenan; D & H: chitosan/dextran sodium sulphate; I: chitosan/GOD; J: κ-carrageenan; K: ι– carrageenan; L: λ–carrageenan M: chitosan; N: glucose oxidase (GOD).

Zeta potential and particle size of the chitosan/carrageenan complex loaded with GOD

Negative zeta potentials were observed for all of the prepared complexes. As shown in Tables 1 and 2, there is no significant difference in the zeta potential between the two charge ratios. Complexes with charge ratio (+/-) = 3 displayed positive zeta potentials > 30 while at charge ratio (+/-) = 5, the complexes exhibited positive zeta potentials > 35. A high zeta potential value, suggests high stability of the complex. The particle size of chitosan/ carrageenan complexes loaded with GOD was higher than that of chitosan/GOD alone. An increase in the particle size was expected at charge ratio (+/-) = 3 as compared to charge ratio (+/-) = 5 because the former contains more carrageenan than the latter. The high values of particle size show that the entrapment of GOD and the complexation of chitosan with carrageenan were achieved.

Table 1. Zeta potential and particle size of GOD loaded chitosan-carrageenan complexes at charge (+/-) ratio of 3.

Charge (+/-) ratio = 3	Zeta Potential (mV)	Particle Size (nm)	Polydispersity Index (SD)
Chitosan/κ-carrageenan	- 47.0 (±1.3)	1288.5 (± 140.71)	0.80 (± 0.02)
Chitosan/ι-carrageenan	- 44.7 (±6.8)	816.5 (± 113.35)	0.55 (± 0.26)
Chitosan/λ-carrageenan	- 47.2 (±5.0)	618.4 (± 76.08)	0.22 (± 0.10)
Chitosan/Dextran sodium sulfate	- 48.6 (±6.6)	1787.3 (± 492.88)	0.50 (± 0.36)
Chitosan	- 47.0 (±1.2)	360.0 (± 5.12)	0.40 (± 0.03)

Table 2. Zeta potential and particle size of GOD loaded chitosan-carrageenan complexes at charge (+/-) ratio of 5

Charge (+/-) ratio = 5	Zeta Potential (mV)	Particle Size (nm)	Polydispersity Index (SD)
Chitosan/κ-carrageenan	- 49.4 (±2.3)	1386.7 (±190.00)	0.40 (± 0.37)
Chitosan/ι-carrageenan	- 48.0 (±8.3)	577.5 (± 74.95)	0.50 (± 0.12)
Chitosan/λ-carrageenan	- 50.0 (±7.6)	600.7 (± 165.82)	0.36 (± 0.18)
Chitosan/Dextran sodium sulfate	- 52.0 (±7.2)	1990.0 (± 489.32)	0.30 (± 0.26)
Chitosan	- 46.0 (±4.7)	360.0 (±5.12)	0.40 (± 0.03)

In vitro release of GOD from the chitosan/carrageenan complex

There are three primary mechanisms by which active agents can be released from a delivery system: diffusion, degradation, and swelling followed by diffusion [50]. In this study, release of GOD was due to the swelling of chitosan/carrageenan complex. The complexes made up of hydrogels swell without dissolving when placed in water or other biological fluids [50]. GOD diffused through the swollen complex into the external environment. In this study, the release profile of GOD from the chitosan/carrageenan complex was evaluated in 0.1 mg/mL solution of chitosanase, pepsin and different physiological solutions. In Figs. 3–5, controlled release of GOD was observed with the lowest rate was exhibited by the chitosan/j-carrageenan complex. The complex of chitosan and dextran sodium sulfate also showed slow release rate of GOD as compared to that of the chitosan/j-carrageenan complex. Chitosan showed the highest release rate of GOD, approximately > 60% after an one-hour incubation in chitosanase and pepsin (data not shown). This suggests that a single polymer (chitosan) was easily degraded by the enzymes as compared to

polymer blends; thus, high release rate was observed. Using different physiological solutions (Fig. 5), controlled release of GOD was also observed. At low pH (1.2–7.0), the cumulative release rate of GOD was <20% while at pH of 7.3, an increase in release rate of GOD was observed. The studies made by Sakiyama et al. [51] for the swelling mechanism of chitosan/carrageenan complex showed that in an acidic solution, no swelling was observed. This is due to the presence of electrostatic bonds between chitosan and carrageenan. The sulfate groups of carrageenan will remain negatively charged and interact with the positively charged amino groups of chitosan hence, the cumulative release rate of GOD was not too excessive. In alkaline solution, the amino groups of chitosan will be neutralized, and the sulfate groups of carrageenan will remain negatively charged therefore, the electrostatic linkage between the two polymers disappears [51]. The electrostatic repulsion between sulfonate groups will contribute to the swelling mechanism [51]; thus, higher release rate of GOD was observed. In alkaline solution, GOD is negatively charged; thus, there is no electrostatic interaction with chitosan. GOD, which has a pI of 4.2 [43], is anionic at physiological pH of 7.4 [2]. Among the prepared complexes, chitosan/λ-carrageenan complex showed the lowest release rate of GOD. This behavior might be due to its chemical structure. Since κ-carrageenan contains only one sulfate group attached to its alternating sugar units, less electrostatic repulsion is expected with fewer ionic binding sites. It also undergoes a helix-coil transition, creating additional cross-links and forming a more rigid system [52]. λ-Carrageenan had the highest release rate of GOD among the three types. This type contains three sulfate groups of the alternating sugar units, creating more electrostatic repulsion

and ionic binding sites. This type does not gel and does not undergo a helix coil transition, which precludes additional cross-links [52].

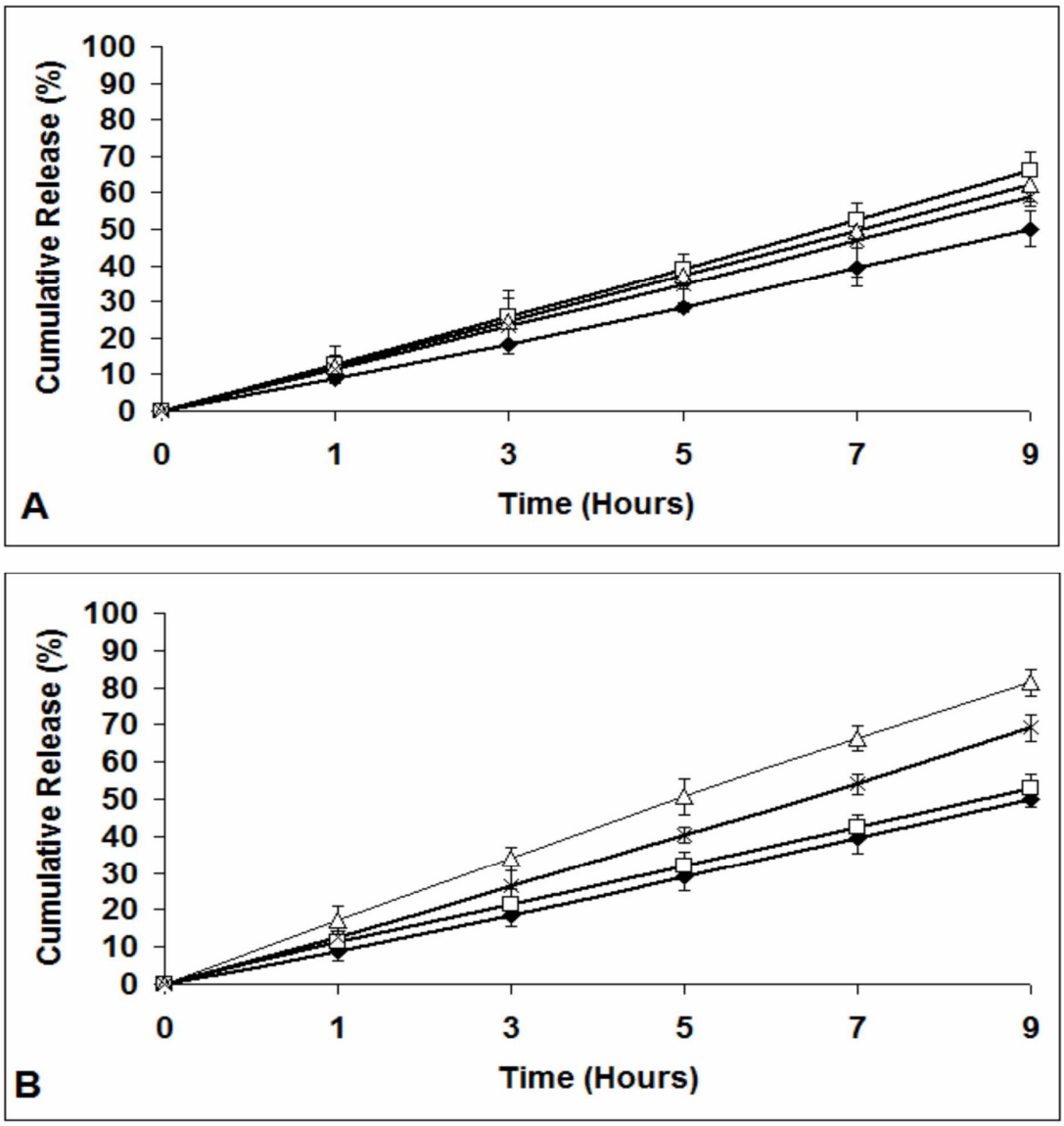


Fig. 3. Cumulative release of GOD from chitosan/carrageenan complex prepared at various charge ratios in chitosanase solution (0.1 mg/mL). A: (+/-) = 3; B: (+/-) = 5; ♦ = chitosan/κ-carrageenan; □ = chitosan/ι-carrageenan; Δ= chitosan/λ-carrageenan; × = chitosan/dextran sodium sulphate. Incubation temperature: 37 ^{0}C. pH: 6.0. The values represent the mean ± SD, *n* = 3. GOD indicates glucose oxidase.

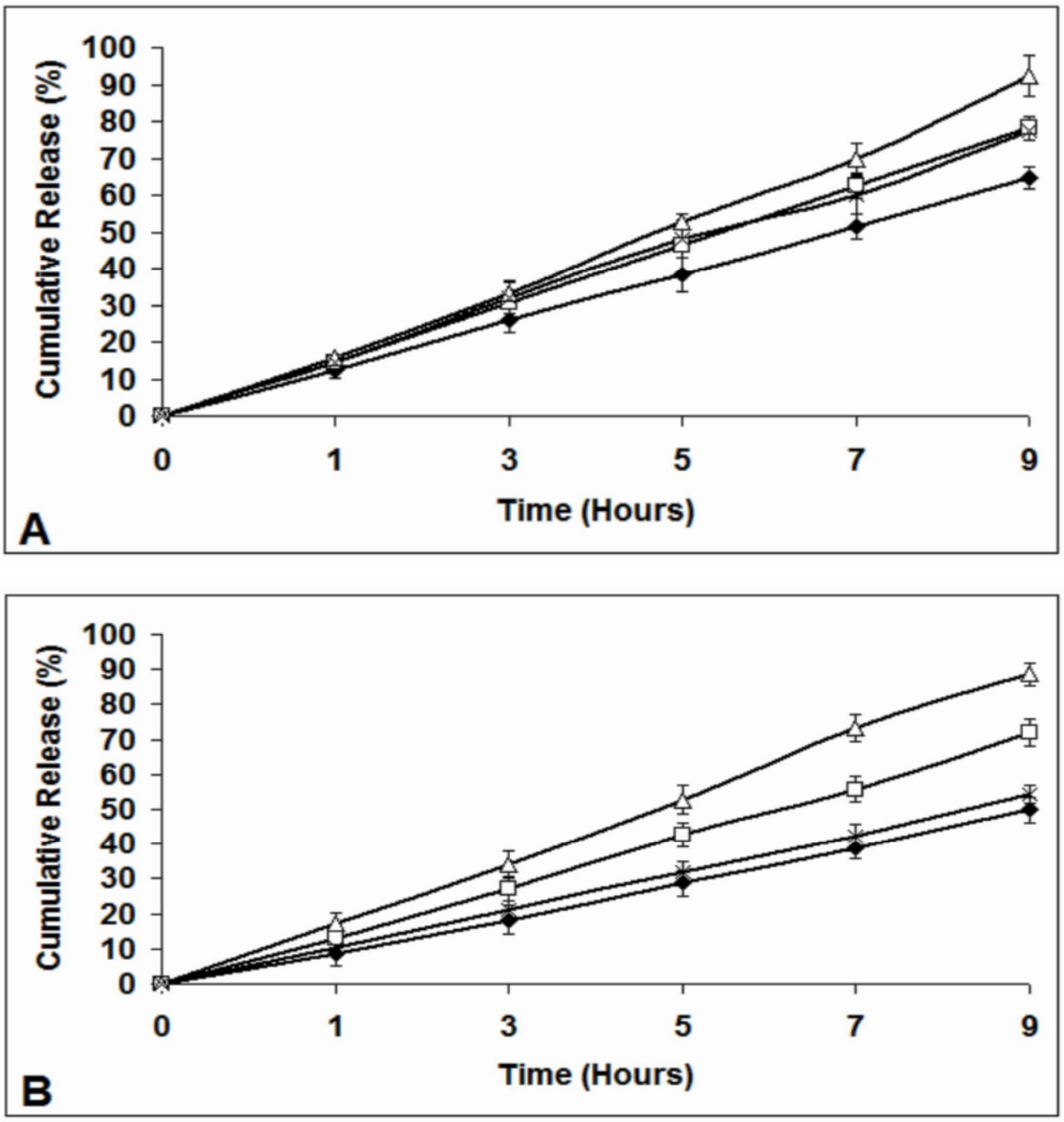


Fig. 4. Cumulative release of GOD from chitosan/carrageenan complex prepared at various charge ratios in pepsin solution (0.1 mg/mL). A: (+/-) = 3; B: (+/-) = 5; ♦ = chitosan/κ -carrageenan; □ = chitosan/ι-carrageenan; Δ= chitosan/λ-carrageenan; × = chitosan/dextran sodium sulphate. Incubation temperature: 37 ^{0}C. pH: 6.0. The values represent the mean ± SD, *n* = 3. GOD indicates glucose oxidase.

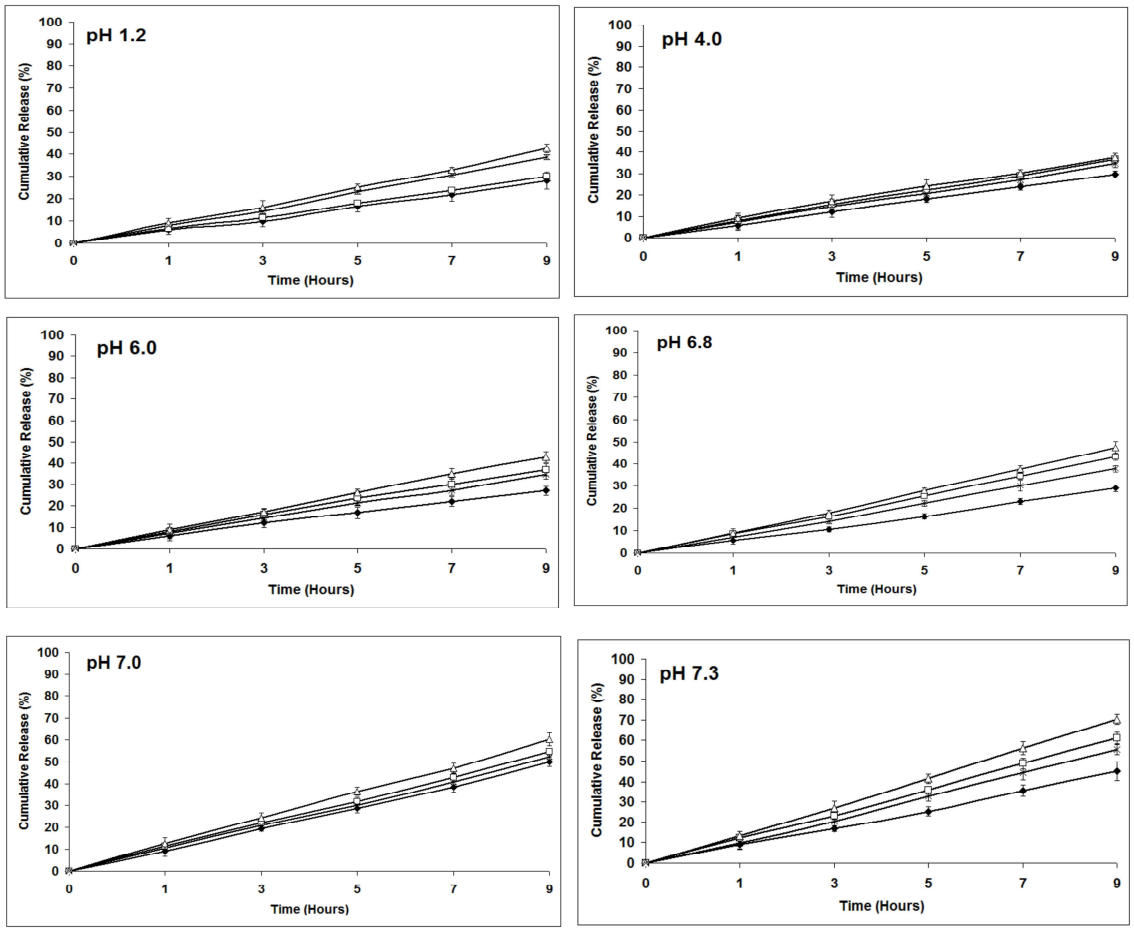


Fig. 5. Cumulative release of GOD from chitosan/carrageenan complex prepared at charge ratio (+/-) of 3 in various physiological solutions. ♦ = chitosan/κ-carrageenan; □= chitosan/ι-carrageenan; Δ = chitosan/λ-carrageenan; × = chitosan/dextran sodium sulphate. Incubation temperature: 37 ^{0}C. The values represent the mean ± SD, *n* = 3. GOD indicates glucose oxidase.

Stability of GOD loaded in the complex

In protein delivery, the protein must be protected by the complex formulation during its application. The stability and integrity of GOD in the complex were evaluated by gel electrophoresis. The use of pepsin solution and pH 1.2 was employed to investigate the integrity of GOD. Fig. 6 shows the SDS–PAGE analysis

of release fractions of GOD in chitosan/carrageenan complexes; free GOD and treated free GOD were used as controls. Analysis showed that the treated free GOD (Fig. 6A, Lane 3) was completely degraded as compared to the GOD released from the polymers and complexes. Pepsin has very high chitosanolytic activity; thus, the degradation of chitosan takes place very quickly during an initial time period and then gradually slows down [53] with optimum pH and temperature of 4.6 and 55 ^{o}C, respectively. Increasing the enzyme or substrate concentration led to the degradation of chitosan. The GOD fractions released from chitosan/carrageenan complex showed partial degradation at a much slower rate, as observed in Fig. 6A. Most of the GOD was still in the well, as indicated by the intensity of the bands, showing controlled release and resistance to degradation. The lanes mostly showed more than one band, showing a slower rate of GOD degradation. The integrity of GOD at pH 1.2 was also investigated by gel electrophoresis. In Fig. 6B, GOD fractions released from the complexes were not degraded as compared to the treated free GOD (Fig. 6B, Lane 3). The GOD was protected from being degraded and hydrolyzed. The use of chitosan/carrageenan complex prevented degradation of GOD in acidic solution.

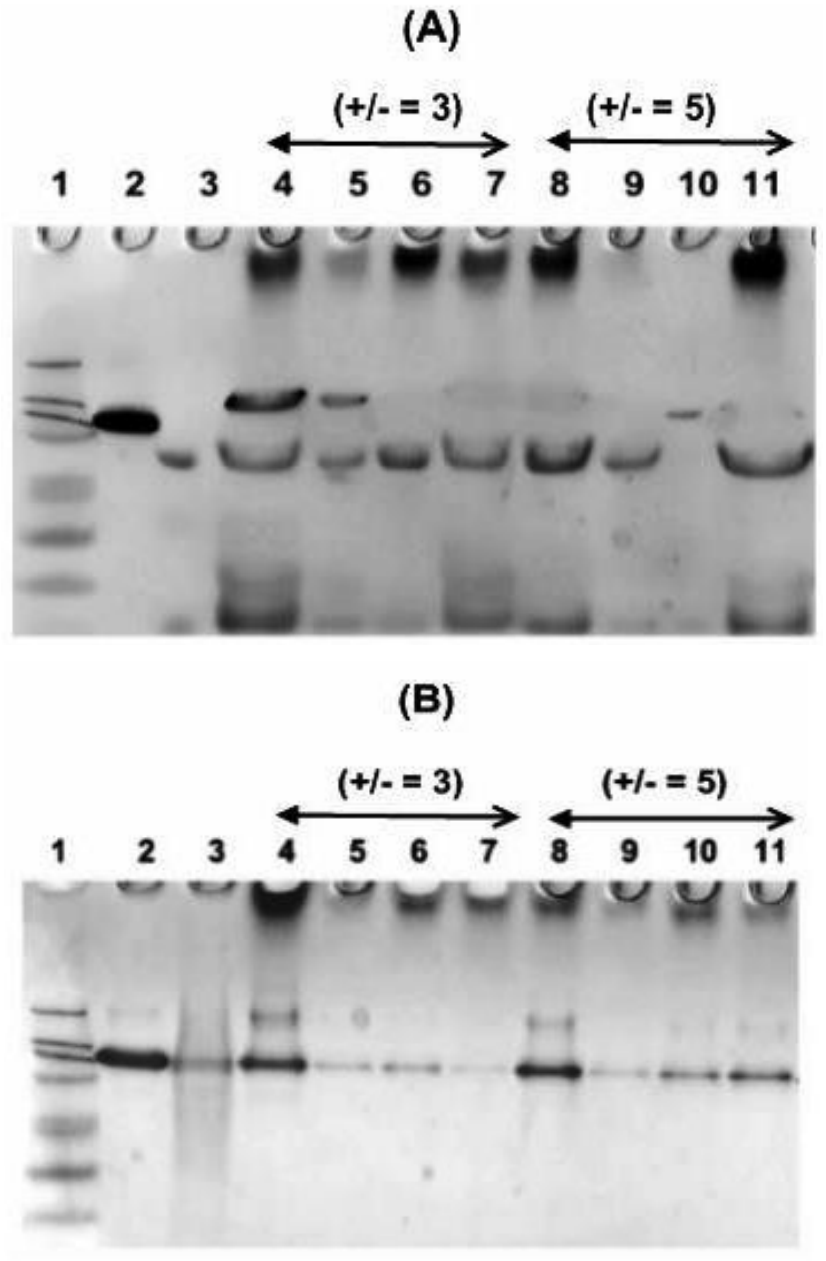


Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis for stability of GOD released from chitosan/carrageenan complex in: (A). pepsin solution (1 mg/mL) and (B). pH 1.2 solution incubated at 37 ^{0}C for one hour. Lane 1: Protein marker; Lane 2: Free GOD; Lanes 3: Treated GOD; Lanes 4 & 8: chitosan/κ-carrageenan; Lanes 5 & 9; chitosan/ι-carrageenan; Lanes 6 & 10; chitosan/λ-carrageenan; Lanes 7 & 11; chitosan/dextran sodium sulphate; GOD indicates glucose oxidase.

GOD activity in the complex

The activity of GOD in the complex is shown in Fig. 7. Evaluation of the activity was done after treating the GOD-loaded complex with chitosanase, pepsin and acidic solutions. Activity was well preserved and showed better H_2O_2 generating behavior than did the GOD-loaded chitosan and treated free GOD. Higher activity reading was observed in an acidic solution than in chitosanase and pepsin solutions. The chitosan/κ-carrageenan complex is the most efficient among the prepared complexes prepared in preserving GOD activity. In acidic solution (Fig. 7B), about 80.3% activity of GOD was preserved by chitosan/κ-carrageenan complex, followed by chitosan/ι-carrageenan complex at 73.3% and chitosan/λ-carrageenan complex at 66.7%. These results are in agreement with the SDS–PAGE results (Fig. 6B). In Fig. 7A, chitosan/κ-carrageenan was able to preserve 73.3% of GOD activity in chitosanase solution and 66.4% in pepsin solution (Fig. 7C), much higher than using chitosan/ι-carrageenan, chitosan/λ-carrageenan and chitosan/dextran sodium sulfate complexes. The activity of free GOD was preserved at <10% and <23% for chitosan in all solutions.

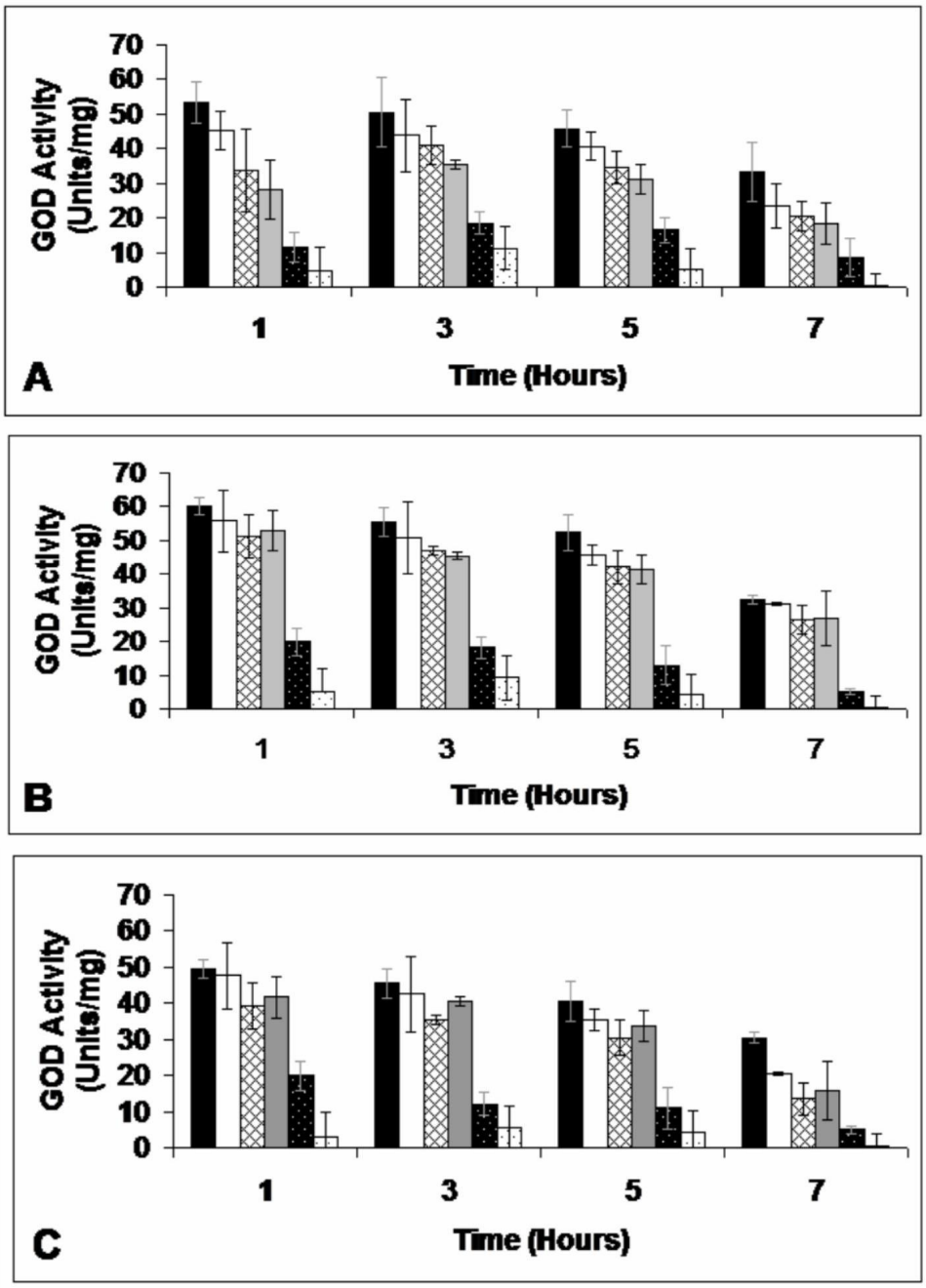
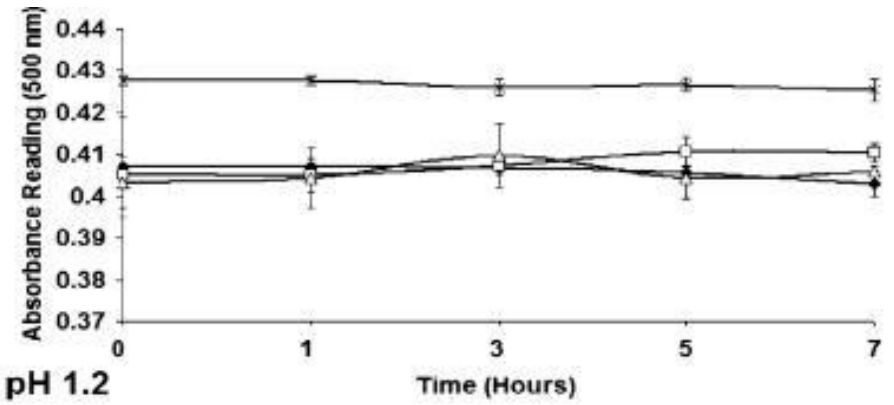
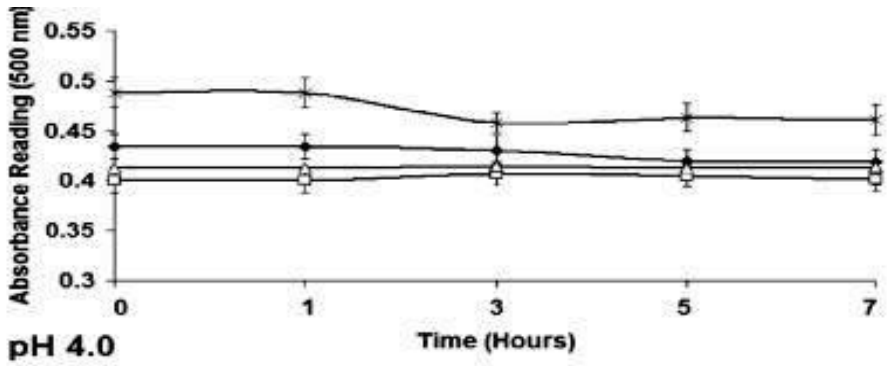


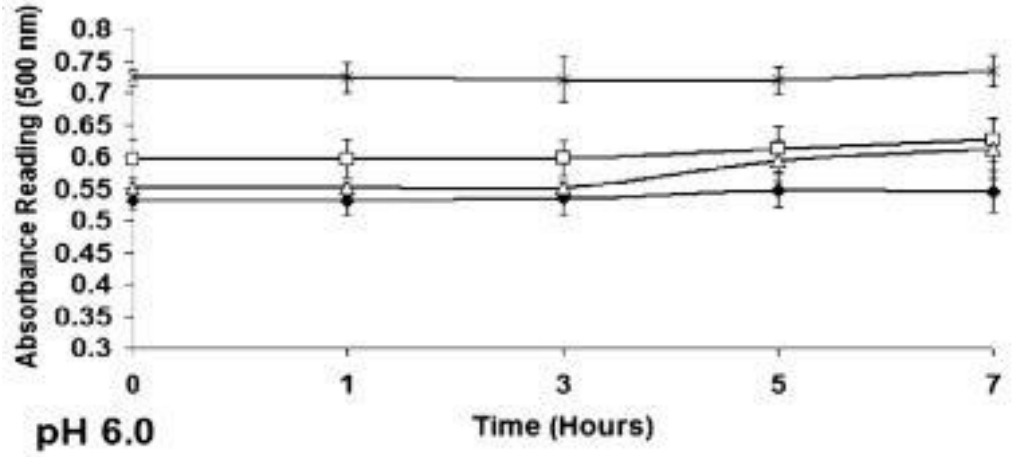
Fig. 7. Activity of glucose oxidase (GOD) encapsulated in chitosan/carrageenan complex at charge ratio (+/-) of 3 treated with A: chitosanase solution (1mg/mL); B: pH 1.2 solution; C: pepsin solution (1 mg/mL); ■: chitosan/κ-carrageenan; □: chitosan/ι-carrageenan; ▩ : chitosan/λ-carrageenan; ▨ : chitosan/dextran sodium sulphate; ■ : treated free GOD; ■: chitosan/GOD. The values represent the mean ± SD, *n* = 3.

Turbidity of the complex loaded with GOD in different physiological solutions

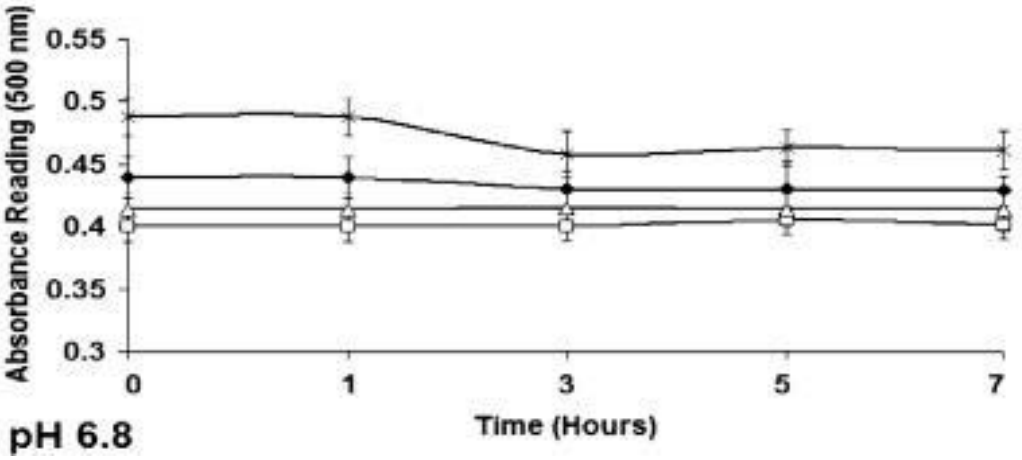
The stabilities of GOD-loaded chitosan/carrageenan complexes were evaluated according to their turbidity readings in various physiological solutions. Fig. 8 shows the turbidity readings at absorbance of 500 nm. The GOD-loaded chitosan/κ-carrageenan complex is the most stable, compared to ι- and λ-type carrageenan and to dextran sodium sulfate in all physiological solutions. The GOD-loaded chitosan/λ-carrageenan is not as stable in low pH solutions of 1.2, 4.0 and 6.0 while GOD-loaded chitosan/ι-carrageenan is not as stable in pH 1.2 and 7.0. This behavior agrees with the observed higher cumulative release of GOD in the said complexes as compared to chitosan/κ-carrageenan complex.







pH 6.0



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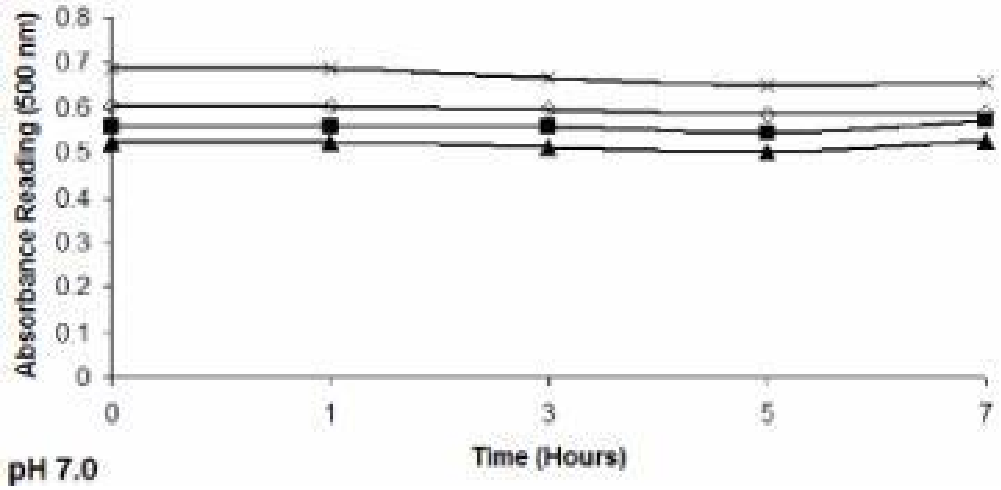

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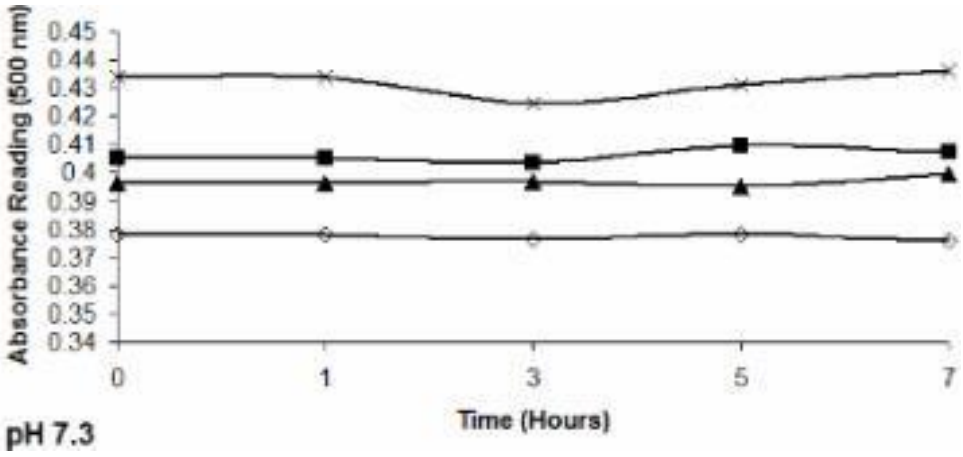

Fig. 8. Turbidity reading of the chitosan/carrageenan complex loaded with GOD at charge (+/-) ratio = 3 in different physiological solutions: pH 1.2: 35 mM sodium chloride (NaCl); pH 4.0: 50 mM acetic acid; pH 6.0: 200 mM sodium chloride (NaCl) in 2-(*N*-morpholino)ethanesulfonic acid. (MES) buffer; pH 6.8: 50 mM potassium phosphate buffer; pH 7.0: H_20; pH 7.3: phosphate buffered saline (PBS, 0.01 g/mL). ♦ = chitosan/κ-carrageenan; □= chitosan/ι-carrageenan; Δ= chitosan/λ-carrageenan; × = chitosan/dextran sodium sulphate. Turbidity was measured at 500 nm.The values represent the mean ± SD, *n* = 3. GOD indicates glucose oxidase.

Chapter 4

MATERIALS AND METHODS

Materials

Chitosan (average MW= 45 kDa, with degree of acetylation of 75.4%) was obtained from YSK Yaizu Suisankagaku Industry Co., Ltd., Japan. κ-Carrageenan (Bengel KK-100, Lot No. XO300-2, average MW= 510 kDa), ι-carrageenan (Benvisco SI-100, Lot No. M1400-1, average MW= 560 kDa) and λ-carrageenan (Benvisco SL-100, Lot No. S2703-2, average MW= 750 kDa) were obtained from Shemberg Biotech Corporation (Carmen, Cebu, Philippines, 6005). Glucose oxidase (GOD) and dextran sodium sulfate (average MW= 5 kDa) were purchased from Wako Pure Chemical Industries, Ltd., Japan. All other chemicals and reagents used were of analytical grade.

Preparation of solution

Chitosan solution was prepared at 1 mg/mL in 2-(N-morpholino) ethanesulfonic acid (MES) buffer solution at pH 6.0. Carrageenan(1 mg/mL), dextran sodium sulfate (1 mg/mL) and GOD (4 mg/mL) solutions were prepared in Milli-Q water.

Preparation of complex

GOD solution was added to chitosan solution, mixed thoroughly and allowed to stabilize at room temperature for 15 min. Carrageenan solution was then slowly added to the mixture, mixed again followed by another 15 min of stabilization at room temperature. The final concentration of GOD in the complex was 1 mg/mL. Thereafter the final mixture was incubated for 24 h at 4 ^{o}C with agitation at 200 rpm.

Encapsulation efficiency (EE)

After incubation, the solution was centrifuged at 10,000 rpm for 20 min at 4 ^{o}C using an Allegra 21R centrifuge (Beckman Coulter). The resulting supernatant was then assayed for its protein content following the manufacturer instruction (Bio-Rad Laboratories, Japan). All experiments were performed in quadruplicate. Encapsulation efficiency was determined according to the following equation:

$$E.E = \frac{C_o - C_r}{C_o} x\,100$$

where C_o is the initial concentration of the protein, and C_r is the protein reading from the assay.

Stability at different physiological solutions

After centrifugation of the solution, the supernatant was removed, and the resulting precipitate was subjected to various simulated physiological solutions: (a) 35 mM NaCl solution adjusted the pH to1.2 by adding 1 N HCl solution; (b) 50 mM acetic acid adjusted the pH to 4.0; (c) 200 mM NaCl in MES buffer adjusted the pH to 6.0 by adding 1 N HCl; (d) 50 mM potassium phosphate buffer adjusted to pH 6.8; (e) Milli-Q H2O; (f) phosphate buffered solution (PBS) solution at pH 7.3 and incubated at 37 ^{o}C with mild agitation. Absorbance was read at 500 nm.

Atomic force microscopy (AFM) measurement

After incubation for 24 h at 4 ^{o}C with agitation (200 rpm), 10 μL of the solution was diluted with MES buffer (pH 6.0) to make 100 μL, then a 20 μL drop was deposited onto the surface of a mica disk as previously described [41]. Following

adsorption for two minutes at room temperature, excess fluid was removed by absorption with filter paper. Then, 50 μL of water was added to the surface to wash excess salt from the PBS solution. The mica surface was subsequently dried at room temperature prior to imaging. A commercial atomic force microscope (SPA-300 system of Seiko Instruments, Inc., Japan) was used for imaging using a Si3N4 tip on the cantilever (100 μm x 400 nm, SN-AF01-A, Olympus Optical Co.) with tapping mode and non-contact recording under ambient condition.

Measurement of zeta potential and particle size

Surface charges of the complexes were evaluated using a Coulter®DELSA 440SX Zeta Potential Analyzer. The complex, after incubation for 24 h at 4 ^{o}C with shaking (200 rpm), was diluted with MES buffer (pH 6.0) to make a final volume of 1.5 mL. Size measurement was determined by using a High Performance Particle Sizer Malvern Instrument, UK), diluting 3 μL of the complex with MES buffer (pH 6.0) to make a final volume of 1.2 mL.

Cumulative release of GOD from the complex after treatment with different physiological solutions

The precipitate formed after centrifugation was treated with 1 mL each of the different physiological solutions at 37 ^{o}C with mild agitation (60 rpm) for 0, 1, 3, 5, and 7 h. After each time interval, the treated samples were assayed for their protein contents as previously described. Cumulative release of GOD from the complex was determined according to the following equation:

$$CR\,[\%] = \frac{C_c - C_r}{C_c} x100$$

where C_c is the concentration of the protein in the complex, and C_r is the protein reading from the assay. Cumulative release of GOD from the complex after treatment with enzymes (chitosanase and pepsin solution at 0.1 mg/mL)

The same procedure was followed as previously described. Release of GOD from the complex was determined by protein assay of the supernatant collected after each time interval of 1, 3, 5, 7, and 9 h.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) analysis

The release and degradation of GOD from the complex, after exposure to pepsin (1 mg/mL) and 35 mM NaCl solution adjusted to pH 1.2, were monitored by SDS–PAGE electrophoresis using Bio-Rad Ready Gels J (161J341 V) in running buffer (1 x tris–glycine, 0.1% SDS) with constant current mode, 20 mA. About 5 μL of sample was applied to each well. A pre-stained protein marker was also used. Coomassie Brilliant Blue was used as the staining solution.

Determination of glucose oxidase (GOD) activity

The activities of free GOD and encapsulated GOD in chitosan/ carrageenan complexes were determined according to manufacturer instruction (Wako Pure Chemical Industries, Ltd.) using a quick endpoint activity assay to determine the relative activity among the complexes. The glucose was oxidized by GOD to D-glucono-d-lactone and hydrogen peroxide. The hydrogen peroxide then oxidized a reduced dye, o-dianisidine, catalyzed by peroxidase. The absorbance change for 5 min at 25 ^{o}C was measured in 436 nm wavelength with water as the control. The

assay consisted of 24 mL of 0.2 mM o-dianisidine solution, 1 mL of 5 mg/mL peroxidase solution and 5 mL of 0.1 g/L glucose aqueous solution, which was prepared 1 h before use. The GOD activity was measured immediately after rinsing the GOD-loaded complex with water.

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CHAPTER 5

Ability of chitosan/carrageenan complex to encapsulate bovine serum albumin (BSA) for potential use in protein delivery

Chapter 5

ABSTRACT

The purpose of this study was to examine the capability of chitosan/carrageenan complex formed by electrostatic interaction as a carrier system for protein delivery. Chitosan/carrageenan complex was formulated at charge ratios (+/-) of 1,3,5,7 while bovine serum albumin (BSA) was the model protein. Microencapsulation of BSA was carried out by mixing BSA with chitosan solution followed by κ-, ι-, or λ-carrageenan then agitated at 200 rpm for 24 hours at 4 ^{o}C. The resulting complex was then evaluated by different physical methods. Results showed that the polyelectrolyte complex with κ-carrageenan had the highest encapsulation efficiency at 74 % with the order of κ>ι>λ and was stable in pH 1.2 to 7.3. The complex of chitosan/carrageenan was more efficient in the encapsulation of BSA than using chitosan or carrageenan alone. Controlled release of BSA from the complex was observed when treated with enzymes and different physiological solutions. Among the complexes prepared, chitosan/κ-carrageenan complex was the most stable releasing the BSA at the lowest rate. SDS-PAGE analysis of the complex showed no degradation of BSA when exposed in pH 1.2 solution 37 ^{o}C for 1 hour while gradual degradation was observed in pepsin solution (1 mg/mL). Zeta potential ranged from -40 mV to -50 mV with z-average size from 334 nm to 782 nm. Atomic Force Microscopy (AFM) images of chitosan/carrageenan complex loaded with BSA showed compact structure with almost uniform circular structure.

Chapter 5

INTRODUCTION

At present, researches being done on protein drugs are directed towards developing effective oral formulations and increasing the oral absorption of intact protein through the use of formulations that protect the macromolecule and/or enhance it's uptake into the intestinal mucosa [1]. In order to effect the delivery of peptides and proteins via the gastrointestinal tract, a carrier system should overcome significant enzymatic and diffusion barriers. The most widely used carrier is the cationic polysaccharide chitosan. It is biodegradable, biocompatible, bioadhesive and has permeation enhancing properties [2]. Previous study revealed that drug release from polyion complexes affords more sustained effect than the preformed complexes [3]. Polymer blends can combine attributes of different polymers to give a superior quality for the dosage form [4]. Since carrageenan reacts with chitosan forming a polyelectrolyte complex [5], the objective of this study is to evaluate the possibility of using polyelectrolyte complexes from chitosan and different types of carrageenan (κ, ι, λ) produced from Philippine *Eucheuma* species as carrier system for protein delivery. The model protein used in this study is bovine serum albumin (BSA). Carrageenan is an anionic polymer extracted from marine red algae and has also been used in many published studies as a carrier system for drug delivery [6,7,8,9,10,]. It shows potential use for new drug delivery system providing more control over the release rate of drugs. Previous studies showed that polyelectrolyte complex (PEC) in the form of beads and microspheres that are formed by cationic polymer(s) and anionic polymer(s) could enhance the controlled or prolonged release of a drug. Examples of PEC for controlling drug release include

alginate/chitosan [5], chitosan-cellulose multicore microparticles [6], chitosan-coated pectin [7], chitosan/poly(acrylic acid) complexes [8], poly(vinyl alcohol)/sodium alginate blend beads [9], poly(methacrylic acid-g-ethylene glycol) particles [10]. The potential use of carrageenan for oral protein delivery will indirectly boost the carrageenan industry in the Philippines since all of the carrageenan samples used in this study were produced in the said country.

RESULTS:

Encapsulation Efficiency

In this study, different charge ratios (+/-) of 1,3,5,7 were prepared to determine the efficiency of the polyelectrolyte complex to encapsulate BSA. As shown in Figure 1A, the complex of chitosan/κ-carrageenan had the highest encapsulation efficiency of 74% followed by chitosan/ι-carrageenan. The least efficient was the λ-type. Chitosan/dextran sodium sulphate complex showed an encapsulation efficiency of 68%. Encapsulation efficiency of each of the polymers was also investigated. ι- carrageenan exhibited the highest efficiency (Fig. 1B) followed by κ-carrageenan and λ-carrageenan. Chitosan only showed an efficiency of about 10%. Since charge ratios of 3 & 5 exhibited high encapsulation efficiency, chitosan/carrageenan complexes prepared at these ratios were furthered investigated and studied according to its morphology, stability and physical properties.

Morphology of the complex

As observed in Figures 1J, 1L, 1M, 1N, AFM images of the polymers (carrageenan, chitosan) and BSA (Figure 1K) showed morphologies that are fibrous in shape. Complexation of chitosan with carrageenan through electrostatic interaction formed a good complex with BSA (Figures 1A-1I). AFM images of chitosan/carrageenan complex loaded with BSA (Figures 1A-1I) showed compact structure with almost uniform circular structure confirming a complex formation. The complexes of chitosan/κ-carrageenan (Figure 1A, AFM image) and chitosan/dextran sodium sulphate (Figs. 1D & 1H, AFM images) showed a good complex formation with BSA as compared to ι-, λ-carrageenan and chitosan (Figs 1B, 1C, 1F, 1G, 1I, AFM images) where some BSA could be seen looping out of the complex. These findings conformed to the results of the encapsulation efficiency (Figures 1A & B) of the polyion complex. As observed in the AFM images, complexes with encapsulation efficiency of < 85% showed some unbound BSA in the background.

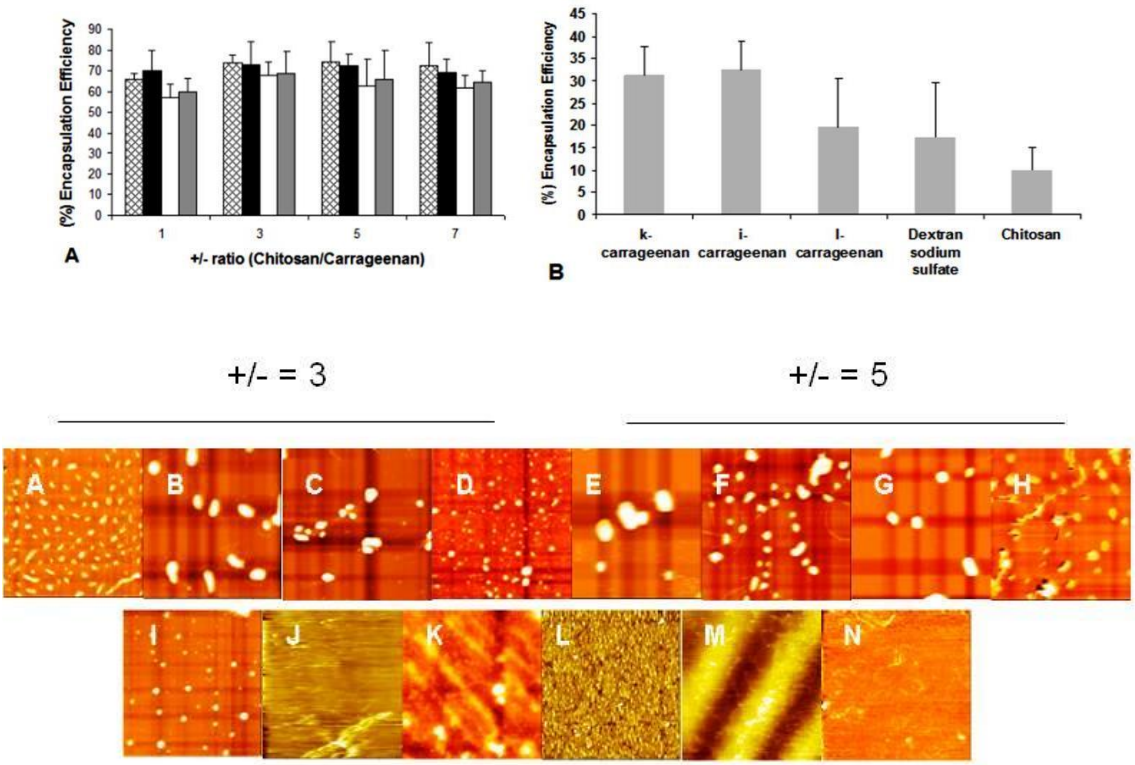


Fig. 1. Efficiency of chitosan/carrageenan complex A and single polymers (B) to encapsulate BSA (1 mg/mL) at different charge (+/-) ratios and AFM images of BSA encapsuated with the complex. ■: chitosan/κ-carrageenan; ■ : chitosan/ι-carrageenan; □ :chitosan/λ-carrageenan; ■: chitosan/dextran sodium sulphate. The values represent the mean ± SD, *n* = 4. BSA bovine serum albumin. AFM images A & E: chitosan/κ-carrageenan; B & F: chitosan/ι– carrageenan; C & G: chitosan/λcarrageenan; D & H: chitosan/dextran sodium sulphate; I: chitosan (with BSA); J: κ-carrageenan; K: BSA; L: ι– carrageenan; M: λ- carrageenan; N: chitosan.

Zeta potential and particle Size

Negative zeta potential (Figure 2) was observed in all of the complexes prepared. There was no significance change in zeta potential in both charge ratios. Charge ratio (+/-) = 3 displayed negative zeta potential at > - 45 while charge ratio

(+/-) = 5 exhibited negative zeta potential of < - 46. High values of zeta potential suggested high stability of the complex. Particle size of chitosan/carrageenan complexes with BSA were higher in values than chitosan/BSA alone. Increase of particle size was expected in charge ratio (+/-) = 3 as compared to charge ratio (+/-) = 5 since the former contained more carrageenan than the latter. The high values of particle size showed that entrapment of BSA and complexation of chitosan with carrageenan were achieved.

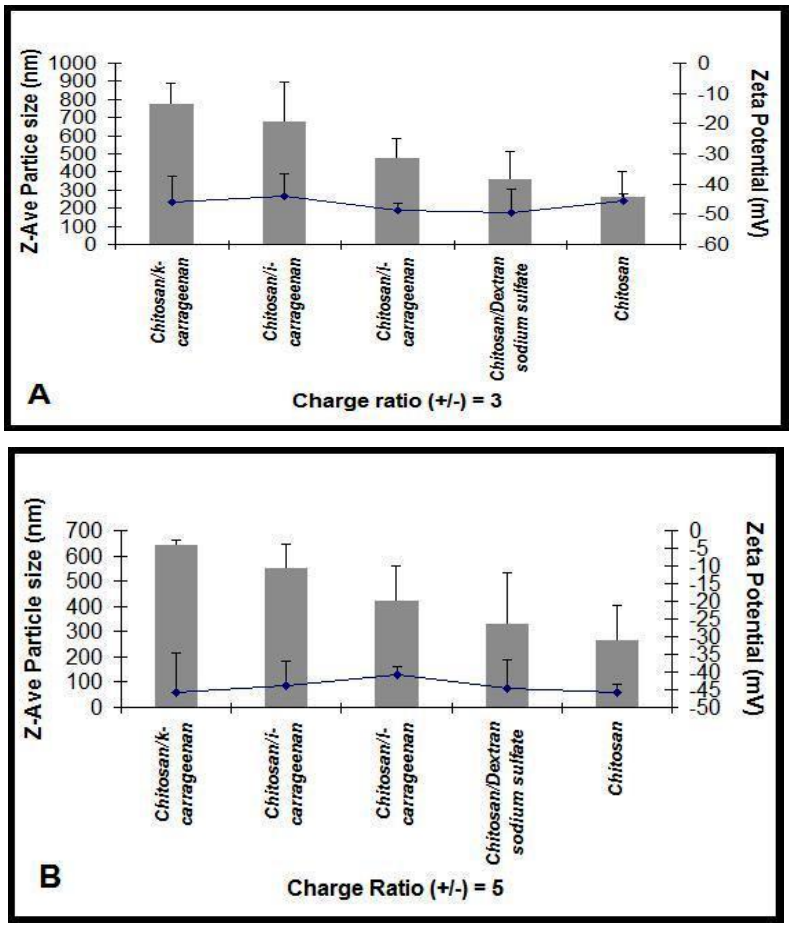
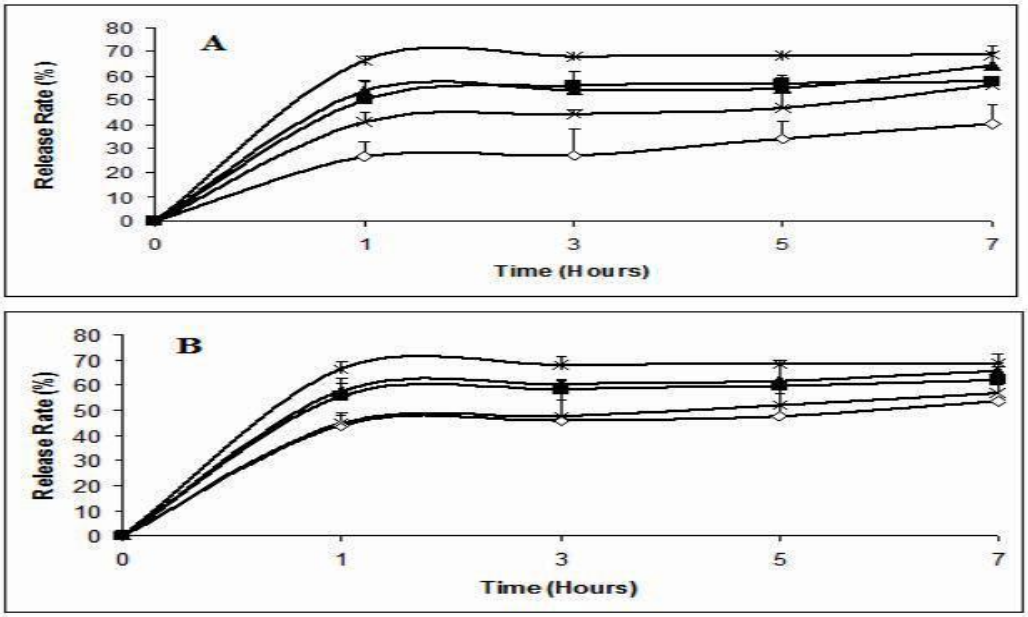
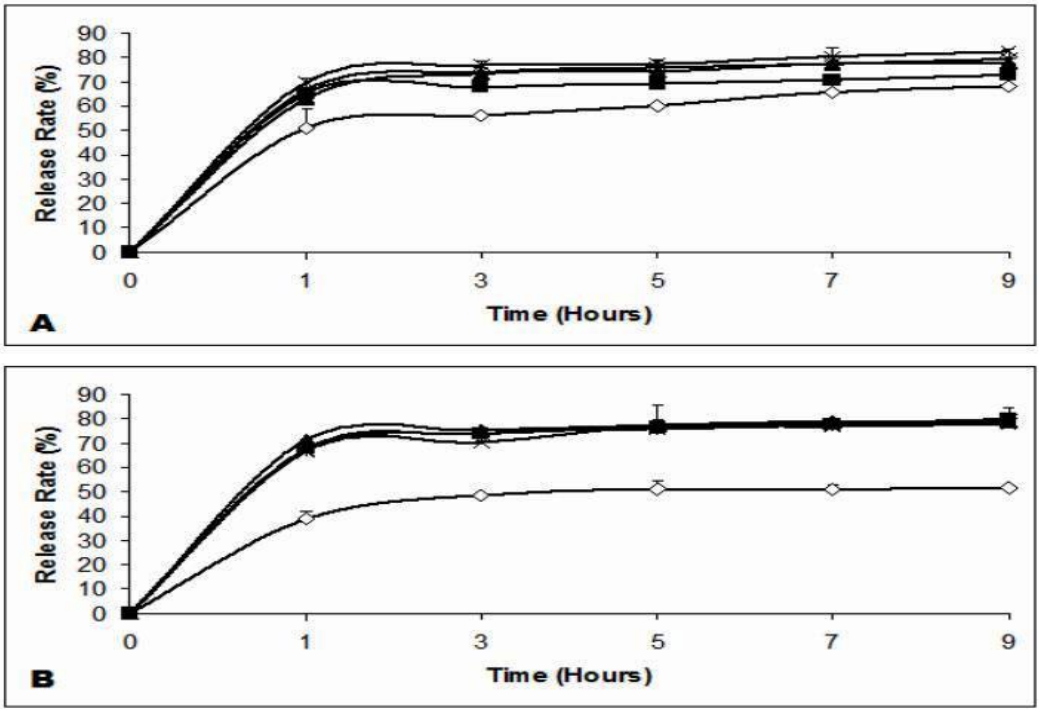


Figure 2. Zeta potential and particle size of the chitosan/carrageenan complex with BSA (1 mg/mL). The values represent the mean ± SD, n = 4. BSA indicates bovine serum albumin.

I-Chitosanase



II-Pepsin



III-Trypsin

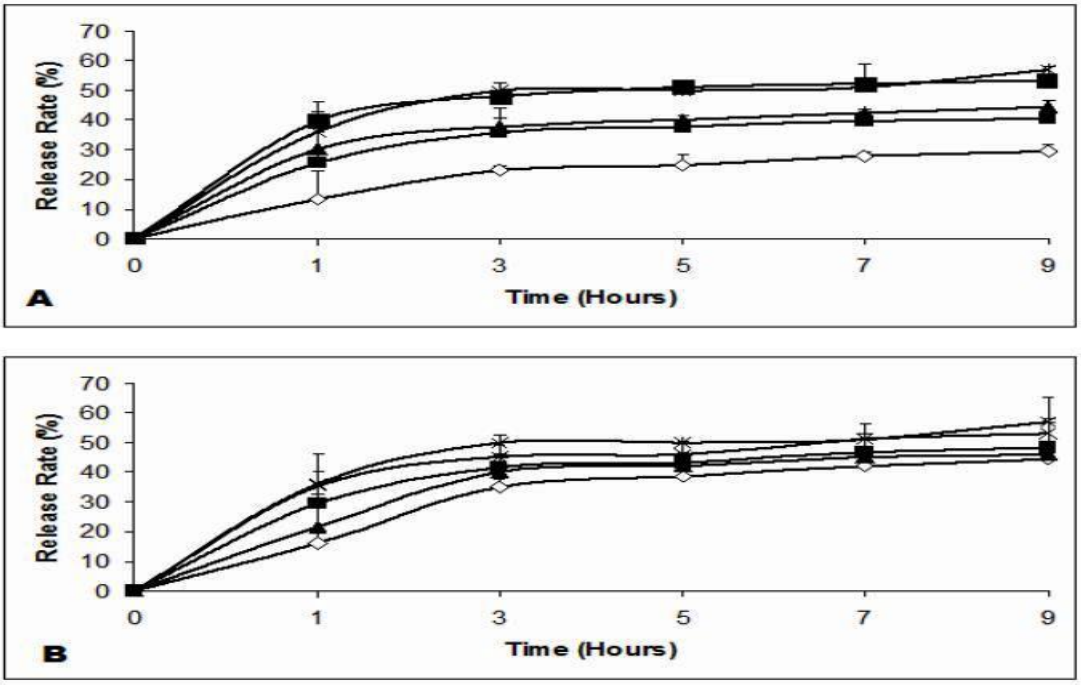
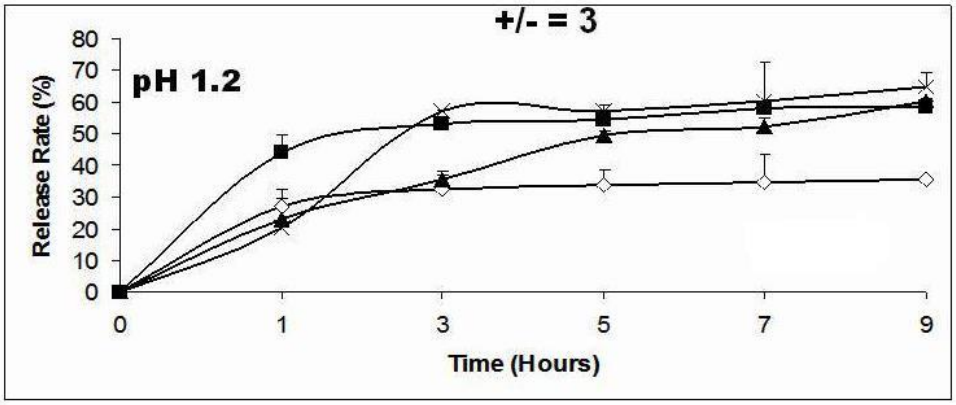


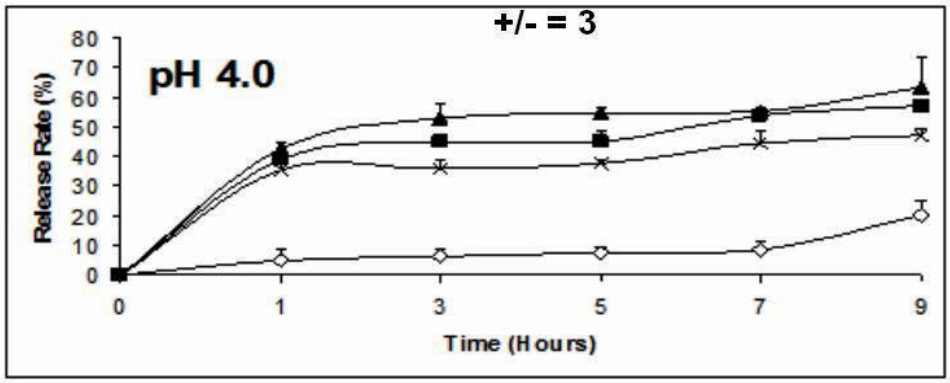
Figure 3. Cumulative release of BSA from chitosan/carrageenan complex prepared at various charge ratios in different enzyme solutions (0.1 mg/mL). A: (+/-) = 3; B: (+/-) = 5; ◊ = chitosan/κ-carrageenan; ■ = chitosan/ι-carrageenan; ▲= chitosan/λ-carrageenan; × = chitosan/dextran sodium sulphate ; □ **=** chitosan. Incubation temperature: 37 ^{o}C. pH: 6.0. The values represent the mean ± SD, *n* = 3. BSA indicates bovine serum albumin.

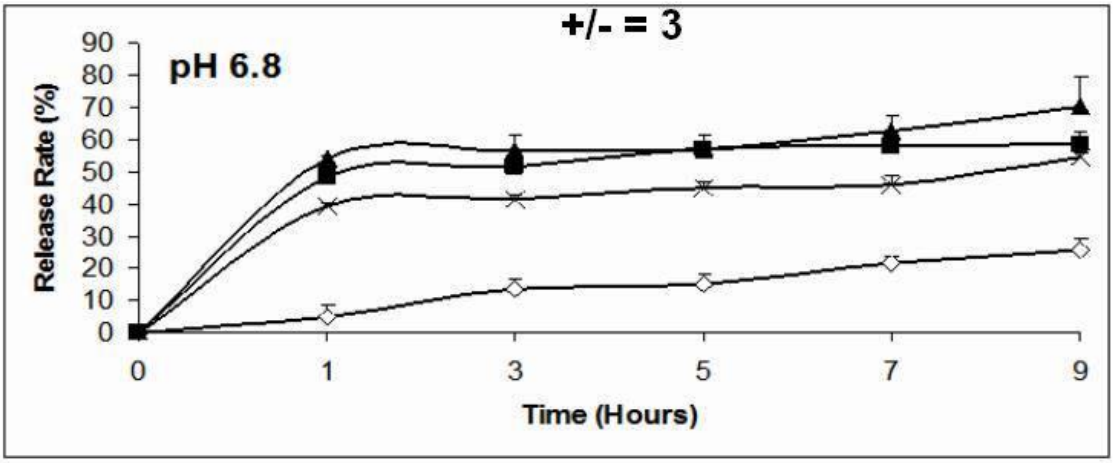
***In Vitro* Release Studies**

The release profile of BSA from the chitosan/carrageenan complex were evaluated in 0.1 mg/mL solution of chitosanase, pepsin and trypsin and different physiological solutions. In Figure 3 controlled release of BSA was observed in which the lowest rate was exhibited by chitosan/κ-carrageenan complex. The electrostatic interaction of the amino group of chitosan with the sulphate group of κ-carrageenan was more stable than the interaction between chitosan to ι- & λ-

carrageenan. The complex of chitosan and dextran sodium sulphate also showed slow release rate of BSA next to chitosan/κ-carrageenan complex. Chitosan showed the highest release rate of BSA at about > 60% after one hour incubation in chitosanase and pepsin (Figure 3). Using different physiological solutions (Figure 4), the chitosan/κ-carrageenan still showed the lowest release rate of BSA. Even at low pH of 1.2, the chitosan/κ-carrageenan complex was the most stable among the complexes releasing the BSA in a much controlled rate. At this condition (below the isoelectric point of BSA), the BSA was positively charged and was bound more to carrageenan making the release rate more slower. Complexation of chitosan with carrageenan controlled the release of BSA at a given time and temperature.



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Release Rate (%)
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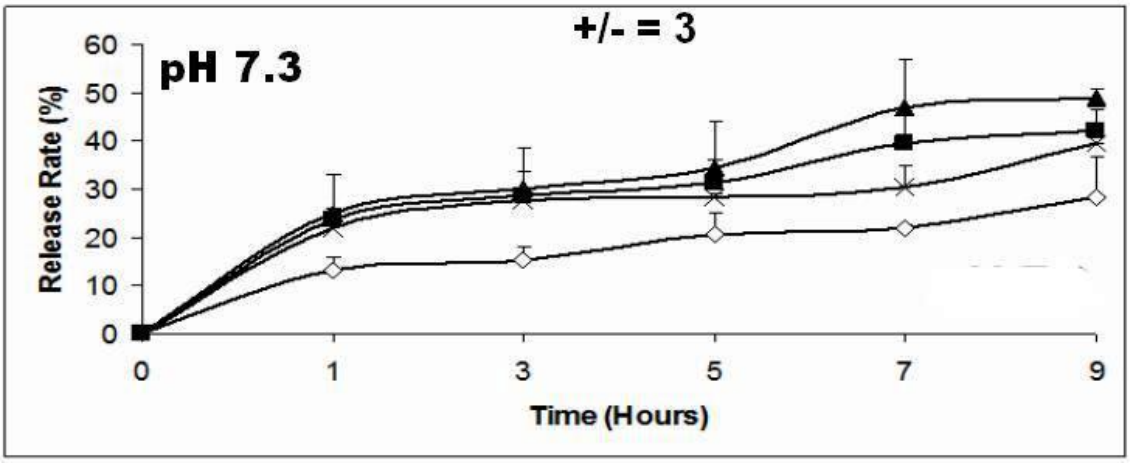
Time (Hours)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pH 7.3
Release Rate (%)
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3
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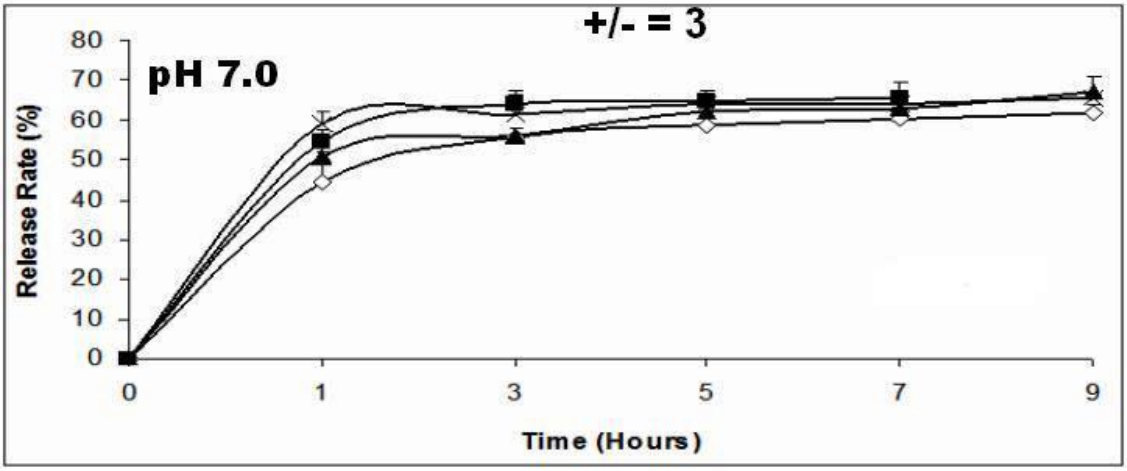
Time (Hours)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pH 7.0
Release Rate (%)
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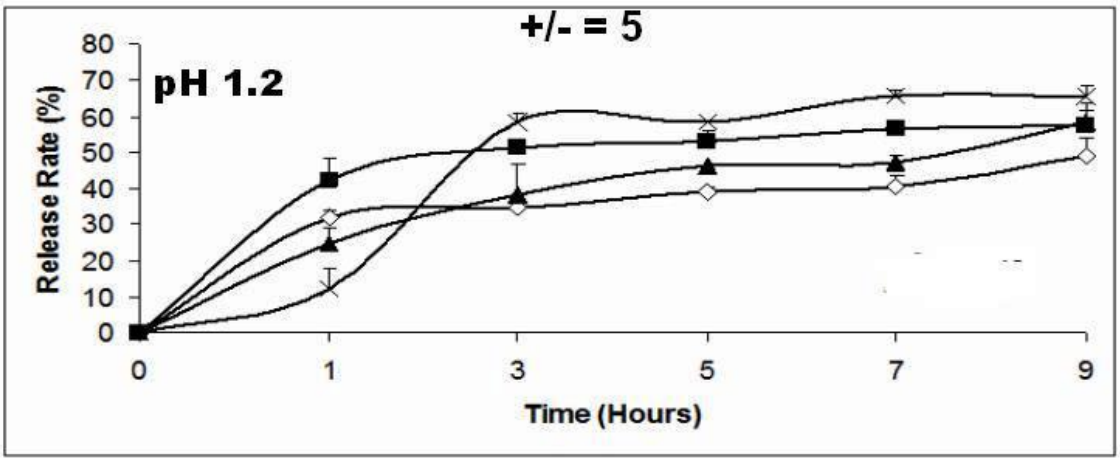
Time (Hours)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Release Rate (%)
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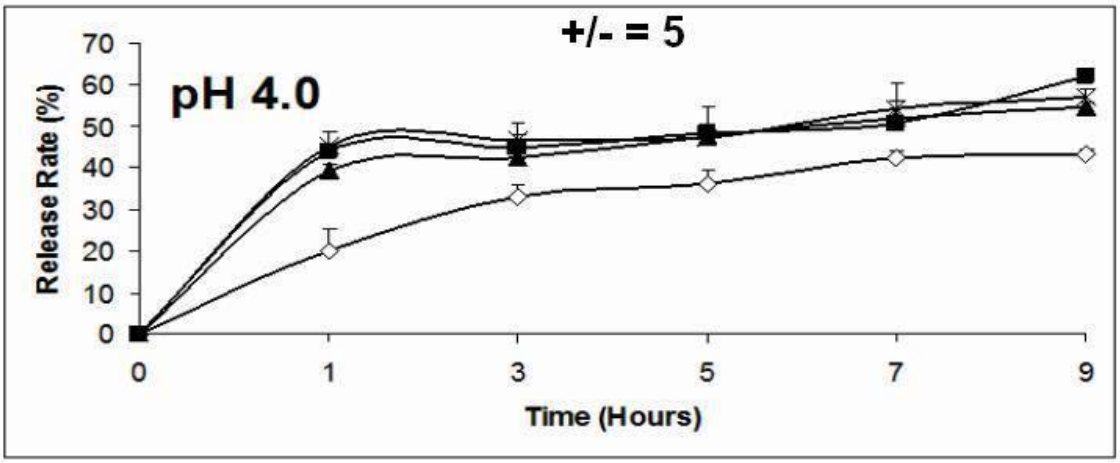
Time (Hours)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Release Rate (%)
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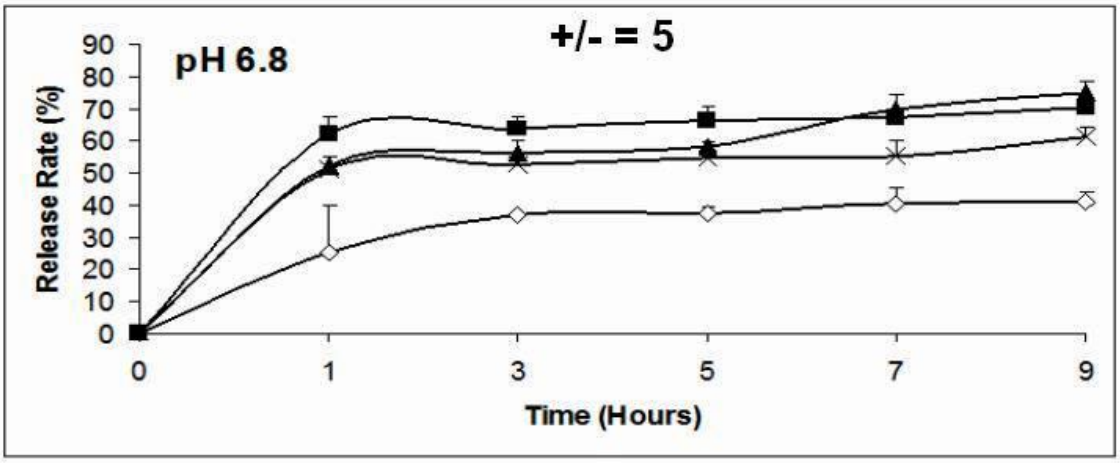
Time (Hours)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pH 6.8
Release Rate (%)
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Time (Hours)

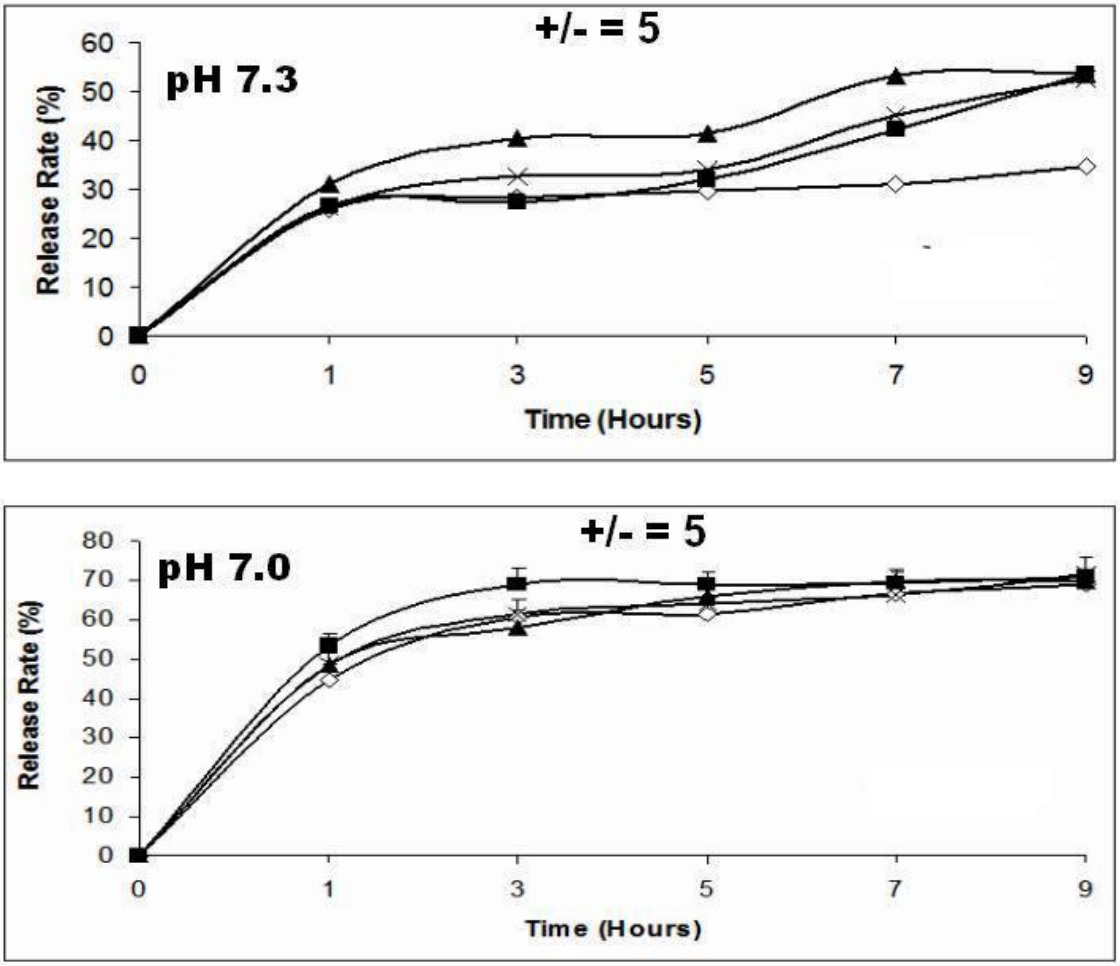
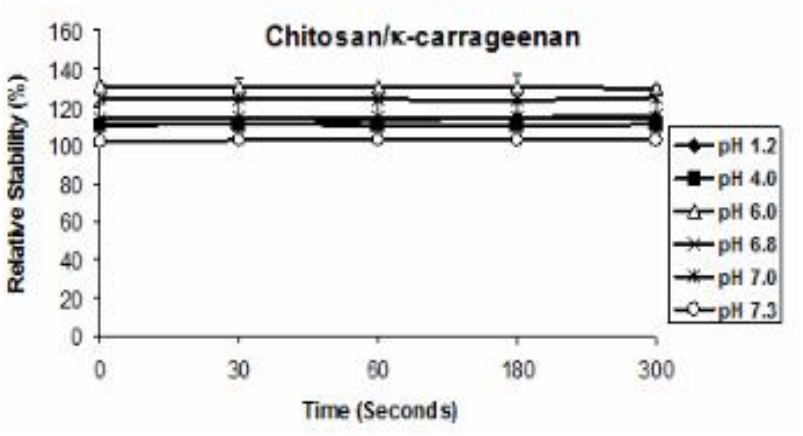


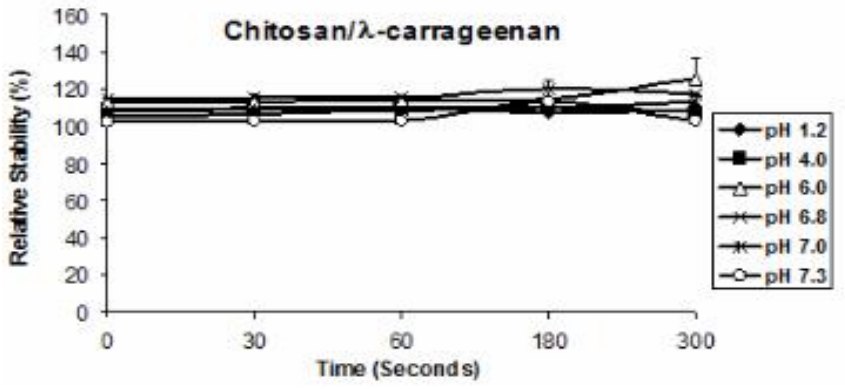
Figure 4. Cumulative release of BSA from chitosan/carrageenan complex prepared at various charge ratios in various physiological solutions. ◊ = chitosan/κ-carrageenan; ■ = chitosan/ι-carrageenan; ▲= chitosan/λ-carrageenan; × = chitosan/dextran sodium sulphate. Incubation temperature: 37 ^{o}C. The values represent the mean± SD, *n* = 3. BSA indicates bovine serum albumin.

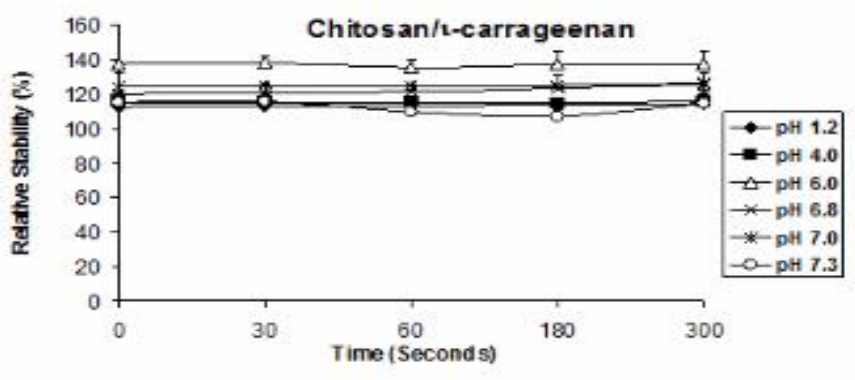
Relative stability at different physiological solutions

The complexes were relatively stable in different physiological solutions at various pH (Figure 5). Among the complexes prepared, chitosan/dextran sodium sulfate has the highest turbidity reading as compared to chitosan/carrageenan complex. The chitosan/λ-carrageenan complex was the most stable as there was no

significant change observed in the turbidity reading at various pH. Turbidity reading increases at pH 6.0 in chitosan/κ-carrageenan, chitosan/ι-carrageenan and chitosan/dextran sodium sulfate complexes.







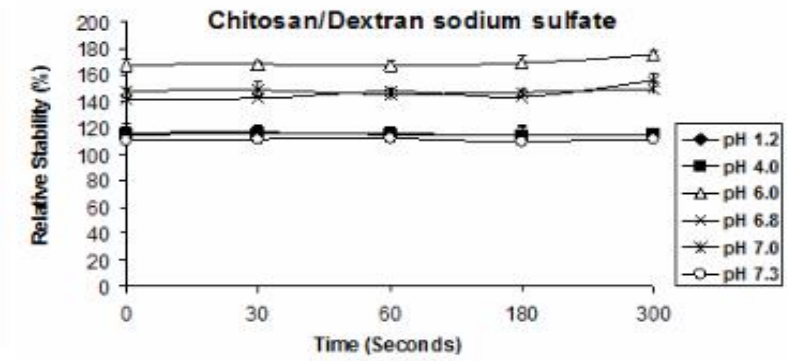
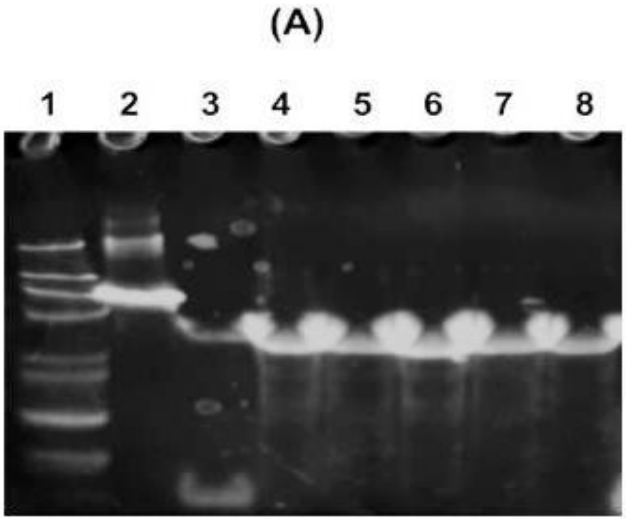


Figure 5. Relative stability of encapsulated BSA in chitosan/carrageenan complex (+/- ratio = 3) using different physiological solutions. pH 1.2: 35 mMNaCl; pH 4.0: 50 mM acetic acid; pH 6.0: 200 mMNaCl in MES buffer; pH 6.8: 50 mM potassium phosphate buffer; pH 7.0: H_20; pH 7.3: PBS (0.01 g/mL). Turbidity of the encapsulated BSA with the different physiological solutions was measured at 500 nm against the control. BSA indicates bovine serum albumin; PBS, phosphate buffered saline; MES, 2-(*N*-morpholino)ethanesulfonic acid.

Stability of BSA in pepsin solution and pH 1.2

In protein delivery, the protein must be protected by the complex formation during its application. The stability and integrity of BSA in the complex were evaluated by gel elecrophoresis. The use of pepsin solution and pH 1.2 was employed to investigate the integrity of BSA. Figure 6 shows the SDS-PAGE analysis of release fractions of BSA in chitosan, carrageenan and dextran sodium sulfate (Figure 6A) and BSA in chitosan/carrageenan complexes (Figure 6B), free BSA and treated free BSA that were used as a control. The study showed that the treated free BSA (Figure 6A & B, Lane 3) was degraded completely as compared to the BSA released from the polymers and complexes. In Figure 6A, the BSA released from chitosan (Lane 8), carrageenan (Lanes 4-6) and dextran sodium sulfate (Lane 7) was degraded in a slower rate based on the formation of more than one band as

from chitosan (Lane 8), carrageenan (Lanes 4-6) and dextran sodium sulfate (Lane 7) was degraded in a slower rate based on the formation of more than one band as compared to treated free BSA in which only one band (Lane 3) was observed. The BSA fractions released from chitosan/carrageenan complex showed also degradation but in a much slower rate. In Figure 6B, most of the BSA were still in the well as observed in the intensity of the bands showing resistance to release and degradation. Most of the lanes showed more than one band showing the slower rate of BSA degradation in the polyion complex than using one polymer in the encapsulation process. The integrity of BSA in pH 1.2 was also investigated by gel electrophoresis. In Figure 7, BSA fractions released from the complexes were not degraded as compared to the treated free BSA (Figure 7, Lane 7). The BSA was protected from being degraded and hydrolyzed. The use of chitosan/carrageenan complex prevented degradation of BSA in acidic solution.



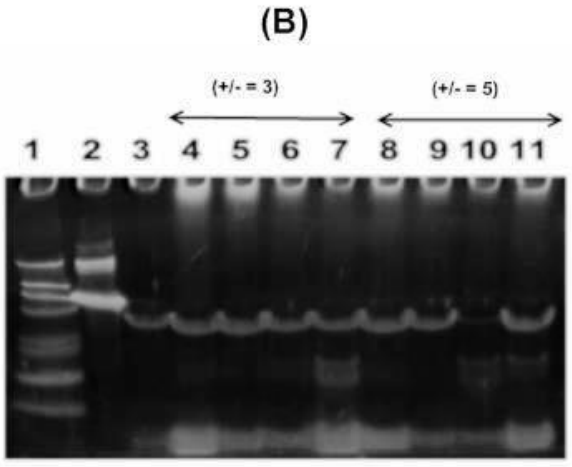


Figure 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis for stability of BSA released from the complex treated with pepsin (1 mg/mL) and incubated at 37 ^{o}C for one hour. A. BSA released in carrageenan, dextran sodium sulphate and chitosan. Lane 1: Protein marker; Lane 2: Free BSA; Lane 3: Treated BSA; Lane 4: κ-carrageenan; Lane 5: ι-carrageenan; Lane 6: λ-carrageenan; Lane 7; Dextran sodium sulphate; Lane 8:Chitosan; B.BSA released in chitosan/carragee nan complex. Lane 1: Protein marker; Lane 2: Free BSA;
Lane 3: Treated BSA; Lanes 4 & 8: chitosan/κ-carrageenan; Lanes 5 & 9; chitosan/ι-carrageenan; Lanes 6 & 10; chitosan/λ-carrageenan; Lanes 7 & 11; chitosan/dextran sodium sulphate; BSA indicates bovine serum albumin.

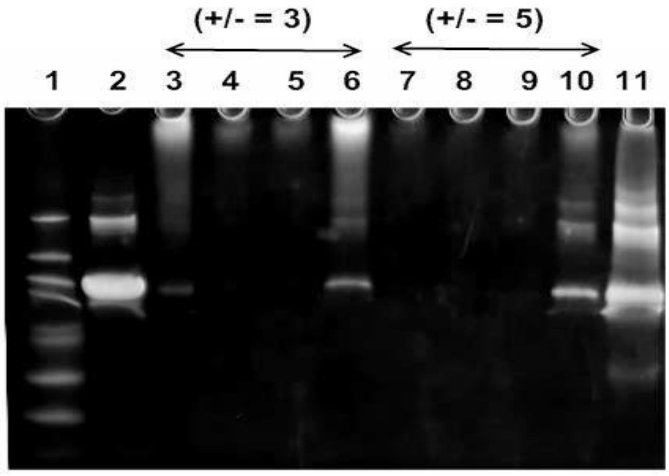


Figure 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis for stability of BSA released from chitosan/carrageenan complex in pH 1.2 incubated at 37 ^{o}C for one hour. Lane 1: Protein marker; Lane 2: Free BSA; Lanes 3 & 7: chitosan/κ-carrageenan; Lanes 4 & 8; chitosan/ι-carrageenan; Lanes 5 & 9; chitosan/λ-carrageenan; Lanes 6 & 10; chitosan/dextran sodium sulphate; Lane 11: Treated BSA; BSA indicates bovine serum albumin.

Chapter 5

DISCUSSION:

The formation of the micro particles of chitosan/carrageenan complex was due to its opposite charges between the positively charged amine groups (NH_3^+) in chitosan and the negatively charged sulphate groups (SO_3^-) in carrageenan. Chitosan/dextran sodium sulphate was used as the control in this study since previous study showed the high efficiency of this complex to entrap BSA and Rhodamine at an efficiency of 98% [12]. The low efficiency of chitosan to encapsulate BSA might be attributed to the condition used in this study wherein no cross linker was used as compared to the previous study which make used of tripolyphosphate (TPP) as cross linker [13]. Here, we were able to show that polyion complex (chitosan/carrageenan) was more efficient than using just one polymer without the use of a crosslinking agent. The electrostatic interaction of the sulphate group of carrageenan and the amine group of chitosan make a better entrapment of BSA than chitosan alone. Charge ratios (+/-) of 3 & 5 were the most efficient as compared to 1 & 7 ratios. The difference of encapsulation efficiency among the three types of carrageenan could be due to its chemical structure and molecular weight. λ- carrageenan had the highest molecular weight of 750 kDa and being the most anionic due to its three sulphate groups of the alternating 1,3-linked β-D-galactopyranosyl and 1,4-linked α-D-galactopyranosyl sugar units while ι-carrageenan has two sulphate groups attached to its alternating sugar [14]. The isoelectric point of BSA is 4.8 [12] and the pH used in this study for complex formation was pH 6.0 which is above the isoelectric point of BSA. At this condition, the BSA would be predominantly negatively charged thus would ionically interact

with chitosan competing with the more anionic carrageenan. The swelling mechanism of chitosan/carrageenan complex showed that in an acidic solution, no swelling was observed [5]. This is due to the presence of electrostatic bonds between chitosan and carrageenan. The sulfate groups of carrageenan will remain negatively charged and interact with the positively charged amino groups of chitosan hence, the cumulative release rate of BSA was not too excessive. In alkaline solution, the amino groups of chitosan will be neutralized, and the sulfate groups of carrageenan will remain negatively charged therefore, the electrostatic linkage between the two polymers disappears [5]. The electrostatic repulsion between sulfonate groups will contribute to the swelling mechanism [5]; thus, higher release rate of BSA was observed. In alkaline solution, BSA is negatively charged; thus, there is no electrostatic interaction with chitosan. BSA, which has a pI of 4.7 [15], is anionic at physiological pH of 7.3. Among the prepared complexes, chitosan/κ-carrageenan complex showed the lowest release rate of BSA. This behavior might be due to its chemical structure. Since κ-carrageenan contains only one sulfate group attached to its alternating sugar units, less electrostatic repulsion is expected with fewer ionic binding sites. It also undergoes a helix-coil transition, creating additional cross-links and forming a more rigid system [16]. λ-Carrageenan had the highest release rate of BSA among the three types. This type contains three sulfate groups of the alternating sugar units, creating more electrostatic repulsion and ionic binding sites. This type does not gel and does not undergo a helixcoil transition, which precludes additional cross-links [16].

MATERIALS AND METHODS

Materials

Chitosan (average MW = 45 kDa, with degree of acetylation of 75.4%) was obtained fromYSK YaizuSuisankagaku Industry Co, Ltd., Japan. κ-carrageenan (Bengel KK-100, Lot No. XO300-2, average MW = 510 kDa), ι-carrageenan (Benvisco SI-100, Lot No.M1400-1, average MW= 560 kDa) and λ-carrageenan (Benvisco SL-100, Lot No. S2703-2, average MW = 750 kDa) were obtained from Shemberg Biotech Corporation (Carmen, Cebu, Philippines, 6005). Bovine serum albumin (BSA) was purchased from Sigma Aldrich Japan K.K while dextran sodium sulphate (average MW = 5 kDa) was purchased from Wako Japan. All other chemicals and reagents used were of analytical grade.

Preparation of solution

Chitosan solution was prepared at 1mg/mL in MES buffer solution at pH 6.0. Carrageenan (1mg/mL), Dextran sodium sulphate (1 mg/mL) and BSA (4 mg/mL) solutions were prepared in MilliQ water.

Preparation of complex

BSA solution was added to chitosan solution, mixed thoroughly and allowed to stabilize at room temperature for 15 minutes. Carrageenan solution was then slowly added to the mixture, mixed again followed by another 15 minutes stabilization at room temperature. Final concentration of BSA in the complex was 1mg/mL. Thereafter the final mixture was incubated for 24 hours at 4 ^{0}C with shaking (200 rpm). The volumes used to prepare the complex at various charge ratios (+/-)

and type of carrageenan are shown in Table 1.

Encapsulation efficiency (E.E.)

After incubation of the complex, the solution was centrifuged at 10,000 rpm for 20 minutes at 4 ^{0}C. The resulting supernatant was then assayed for its protein content following the manufacture's instruction (Bio-Rad Laboratories, Japan). All experiments were performed in triplicate. Encapsulation efficiency was determined according to the following equation:

$$E.E. = \frac{Co - Cr}{Co} \times 100$$

where where *Co* is the initial concentration of the protein, and *Cr* is the protein reading from the assay.

Relative stability at different physiological solutions

After centrifugation of the solution, the supernatant was removed and the resulting precipitate was subjected to various simulated physiological solutions: a. 35 mMNaCl solution adjusted to pH 1.2; b. 50 mM Acetic acid adjusted to pH 4.0; c. 200 mMNaCl in MES buffer adjusted to pH 6.0; d. 50 mM Potassium phosphate buffer adjusted to pH 6.8; e. MilliQ H_2O; f. PBS solution at pH 7.3 The relative stability was calculated according to the following equation:

$$\text{Relative Stability} = \frac{\text{Turbidity reading at 500 nm of the complex in physiological solution}}{\text{Turbidity reading at 500 nm of 0 mMNaCl, MES buffer solution, pH 6.0}} \times 100$$

Atomic force microscopy (AFM) measurement

After incubation for 24 hours at 4 ^{o}C with shaking (200 rpm), 10 uL of the

solution was diluted with MES buffer (pH 6.0) to make 100 uL then applied 20 uL drop deposition onto the surface of a mica disk as previously described [11]. Following adsorption for 2 minutes at room temperature, excess fluid was removed by absorption with filter paper. Then 50 μL of water was added to the surface to wash excess salt from the PBS solution. The mica surface was subsequently dried at room temperature prior to imaging. A commercial atomic force microscope (SPA-300 system of Seiko Instruments, Inc. Japan) was used for imaging using a Si_3N_4 tip on the cantilever (100 μm x 400 nm, SN-AF01-A, Olympus Optical Co.) with tapping mode and non-contact recording under ambient condition.

Measurement of zeta potential and particle size

Surface charges of the complexes were evaluated using a Coulter® DELSA 440SX Zeta Potential Analyzer. The complex after incubation for 24 hours at 4 ^{o}C with shaking (200 rpm) was diluted with MES buffer (pH 6.0) to make a final volume of 1.5 mL. Size measurement was determined by using a High Performance Particle Sizer (Malvern Instrument, UK) diluting 3 uL of the complex with MES buffer (pH 6.0) to make a final volume of 1.2 mL.

Cumulative release of BSA from the complex after treament with different physiological solutions

The precipitate formed after centrifugation was treated with 1 mL each of the different physiological solutions at 37 ^{o}C with mild agitation (60 rpm) for 10, 30, 60, 120, 180 minutes. After each time interval, the treated samples were assayed for its protein content as previously described.

Cumulative release of BSA from the complex was determined according to the following equation:

$$CR\,[\%] = \frac{C_c - C_r}{C_c} x100$$

where C_c is the concentration of the BSA in the complex, and C_r is the BSA reading from the assay.

Cumulative release of BSA from the complex after treatment with enzymes (Chitosanase & Papain, 1 mg/mL, Pepsin & Trypsin, 0.1 mg/mL)

The same procedure was followed as previously described. Release of BSA from the complex was determined by protein assay of the supernatant collected after each time interval of 1, 3, 5, 7, 9 hours.

SDS PAGE analysis

Release and degradation of BSA from the complex after exposure to pepsin (1 mg/mL) and pH 1.2 was monitored by SDS-PAGE electrophoresis using Bioradredy gels J (161J341V) in running buffer (1 x Tris-Glycine, 0.1% SDS) with constant current mode, 20 mA. About 5 uL of sample was applied in each well. A pre-stained protein marker was also used. Staining solution used was Coomasie Brilliant Blue.

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CHAPTER 6

Structural Studies on ι-Carrageenan Derived Oligosaccharides and Its Application

Chapter 6

ABSTRACT

Mild hydrochloric acid hydrolysis of ι–carrageenan from *Eucheuma spinosum* yielded two oligosaccharides of sulfated tetrasaccharide structure. These were characterized by Fourier Transform Infrared Spectroscopy (FT-IR), Nuclear Magnetic Resonance (NMR) and Electrospray Ionization Mass Spectrometry (ESIMS). Both oligosaccharides have structure of β-D-galactopyranose(Gal*p*)4S-(1→4)-*α*-D-AnGal*p*2S-(1→3)-β-D-galactopyranoseGal*p*)4S-(1→4)-*α*-D-AnGal*p*2S-(1→3).

Application of the resulting oligosaccharides on protein delivery system in terms of encapsulation efficiency was performed.

Chapter 6

INTRODUCTION

Oligosaccharides which are sugars of shorter chains derived from larger polysaccharides or being synthesized are now of scientific interest for therapeutics and medical applications [1-8]. It can be obtained by direct extraction from natural sources, or chemical processes such as hydrolyzation of polysaccharides or chemical synthesis from disaccharides [9]. In this paper, oligosaccharides were derived by mild hydrolysis from ι-carrageenan. Previous studies showed that by using mild hydrolysis, oligosaccharides can be derived from κ-carrageenan [10,11]. There were no studies reported yet on deriving oligosaccharides from ι-carrageenan using mild acid hydrolysis. The published method was by enzymatic hydrolysis [12] and also used in κ-carrageenan and λ-carrageenan [13, 14]. Hence, the aim of this study is to prepare oligosac- charides from ι-carrageenan by mild acid hydrolysis and evaluate its potential use for protein delivery system.

Numerous studies were also made on the use of carrageenan for controlled release of drugs [15-21]. Carrageenan has also been cross-linked with polyacrylic acid co-poly-2-acrylamido-2-methylpropanesulfonic acid (PAA—co-PAMPS) to form a biodegradable hydrogel as a candidate for a drug delivery system [22] and interacted with chitosan to form a polyelectrolyte complex to en- capsulate GOD [23]. There has been no study done on the use of oligosaccharides derived from ι-carrageenan for protein delivery system thus in this study, its application for protein delivery system was preliminary investigated.

RESULTS AND DISCUSSION

Characterization of Oligosaccharides

The structure of ι-carrageenan is composed of (1→3)-β-D-galactopyranose(Gal*p*)4S-(1→4)-α-D-AnGal*p*2S-(1→) n as shown in **Figure 1** [26] while oligosaccharides are composed of longer chains of monosaccharide (between two and nine) units bound together by glycosidic bonds. In this study, two oligosaccharides (F1 & F2) were separated and purified. Prior to characterization of the derived oligosaccharides, the samples were subjected to thin layer chromatography analysis. Single spot was observed (data not shown) for both fractions illustrating a pure compound. IR analysis showed that the oligosaccharides contain the basic sugar unit 3,6-anydrogalactose and sulphated galactose as shown in Table 1. The pre-sence of ester sulfates was also confirmed.

The ESIMS spectra gave molecular ions for the sodium salt forms of both sulphated oligosaccharides. ESIMS analysis of compound F1 shown in Figure 2 and Table 2 displayed a molecular ion (M+ Na+) at m/z 940.4 corresponding to a mass of 917.4 while compound F2 exhibited a mass of 944.1 at m/z 967.1 (refer to Table 2 and Figure 2) suggesting that both oligosaccharides are of tetrasaccharide containing two galactopyranose (Gal*p*) residues and two 3,6 anhydrogalactopyranose (AnGal*p*) with four *O*-sulfo groups. Fragmentation analysis of carrageenan oligosaccharides is illustrated in Figure 3. The fragmentation was consistent with each of the three galactose residues carrying one *O*-sulfo group. In ^{13}C NMR analysis of the oligosaccharides, the region of 105 to 90 ppm (Figure 4), is the most informative since it is where the anomeric carbon signals are

observed [12]. Unhydrolyzed *iota* carrageenan displays signals at around 103 ppm for the β-Gal*p*4S and at around 93 ppm for the AnGal*p*2S [12]. Both oligosaccharides exhibited peaks at the 105 ppm for β-Gal*p*4S and 94.65 ppm for AnGal*p*2S (Table 3). This signal at around 94.65 ppm may be attributed to the anomeric carbon of the galactopyranosyl-4-sulfate residue at the reducing end of the chain which may have either α or β conformations as observed [27]. No peaks are present at 66.4 ppm corresponding to C-4 of unsulfated Gal*p* residues; thus, the ^{13}C spectra (Figure 4) confirm that all Gal*p* residues contained a 4-*O*-sulfo group [11]. The ^{13}C NMR spectra of the degraded products clearly showed that besides the known splitting of 3,6-anhydrogalactosidic linkages, the linkage between alpha-D-galactose 2,6-disulphate and beta-D-galactose 4-sulphate is also cleaved. One-dimensional 1H NMR confirmed the high level of purity of both oligosaccharides (Figures 5 & 6). NMR and MS analysis definitively establish the structures of the oligosaccharides F1 & F2 to be sulfated tetrasaccharide of the structure: β-D-galactopyranose (Gal*p*) 4S-(1→4)-α-D-AnGal*p*2S-(1→3)-β-D-galactopyranose(Gal*p*)4S-(1→4)-)-α-D-AnGal*p*2S-(1→3).

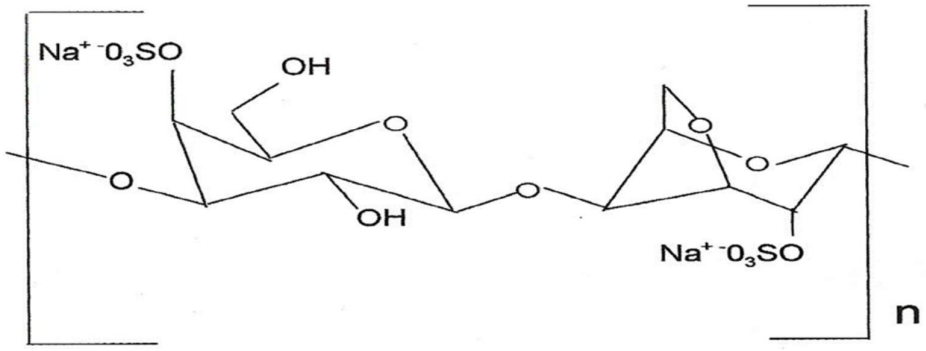

Figure 1. Disaccharide group of ι-carrageenan [26].

Table 1. Molecular assignment of carrageenan oligosaccharides by IR analysis

	Literature values [27]	*Iota* carrageenan (Unhydrolyzed)	Carrageenan oligosaccharide (F1)	Carrageenan oligosaccharide (F2)
Ester sulfate	1220-1260 cm^{-1}	1254.31 cm^{-1}	1231.70 cm^{-1}	1232.29 cm^{-1}
3,6-anhydrogalactose	928-933 cm^{-1}	930.94 cm^{-1}	927.99 cm^{-1}	928.10 cm^{-1}
Galactose -4-sulfate	840-850 cm^{-1}	849.07 cm^{-1}	847.82 cm^{-1}	-
3,6 anhydrogalactose -2-sulfate	800-805 cm^{-1}	804.33 cm^{-1}	804.01 cm^{-1}	802.77 cm^{-1}

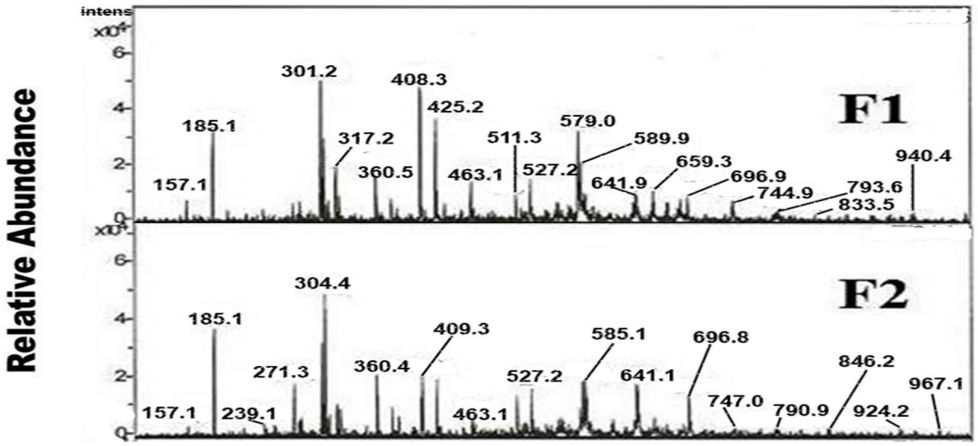

Figure 2. ESIMS analysis of oligosaccharides F1 & F2.

Table 2. Assignment of ESIMS of ι-carrageenan oligosaccharides (F1 & F2)

Ion	Carrageenan oligossacharide	
	F1	F2
$M+Na^+$	940.4	967.1
$M-6Na^+-_2H_20$	833.5	
$M-Na^+H_20$		924.2
$M-2NA^+-_2H_20$		846.2
$M-9Na^+-6H_20$	696.7	696.7
M-Galρ4SAnGalρ2S-Na^+-$2H_20$	511.3	511.3
Galρ4SAnGalρ2SGalρ4SAnGalρ2S-$4Na^+$	940.4	
Galρ4SAnGalρ2SGalρ4SAnGalρ2S-$3Na^+$		967.1
Galρ4SAnGalρ2S-N^+-$2H_20$	527.2	527.2
Galρ4S-Na^+-H_20	239.2	
Galρ4S-$2Na^+$-H_20		239.2
AnGalρ2S-$NaS0_3$+H^+	157.1	157.1

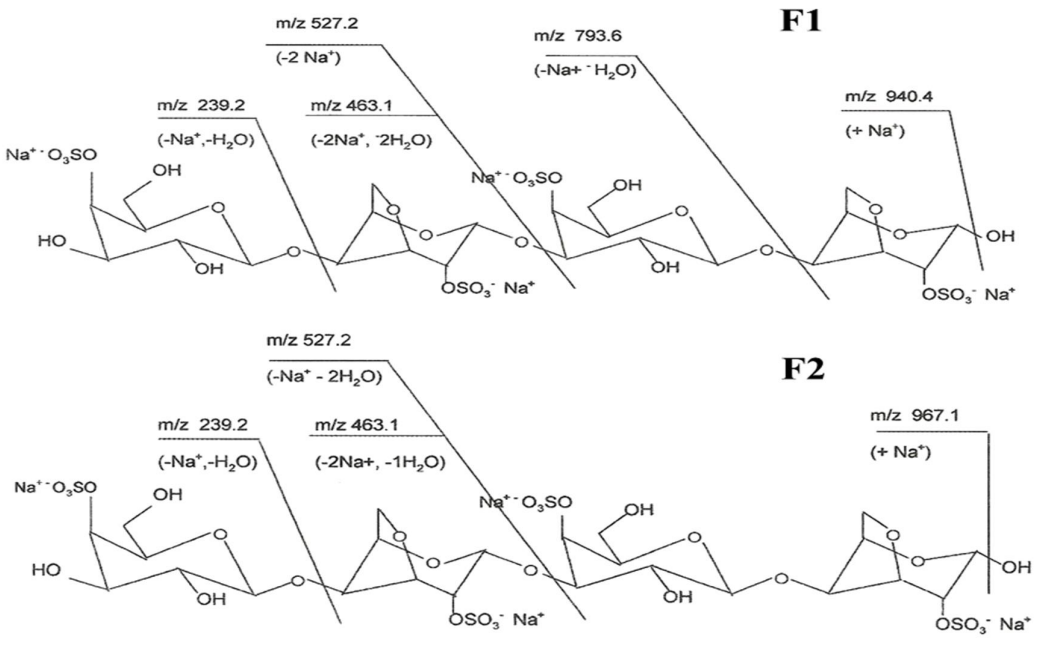

Figure 3. Fragmentation analysis of oligosaccharides F1 & F2.

Table 3. ^{13}C NMR chemical shifts of the ι-carrageenan oligosaccharides (F1 & F2)

Residue	Chemical shift (ppm)					
	C1	C2	C3	C4	C5	C6
Oligosaccharide F1						
β-Galρ4S	105.08	71.60	77.30	74.50	79.41	63.70
AnGalρ2S	94.64	72.31	80.90	80.27	77.40	63.78
Oligosaccharide F2						
β-Galρ4S	105.05	71.60	77.29	74.59	79.40	63.70
AnGalρ2S	94.65	72.23	80.91	80.30	77.39	64.01

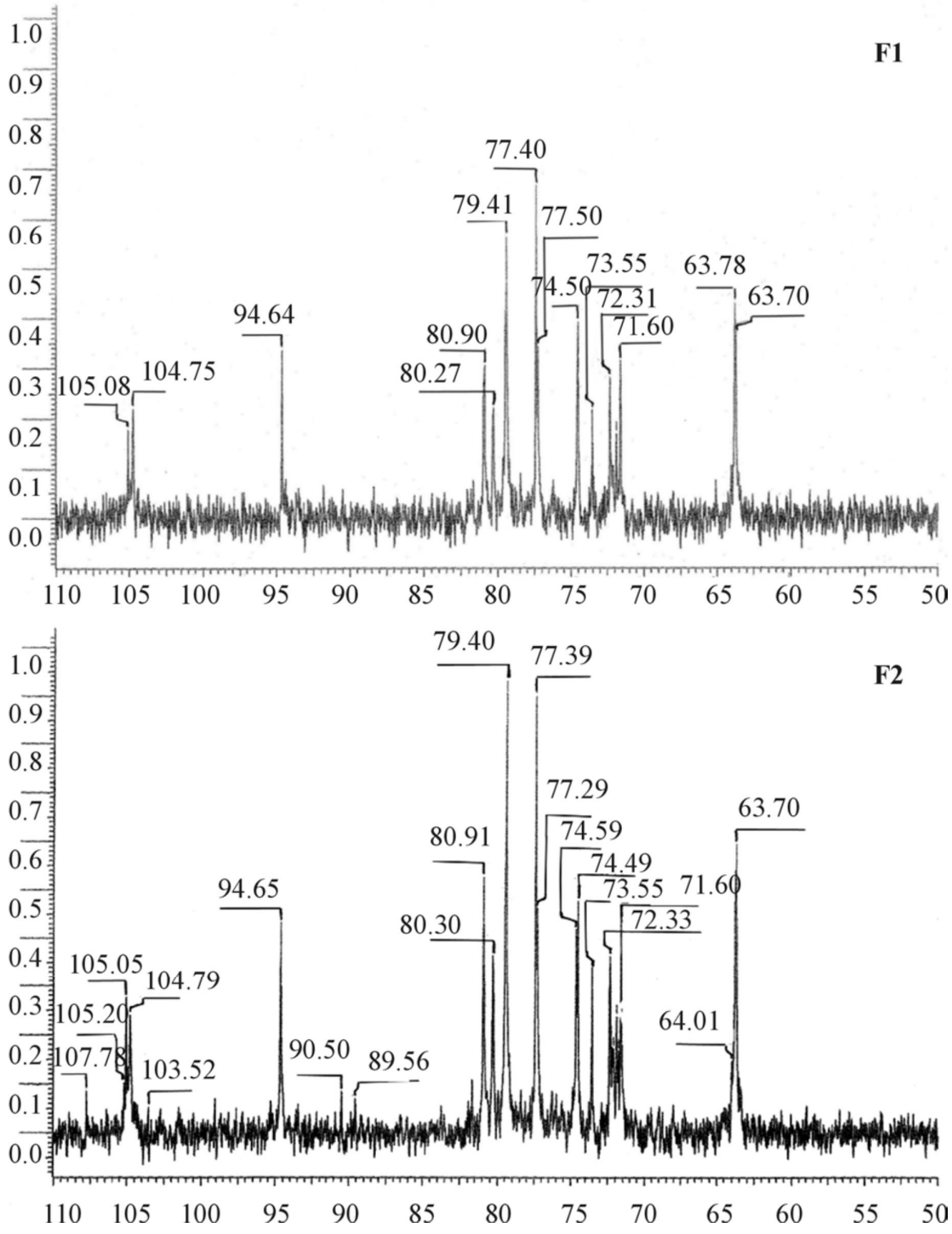

Figure 4. **^{13}C NMR spectra of the ι-carrageenan oligosaccharides (F1 & F2).**

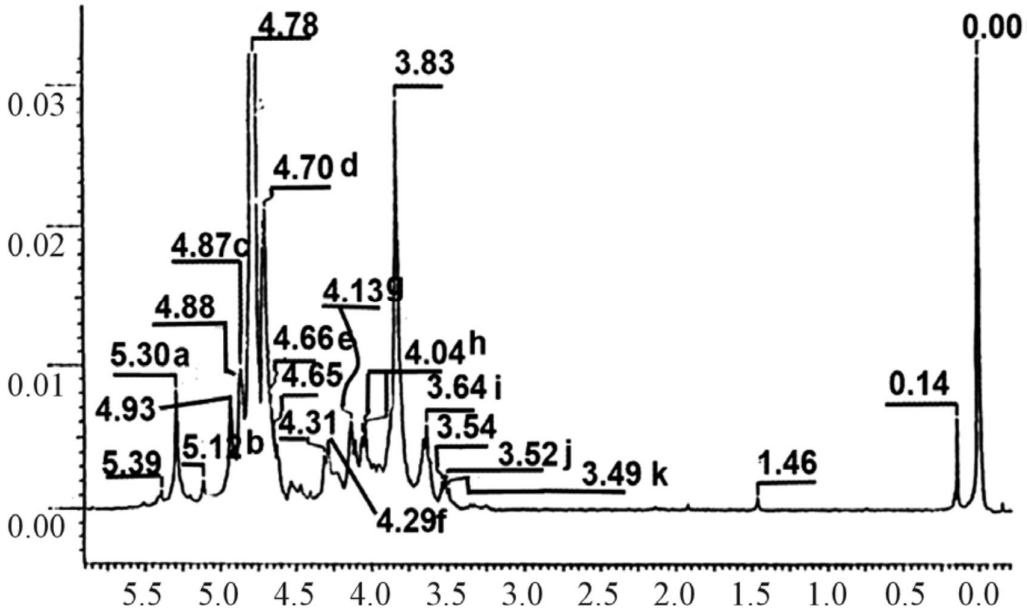

Figure 5. One dimensional 1H NMR spectrum of ι-carrageenan oligosaccharide F1. Signals: (a) α-Gal*p*-4S H-1; (b) AnGal*p*2S H-1; (c) α-Gal*p*-4S H-4; (d) β-Gal*p*-4S H-4; (e) β-Gal*p*-4S H-1; (f) AnGal*p*2S H-6b; (g) AnGal*p*2S H-2; (h) AnGal*p*2S H-6a; (i) β-Gal*p*-4S H-5; (j) β-Gal*p*-4S H-2.

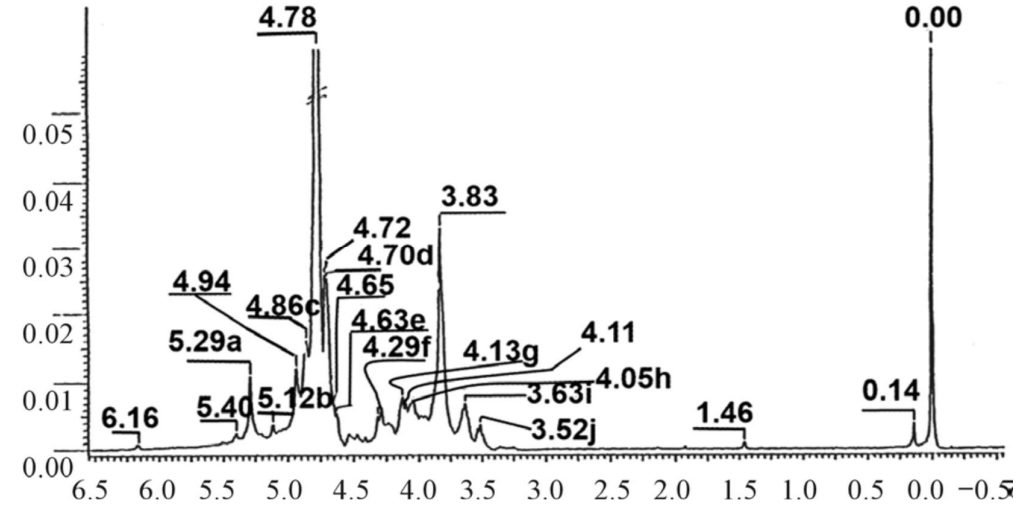

Figure 6. One dimensional 1H NMR spectrum of ι-carrageenan oligosaccharide F2. Signals: (a) α-Gal*p*-4S H-1; (b) AnGal*p*2S H-1; (c) α-Gal*p*-4S H-4; (d) β-Gal*p*-4S H-4; (e) β-Gal*p*-4S H-1; (f) AnGal*p*2S H-6b; (g) AnGal*p*2S H-2; (h) AnGal*p*2S H-6a; (i) β-Gal*p*-4S H-5; (j) β-Gal*p*-4S H-2.

Application of oligosaccharides in protein delivery system

The oligosaccharides were evaluated for their ability to form a complex with chitosan to encapsulate Bovine Serum Albumin (BSA) as the protein model. Chitosan is known to form stable complexes with carrageenans [28]. The maximum yield of complex formation was observed for the molar mixing ratio of 1:1, which suggests a stoichiometric anion-cation interaction [28]. The complexation of chitosan and carrageenan was due to the opposite charges between the positively charged amine groups (NH_3^+) in chitosan and the negatively charged sulfate groups (SO_3^-) in carrageenan. In this study, different charge ratios (+/−) of 0.5, 1, 3, 5, 7 were prepared to determine the encapsulation efficiency of the polyelectrolyte complex of ι-carrageenan oligosaccharide/chitosan. In previous study [25], chitosan and carrageenan complex were formed by electrostatic interaction without using a cross-linker. Here, a crosslinker which is trisodium citrate was used in the preparation of the complex. As shown in Figure 7, the complex of chitosan/ι-carrageenan oligosaccharide had the highest encapsulation efficiency of 67.0% at a charge ratio of 5 while chitosan with the cross-linker had the highest encapsulation efficiency of 67.14% at a charge ratio of 1. In previous study, the encapsulation efficiency of chitosan without the cross linker is only 10% while the unhydrolyzed ι-carrageenan is 24% [25]. With the use of a cross linker, the encapsulation efficiency increases. However, the complex of chitosan with unhydrolyzed ι-carrageenan has an encapsulation efficiency of 73.0% at charge ratio of 3 [25].

The encapsulation efficiency of BSA using the complex of chitosan and carrageenan (hydrolyzed and unhydrolyzed) is dependent on the charge ratios and its molecular weight. The polyion complex (chitosan/carrageenan) is more efficient than using just one polymer without the use of a crosslinking agent. The electrostatic interaction of the sulphate group of carrageenan and the amine group of chitosan makes a better entrapment of BSA than chitosan alone. Charge ratios (+/−) of 3 & 5 were the most efficient as compared to 1 & 7 ratios using unhydrolyzed carrageenan [25] while the ι-carrageenan oligosaccharide tends to favor 5 & 7 charge ratios (Figure 7). The entrapment of BSA with the complex is due to ionic interaction. The isoelectric point of BSA is 4.8 [12] and the pH used in this study for complex formation was pH 6.0 which is above the isoelectric point of BSA. At this condition, the BSA would be predominantly negatively charged thus would ionically interact with chitosan competing with the more anionic carrageenan.

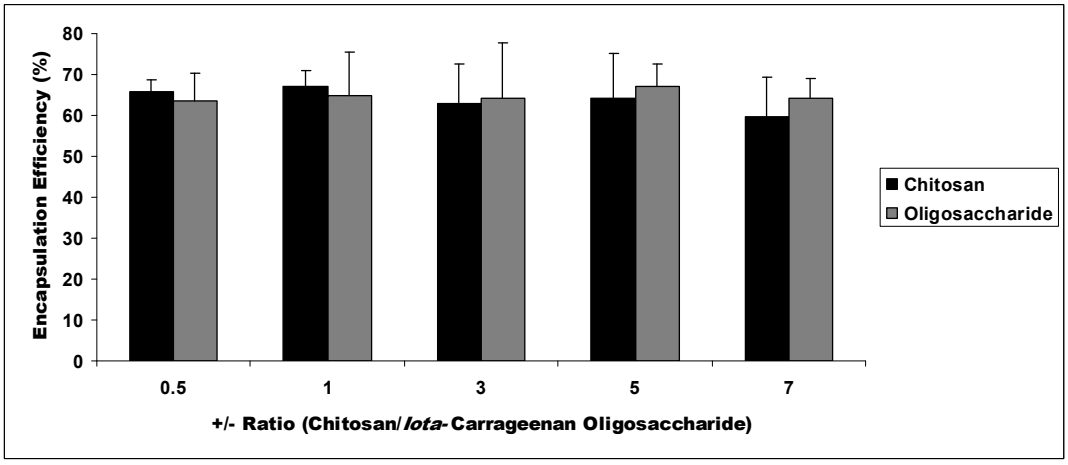

Figure 7. Efficiency of chitosan/ι-carrageenan oligosaccharide complex to encapsulate BSA (1 mg/mL) at different charge (+/−) ratios. The values represent the mean ± SD, n = 4. BSA indicates bovine serum albumin.

MATERIALS AND METHODS

Mild hydrolysis of ι-carrageenan

ι-Carrageenan (Benvisco SI-100, Lot No. M1400-1, average MW= 560 kDa) was obtained from Shemberg Biotech Corporation (Carmen, Cebu, Philippines, 6005). Hydrolysis was carried out by dissolving 10g of ι-Carrageenan with 500 mL of 0.1M hydrochloric acid and heated at 60 ^{o}C for 3 hours. The degradation was terminated by neutralization with 0.1 M NaOH, then filtered followed by desalting and freeze drying. Separation and purification of ι-carrageenan oligosaccharides were done following the method of Guangli Yu *et al* [11].

Characterization of oligosaccharides

The analysis of physicochemical properties of the oligosacchrides were based on the procedures found in USP XXII [24]. Fourier Transform Infrared Spectra (FT-IR) for oligosaccharides were measured using RX-1 Perkin Elmer FTIR spectrometer prepared as thin film on a potassium bromide pellet. The oligosaccharides were also analyzed by ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopy (JEOL Lambda LA 400 NMR spectrometer) and Electrospray Ionization Mass Spectrometry (ESIMS) 3000 plus Esquire mass selective detector (Bruker Daltonics) using a 1:1 mixture of water and acetonitrile.

Application of oligosaccharides in protein delivery system

The oligosaccharide solution and protein complex was prepared following the procedure of A.V. Briones and T. Sato [25] with some modification. Chitosan (45

KDa) solution was prepared at 1 mg/mL in Phosphate Buffer Solution (PBS) at pH 6.5. Carrageenan (1 mg/mL), Trisodium Citrate (1 mg/mL) and BSA (4 mg/mL) solutions were prepared in MilliQ water. BSA solution was added to chitosan solution, mixed thoroughly followed by addition of trisodium citrate solution and allowed to stabilize at room temperature for 15 minutes. Carrageenan solution was then slowly added to the mixture, mixed again followed by another 15 minutes stabilization at room temperature. Final concentration of BSA in the complex was 1 mg/mL. Thereafter the final mixture was incubated for 24 hours at 4^{o}C with shaking at 200 rpm [25]. After incubation of the complex, the solution was centrifuged at 10,000 rpm for 20 minutes at 4^{o}C. The resulting supernatant was then assayed for its protein content following the manufacture's instruction (Bio-Rad Laboratories, Japan). All experiments were performed in triplicate. To determine the efficacy of the complex of ι-carrageenan oligosaccharide and chitosan to entrap the protein (BSA) the encapsulation efficiency (E.E.) was measured according to the following equation:

$$E.E. = \frac{Co - Cr}{Co} \times 100$$

Where *Co* is the initial concentration of protein (amount of BSA used, 1 mg/mL); and Cr is the protein reading from the assay (amount of BSA recovered from the complex).

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CHAPTER 7

Formation of Polyethylenimine/Carrageenan Bi-layer as Monitored by Atomic Force Microscopy and Biomolecular Interaction Analysis

Chapter 7

ABSTRACT

Atomic force microscopy (AFM) was used to monitor the formation of polyethylenimine (PEI)/carrageenan bi-layer for potential use as anti-bacterial coating on biomaterial surfaces. All samples were prepared in phosphate buffer solution and applied to mica disk alternately. The micrographs showed the formation of bi-layer of polyethylenimine and carrageenan (κ, ι, λ) as observed in the change of height of the layer and surface morphology. The bimolecular binding of carrageenan with polyethylenimine was also investigated using a biosensor. The sensorgram showed that PEI interacted molecularly with carrageenan. Results were: 1,916.08 pg/nm^2 for *kappa* type; 1,844.1 pg/nm^2 for *iota* type and 6,074.24 pg/nm^2 for *lambda* type.

Chapter 7

INTRODUCTION

The use of layer-by-layer technique as anti-bacterial coating on biomaterial surfaces is becoming popular. This technique is based on alternating deposition of oppositely charged synthetic or natural polyelectrolytes to form a thin film layer-by-layer assembly [1-3]. One of the key advantages of layer-by-layer assembly is that the films often display close to identical properties after deposition of the first few layers, even if films are deposited on very different surfaces [4]. Previous studies done using this technique was the use of chitosan/κ-carrageenan multi-layers [5].

In this study, polyethylenimine [PEI] was used instead of chitosan. It was then layered with the three types of carrageenan. Studies showed that PEI prepared in nanoparticles were incorporated in dental provisional cements and found to had antibacterial effect against *Streptococcus mutans* [6]. Insoluble cross-linked quaternary ammonium polyethylenimine (PEI) nanoparticles incorporated at 1 or 2% w/w in a resin composite also has antimicrobial effect against *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Pseudomonas aeruginosa* and *Escherichia coli* [7]. Carrageenan is a family of natural, sulfated polysaccharides that are biocompatible, biodegradable, non toxic, cheap and gel-forming [8]. It has been shown that variation of carrageenan type for multilayers buildup allows formation of films differing in thickness, surface morphology, and elasticity [9], although the use of it for coatings is limited so far [9-10].

Atomic force microscopy [AFM] imaging was done to determine the morphology of bi-layers. AFM [11] was previously used in lipid fims research due to its unique advantages over other techniques [12]. These include: capacity to study

in real time and in aqueous environment, the surface nanostructure of the films and the conformational changes of individual molecule; ability to directly measure physical properties at high spatial resolution; possibility to perturb film structure and biophysical processes in a controlled way [12].

The bimolecular binding of carrageenan with polyethylenimine was also investigated using a biosensor based on surface plasmon resonance (SPR) technology. According to Tanious et al. [13], the surface plasmon resonance (SPR) biosensor method has emerged as a very flexible and powerful approach for detecting a wide diversity of biomolecular interactions. SPR monitors molecular interactions in real time and provides significant advantages over optical or calorimetric methods for systems with strong binding and low spectroscopic signals or reaction heats [13].

RESULTS AND DISCUSSION

Morphologies of κ, ι, λ type carrageenan are shown in Fig. 1. κ-carrageenan (Fig. 1A) forms an aggregated network in conformity with the results of McIntire and Brant, 1999 [14] who uses water to dilute the carrageenan samples and prepared by aerosol spray deposition. Polymer chains are separated with some overlap. The network is composed of a continuous bifurcated fibrous structure [15].

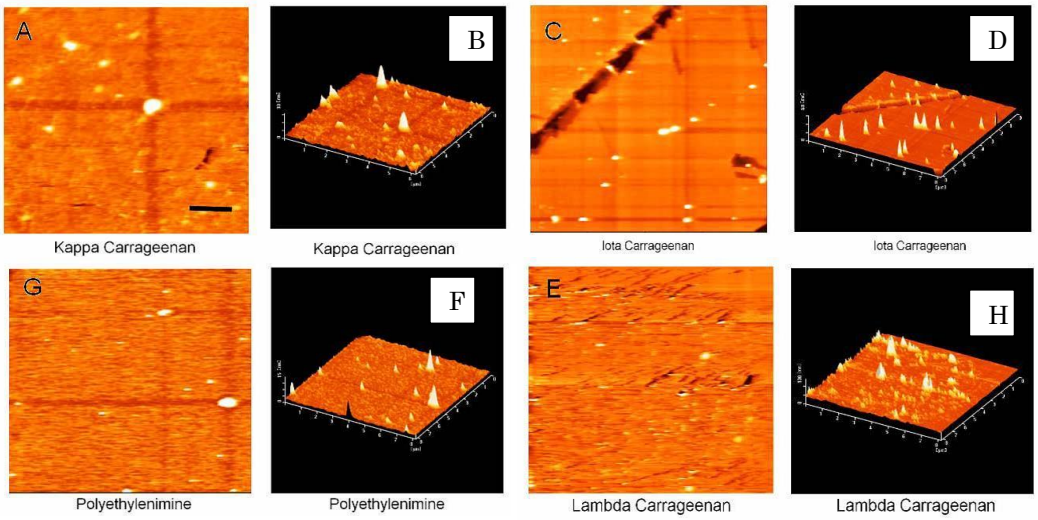

Figure 1. AFM micrographs of κ, ι, λ carrageenan and polyethylenimine. Scale bar: 2 μm.

ι-carrageenan forms a compact network as compared with κ-carrageenan. As seen in Figure 1C, there was no separation of polymer chains since the molecular structure of ι-carrageenan has “kinks” that increase its flexibility and reduce the space occupied by the molecule [15], this might explain the irregularities of the structure. It forms a homogeneous and continuous polymer network. In λ-carrageenan (Fig.1E) the structure exhibited a network or typical chains for the

polymeric molecule. It has the same morphology as described by Oliva *et al*, 2003 [16] in their studies. PEI film (Fig. 1G) shows a network that is similar to κ-carrageenan but its polymer chains are arranged uniformly.

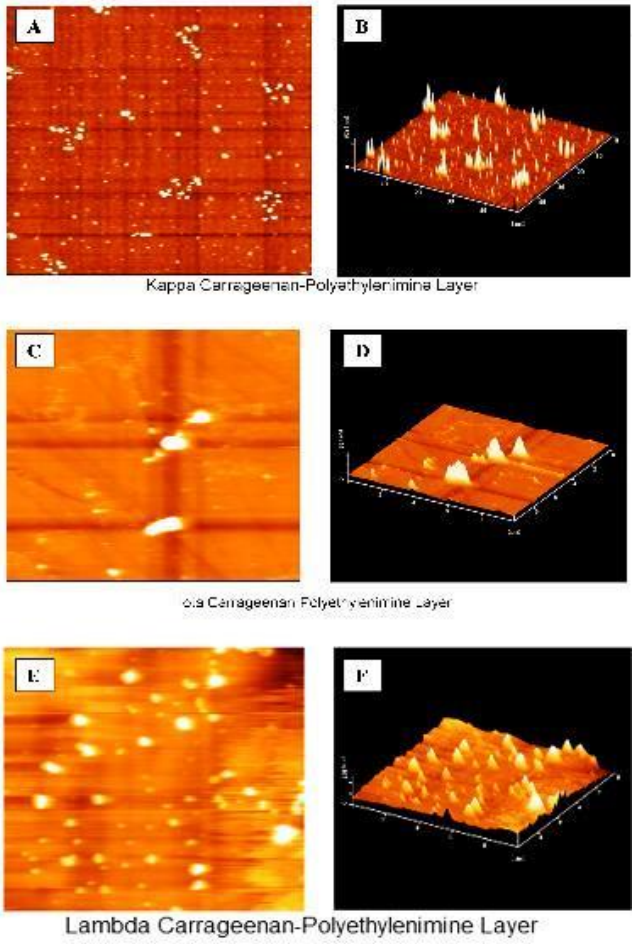

Figure 2. AFM micrographs of PEI/carrageenan layer.

Figure 2 shows the AFM micrographs of the PEI/carrageenan bi-layers. The images (Fig.2 A,C,E) shows the formation of layer -by- layer assembly as observed in the change of height. The change in height of the bi-layer film was observed in the images (Fig. 2B,D,F) as compared to the images of single polymer (Fig. 1 B, D, F,H). Table 1 shows the change in height of the single polymer layer and bi-layers.

The formation of bi-layer was further analyzed using a biosensor (BIACORE X). Figure 3 shows the sensorgram of the interaction between different types of carrageenan and PEI. The binding capacity of PEI with carrageenan is tabulated in Table 2. Results showed that PEI interacted molecularly with carrageenan.

Table 1. Height of single polymer and bi-layer of PEI and carrageenan

	Height (nm)
Kappa carrageenan	30
Iota carrageenan	60
Lambda carrageenan	100
Polyethylenimine (PEI)	15
Kappa carrageenan/PEI bi-layer	150
Iota carrageenan/PEI bi-layer	60
Lambda carrageenan/PEI bi-layer	200

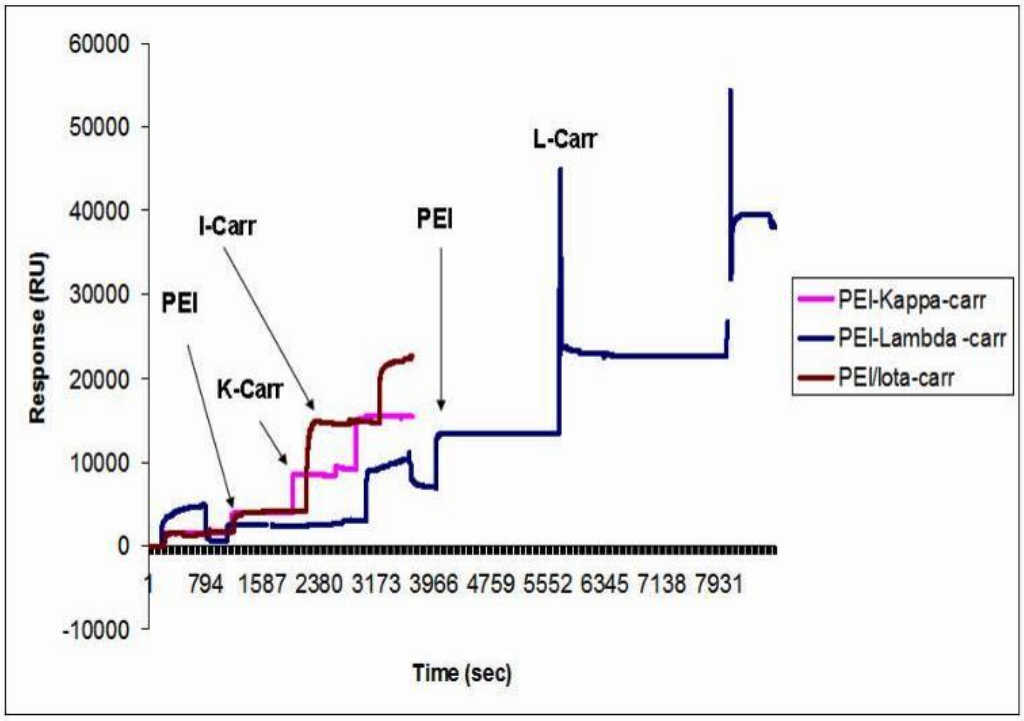

Figure 3. Sensorgram of the interaction of PEI and different types of carrageenan.

Table 2. Binding capacity of PEI with different types of carrageenan

	Binding capacity (pg/nm^2)
Kappa carrageenan	1,916.08
Iota carrageenan	1,844.1
Lambda carrageenan	6,074.24

MATERIALS AND METHODS

Materials

PEI (average MW=25KDA, branched with degree of polymerization of 580) was obtained from Aldrich Company (St Quentin Fallavier, France). κ-carrageenan (Bengel KK-100, Lot No. XO300-2), ι-carrageenan (Benvisco SI-100, Lot No.M1400-1) and λ-carrageenan (Benvisco SL-100, Lot No. S2703-2) was obtained from Shemberg Biotech Corporation (Carmen, Cebu, Philippines, 6005). Dulbecco's PBS (-) was obtained from Nissui Pharmaceutical Co. Ltd (Japan).

Sample preparation

κ, ι, λ carrageenan and PEI were dissolved in PBS (-) solution then mixed using a vortex machine, followed by ultrasonication and shaken at 200 rpm for 8 hours. Final concentration of each sample is: 1 mg/mL.

Preparation of sample for AFM analysis

The silica glass was dipped in carrageenan solution then allowed to dry at room temperature followed by washing with water to remove excess buffer solution and allowed to dry again. The dried coated silica glass was immersed in PEI solution, dried and washed with water followed by final drying at room temperature.

Atomic force microscopy (AFM) measurement

Imaging was done through a commercial SPA-300 system of Seiko Instruments, Inc. Japan. A Si_3N_4 tip on the cantilever with a length of 100μm and a depth of 400 nm (SN-AF01-A, Olympus Optical Co.) was used with tapping mode. Imaging was recorded in non-contact mode under ambient condition. Solutions of PEI and carrageenan on the mica disk were observed as control.

Biomolecular interaction analysis

Analysis of biomolecular binding of carrageenan and PEI was done using BIACORE X, Pharmacia Biosensor AB, Uppsala, Sweden equipped with sensor chip C1 BIACORE Lot no. 10020781. Samples of κ, ι, λ-carrageenan solution and PEI solution at 1 mg/mL concentration was injected at 120 μL with a flowrate of 10 μL/min.

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CHAPTER 8

Antibacterial activity of polyethylenimine/carrageenan multi-layer against pathogenic bacteria

Chapter 8

ABSTRACT

The multi-layer of polyethylenimine (PEI) and carrageenan (κ, ι, λ) formed by layer-by-layer assembly was investigated for its antibacterial activity against *Enterobacter cloaceae*, *Staphylococcus aureus* and *Enterococcus faecalis* 29505 for potential use as coating on biomaterial surface. All the multi-layers exhibited growth inhibition. PEI/ι-carrageenan multi-layer is effective in inhibiting the growth of the *E. cloaceae, S. aureus* and *E. faecalis* while PEI/λ-carrageenan is effective in inhibiting the growth of *E. cloaceae*. Results of the paper strip test for combined action of carrageenan and PEI showed synergism with regards to bacterial growth inhibition. The multi-layers have also contact-killing effect with the test organisms. The multi-layers were also characterized by atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS) and biomolecular interaction analysis.

Chapter 8

INTRODUCTION

Designing an antibacterial coating on biomaterial surfaces using layer-by-layer technique is becoming popular. This technique is based on alternating deposition of oppositely charged synthetic or natural polyelectrolytes to form a thin film layer-by-layer assembly which is a cheap method eliminating complicated multistage covalent grafting process that produce materials with controllable adhesive properties [1-3]. There are a lot of antibacterial coatings developed based on hydrophobicing agents, application of positive charge, polymer brush coatings [4-5], thermoresponsive polymers [6], drug eluting coatings, silver coatings, natural [7] or synthetic polymeric biocides [8-9]. However, clinical efficacies of these many coatings still have to be validated. Coatings should not negatively impact tissue integration of a prosthesis which would be the best guarantee for a long-term functioning of an implanted prosthesis [10].

Previous studies done using this technique was the use of chitosan/hyaluronan[3] and chitosan/κ-carrageenan multi-layers [11]. In this study, polyethylenimine (Figure 1) was used instead of chitosan then layered with the three types of carrageenan (Figure 1). There have been no studies reported yet on the use of PEI-carrageenan multi-layer for use as anti-bacterial coating without modifying the structure of PEI. Hence, the aim of this study is to evaluate the potential of PEI-carrageenan multi-layer as antibacterial coating.

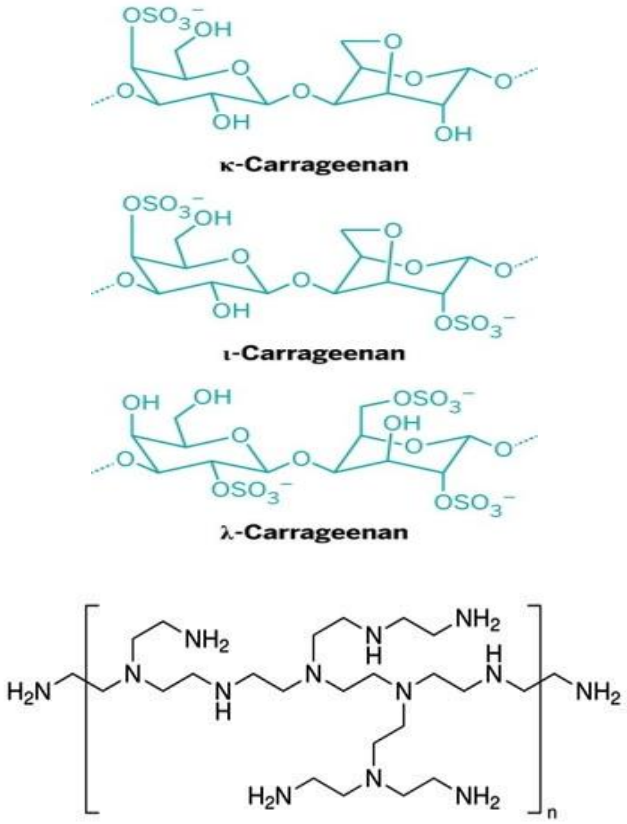

Figure 1. Chemical structure of polyethylenimine and carrageenan.

Polyethylenimine (PEI) is a weakly basic, aliphatic, nontoxic synthetic polymer which is polycationic owing to the presence of primary, secondary, and tertiary amino groups [12]. It has a strong permeabilizing effect but no bactericidal effect on gram-negative bacteria [13]. PEI prepared in nanoparticles were incorporated in dental provisional cements and found to had antibacterial effect against *Streptococcus mutans* [14]. Insoluble cross-linked quaternary ammonium polyethylenimine (PEI) nanoparticles incorporated at 1 or 2% w/w in a resin composite also has antimicrobial effect against *Staphylococcus aureus, Staphylococcus epidermidis,*

antimicrobial effect against *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Pseudomonas aeruginosa* and *Escherichia coli* [15].

Carrageenan is classified as a food additive [16] and shows potential use for a new drug delivery system providing more control over the release rate of drugs. Being a sulfated polysaccharide, carrageenan is biocompatible, biodegradable, nontoxic, cheap and gel forming [17], however, the use of carrageenan as antibacterial coating is limited [18-19].

In one study, oligosaccharides from κ- carrageenan has antibacterial activities against *E. coli; S. aureus; S. cere; P.citr* and *Mucor sp.*[20].

RESULTS AND DISCUSSION

Formation of multi-layers

The multi-layers were formed by electrostatic interaction between the cationic PEI and anionic carragenan using the layer-by-layer self-assembly technique [23-24]. The interaction of PEI and carrageenan was due to the opposite charges between the positively charged amine groups (NH_3^+) in PEI and the negatively charged sulfate groups (SO_3^-) in carrageenan. This method is very useful in many applications that require multilayer thin films. The process is relatively inexpensive with no complex reaction mechanisms. The process is simple just to dip a substrate into alternating positive and negative charge containing solutions to form uniform and stable layers.

The formation and build-up of multilayers were monitored using an atomic force microscope (AFM, SPA-300 system of Seiko Instruments, Inc. Japan). The results were previously published in International Proceedings of Chemical, Biological,

Environmental Engineering Journal [21]. The AFM micrographs of the PEI/carrageenan bi-layers showed the formation of layer - by- layer assembly [21]. The AFM micrographs also showed the change in height of the bi-layer film as compared to the images of single polymers (Carrageenan and PEI) [21]. The formation of bi-layer was further analyzed using a biosensor (BIACORE X). The results were also previously published in International Proceedings of Chemical, Biological, Environmental Engineering Journal [21]. The results showed that PEI interacted molecularly with carrageenan with PEI [21]. Binding capacity (pg/mm^2) for each type of carrageenan with PEI was also shown [21]. The multi-layer of PEI/λ -carrageenan had the highest binding capacity of 6,074. 24 pg/mm^2 followed by PEI/κ-carrageenan (1,916.08 pg/mm^2) and PEI/ι-carrageenan multi-layers (1,844.1 pg/mm^2) [21]. Based on the binding curves of all multi-layer [21], a single injection of PEI apparently reached binding equilibrium with the membrane. Then after injection with carrageenan the signal increased, reaching a higher plateau confirming binding of the two polymers as observed in the staircase- like binding curves in all multi-layers [21].

Surface composition of the multi-layer by XPS

The XPS survey spectra of the multilayer of carrageenan and polyethylenimine is shown in Figure 2. All multilayer showed similar spectra regardless of the mode of deposition. XPS is one of the most commonly used techniques of surface analysis [25]. Upon exposure of the sample to an X-ray beam, the binding energies of characteristically emitted photoelectrons are measured, providing information on the elements from which they originate, as well as their

chemical bonding [25]. As shown in Figure 3, all spectra did not show peaks at 285.0 eV which represent saturated hydrocarbons suggesting that no carbon contamination for all the surfaces. The surfaces of PEI/ι-carrageenan and PEI/λ-carrageenan have peaks in 286.8 eV and 286.6 eV (Figure 3) respectively due to C-N groups of PEI and the residual amide groups while PEI/κ-carrageenan tend to shift to the lower side and has a peak at 287.2 eV.

The peak observed at 287.5 for both PEI/ι-carrageenan and PEI/λ-carrageenan is due to the O-C-O groups of the saccharide unit. The O-C-O groups of PEI/κ-carrageenan is observed at 288.2 eV. Among the three surfaces, PEI/λ-carrageenan showed a peak at 288.4 eV due to oxidized saccharide species. PEI/κ-carrageenan (1,916.08 pg/mm^2) and PEI/ι-carrageenan

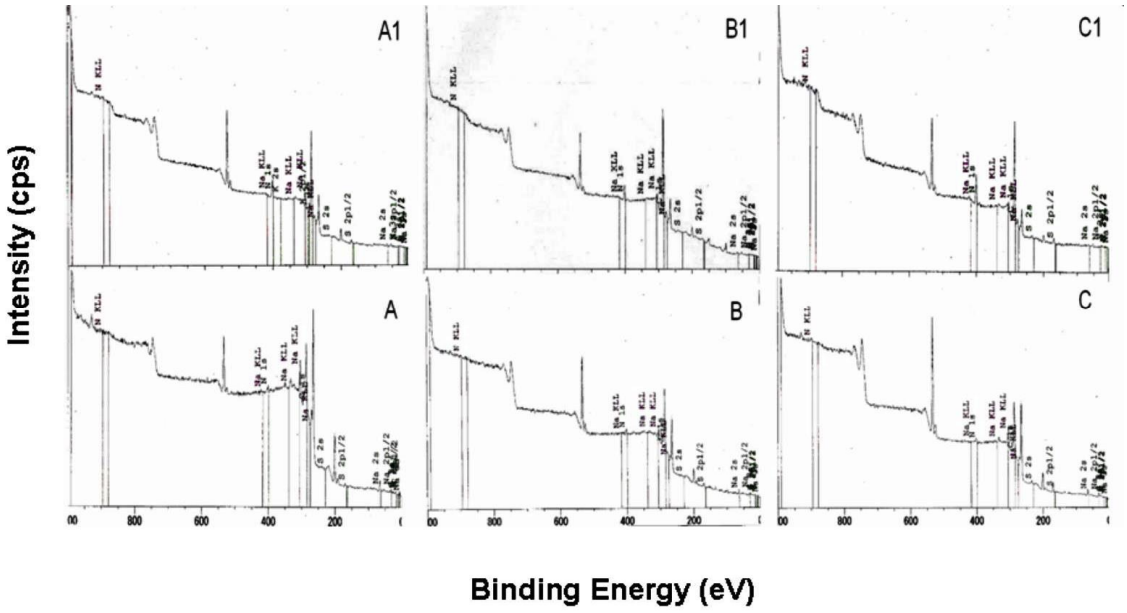

Figure 2. XPS spectra of multi-layers of PEI/Carrageenan. A. κ-carrageenan B. ι-carrageenan, C. λ-carrageenan, A1. PEI/κ-carrageenan, B1. PEI/ι-carrageenan, C1. PEI/λ-carrageenan.

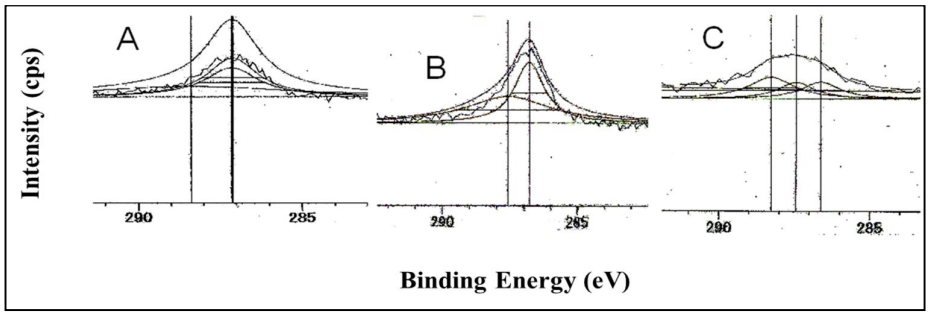

Figure 3. XPS carbon spectra of multi-layer. A. PEI/κ-carrageenan, B. PEI/ι-carrageenan, C. PEI/ PEI/λ-carrageenan

Bacterial growth inhibition

The results of bacterial growth inhibition is shown in Figures 4-6. All the multi-layers exhibited growth inhibition. The multi-layer of PEI/λ-carrageenan showed the highest growth inhibition of *E. cloaceae* of 84.6% followed by PEI/ι-carrageenan of 81.32% and PEI/κ-carrageenan of 69.12% (Figure 4). Against *S. aureus* the multi-layer of PEI/ι-carrageenan showed the highest inhibition growth of 66.84% followed by PEI/κ-carrageenan of 38.86% and PEI/λ-carrageenan of 38.35% (Figure 5). The multi-layers were also tried against *E. faecalis*. This organism is known to be involved in the clogging of biliary stents [1]. Again the multi-layer of PEI/ι-carrageenan showed the highest inhibition growth of 77.66% followed by PEI/κ-carrageenan of 62.17% and PEI/λ-carrageenan of 57.28% (Figure 6). In summary, PEI/*Iota* carrageenan is effective in inhibiting the growth of the *E. cloaceae, S. aureus* and *E. faecalis* while PEI/λ-carrageenan is effective in inhibiting the growth of *E. cloaceae*. The PEI and carrageenan alone were also investigated for its ability to inhibit bacterial growth. Shown in Figure 7 are the results of bacterial count after

24 hours incubation period with PEI and carrageenan. PEI has zero bacterial growth (100% growth inhibition) when exposed to *S. aureus* and *E. cloaceace* (Figure 7*)* but when exposed to *E. faecalis*, inhibition growth is only about 76.2% (Figure 7). Carrageenan showed bacterial growth inhibition in *S. aureus* with the highest inhibition growth of 25.0% by λ-carrageenan (Figure 7) while ι-carrageenan displayed the highest inhibition growth of 48.20% to *E. faecalis* and 4.1% inhibition growth to *E. cloaceae*. λ-carrageenan did not show any inhibition growth for both *E. cloaceae* and *E. faecalis* (Figure 7) instead it increased the growth of *E. faecalis* by 29.5% (Figure 7). κ-carrageenan and λ-carrageenan showed an increase of growth in *E. cloaceae* by 18.0% and 17.44% respectively (Figure 7). Although the results of bacterial growth inhibition obtained from carrageenan were not that quite high for *S.aureus*, it indeed showed that it has bacterial activity as previously reported [20].

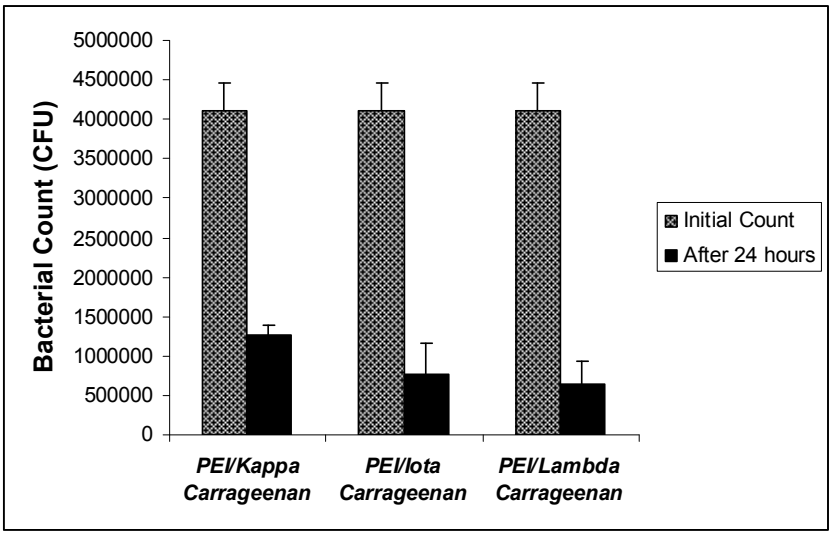

Figure 4. Bacterial growth of *Enterobacter cloaceae* after 24 hours exposure to multi-layers of PEI and carrageenan.

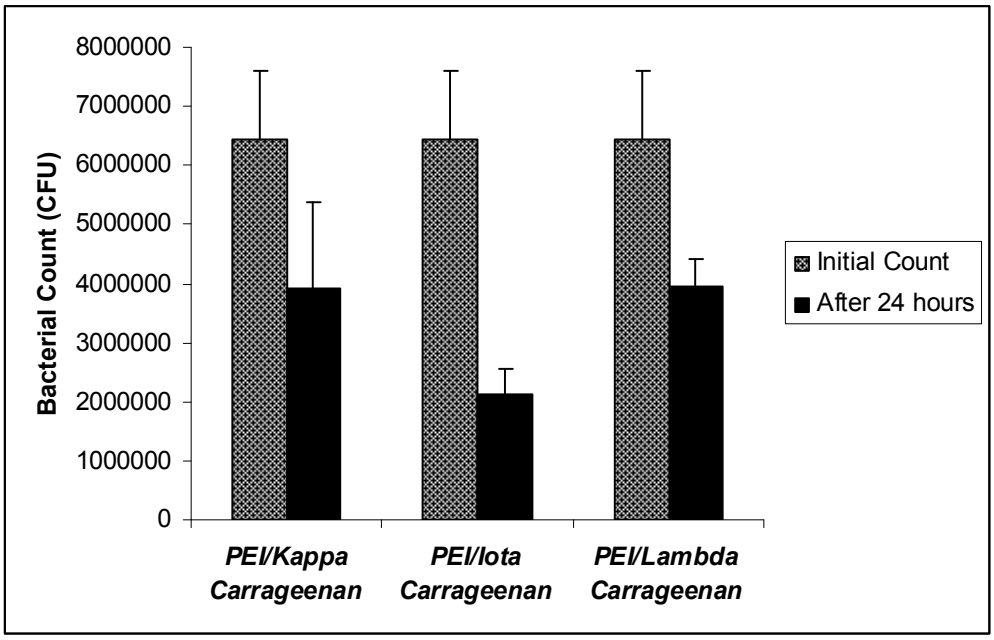

Figure 5. Bacterial growth of *Staphylococcus aureus* after 24 hours exposure to multi-layers of PEI and carrageenan.

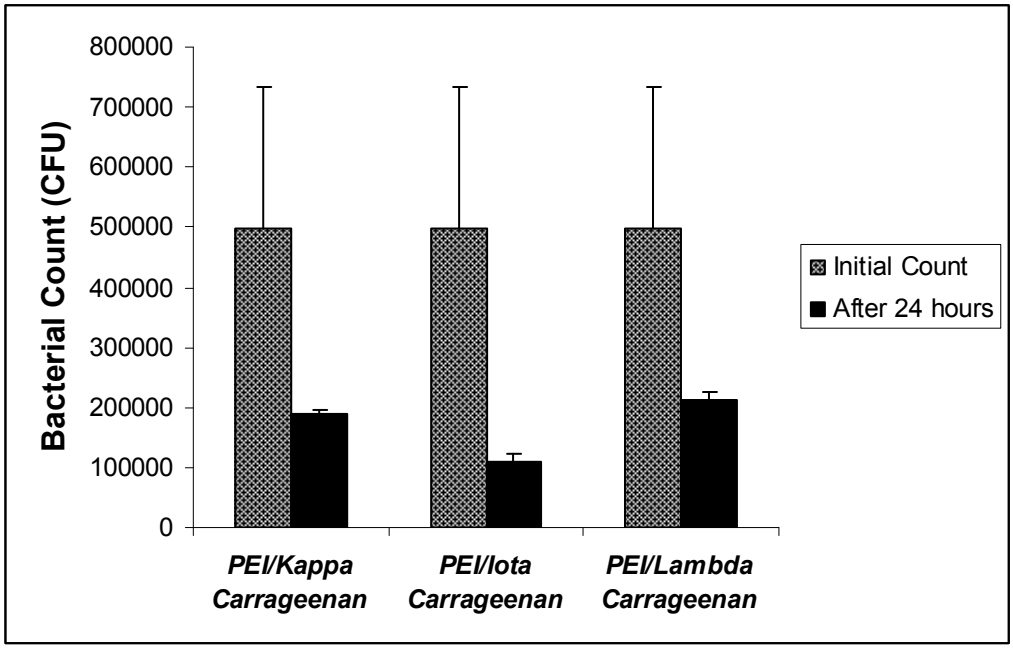

Figure 6. Bacterial growth of *Enterococcus faecalis* (29505) after 24 hours exposure to multi-layers of PEI and carrageenan.

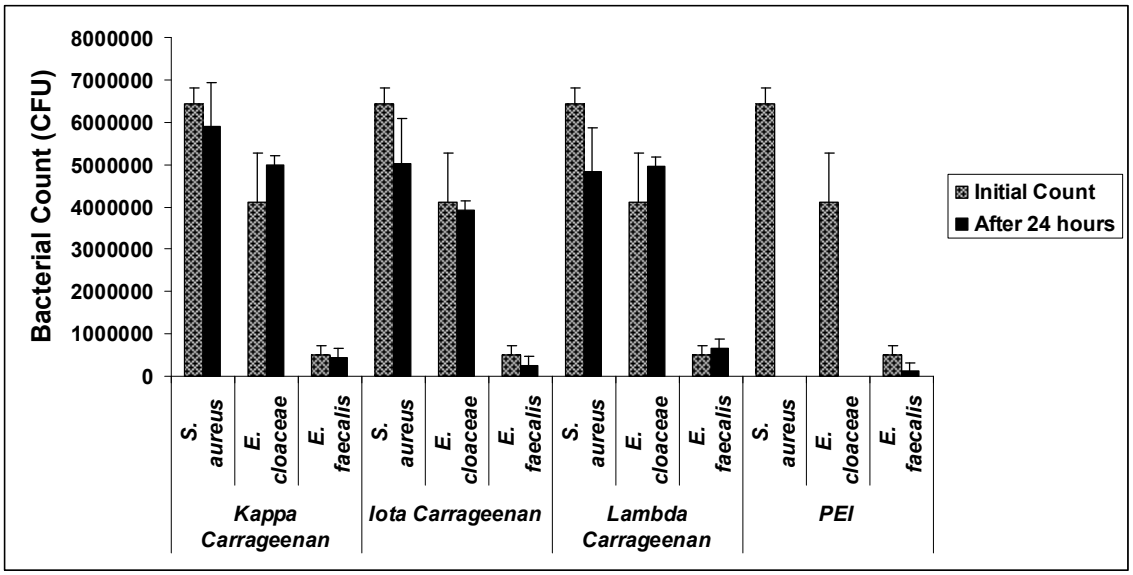

Figure 7. Bacterial growth of *Enterococcus faecalis* (29505) after 24 hours exposure to PEI and carrageenan.

Paper strip test for combined action

Figures 8-9 showed the paper strip test for combined action of double layer and multi-layers of carrageenan and PEI. The test was done to determine the antagonizing effect of PEI with carrageenan. During incubation PEI diffused together with carrageenan and the shape of inhibition area within the area of overlap indicates whether stimulation or antagonism has taken place. The results showed that PEI and carrageenan have synergistic effect with regards to antibacterial activity and does not antagonize nor create competitive reversal of bacterial growth inhibition of *S. aureus* and *E. cloaceae.* Synergism is displayed by a clear area devoid of growth in the angle between PEI (vertical) and carrageenan (horizontal) strips as observed in both Figures 8 -9. Lack of synergism is displayed by growth of organism in the zone of inhibition which is not observed in both Figures.

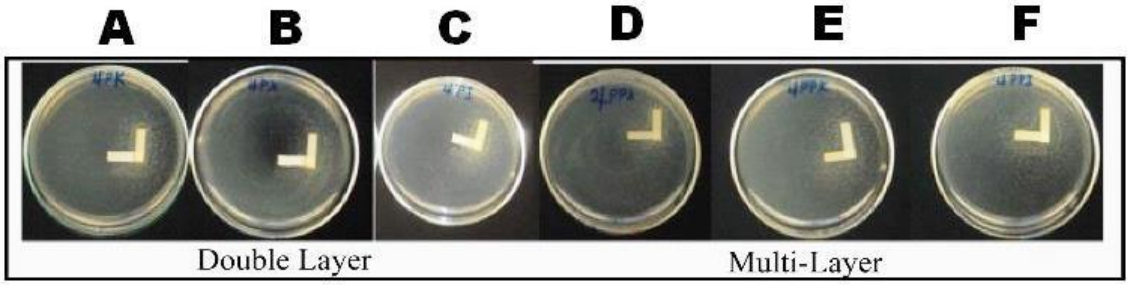

Figure 8. Paper strip test for combined action of the Double and Multi-layers of Carrageenan and Polyethylenimine in *S. aureus.* A & E. PEI/κ-carrageenan. B & D. PEI/ λ-carrageenan. C & F. PEI/ι-carrageenan. All experiments were done in triplicate.

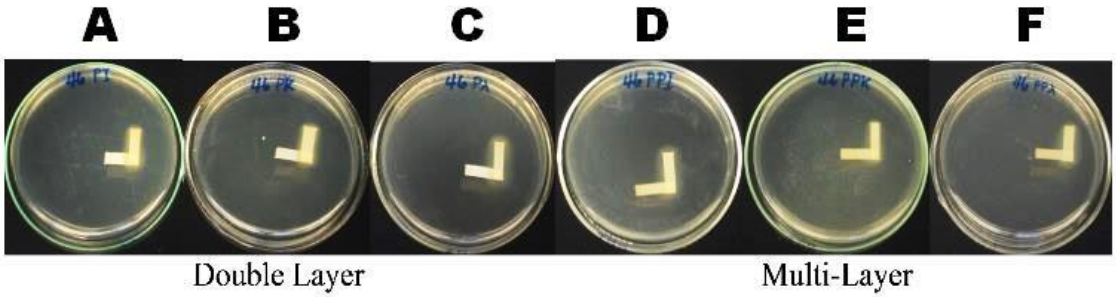

Figure 9. Paper strip test for combined action of the Double andMulti-layers of Carrageenan and Polyethylenimine in *E. cloaceae.* A & D. PEI/ι-carrageenan. B & E. PEI/κ-carrageenan. C & F. PEI/λ-carrageenan . All experiments were done in triplicate.

Zone of inhibition test

The result of zone of inhibition test is found in Figure 10. The multi-layers of PEI/Carrageenan were tested against *E. coli, E. cloaceae* and *S. aureus.* The zone

of inhibition were observed after 24 and 48 hours. As shown in Figure 10a, there was no growth observed for all the test strips after 24 hours of incubation. Extending the incubation to 48 hours of exposure, still no growth was observed and the zone of inhibition is more distinct than overnight incubation as observed in *S. aureus* (Figure 10b). The test clearly showed that the multi-layers of PEI/Carrageenan have contact-killing effect with the test organisms.

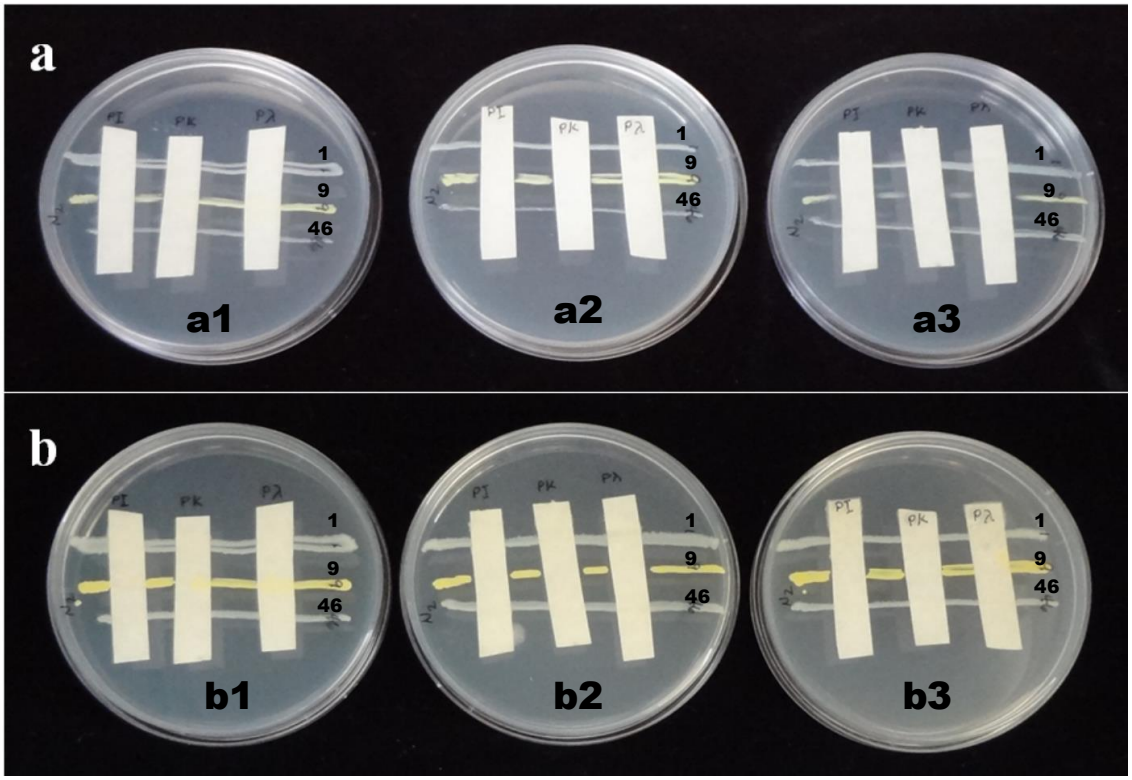

Figure 10. Zone inhibition test against *E. coli* (1*), E. cloaceae* (46) and *S. aureus* (9). a. 24 hours incubation. b. 48 hours incubation. All experiments were done in triplicate.

MATERIALS AND METHODS

Materials

PEI (average MW=25KDA, branched with degree of polymerization of 580) was obtained from Aldrich Company (St Quentin Fallavier, France). κ-carrageenan (Bengel KK-100, Lot No. XO300-2), ι-carrageenan (Benvisco SI-100, Lot No.M1400-1) and λ-carrageenan (Benvisco SL-100, Lot No. S2703-2) was obtained from Shemberg Biotech Corporation (Carmen, Cebu, Philippines, 6005). Dulbecco's PBS (-) was obtained from Nissui Pharmaceutical Co. Ltd (Japan).

Sample preparation

κ, ι, λ carrageenan and PEI were dissolved in PBS (-) solution then mixed using a vortex machine, followed by ultrasonication and shaken at 200 rpm for 8 hours. Final concentration of each sample is: 1 mg/mL.

Preparation of multilayers

Multilayers were prepared by successive immersion of a substratum to solutions of carrageenan and PEI for 10 minutes then allowed to dry at room temperature followed by washing with water to remove excess buffer solution and allowed to dry again. This was done alternately for each polymer. The process was repeated thrice each for every solution to make a final of 6 coatings with alternating layer of carrageenan and PEI solution.

Atomic force microscopy (AFM) measurement

This method was previously published in International Proceedings of Chemical, Biological, Environmental Engineering Journal [21]. Imaging was done through a commercial SPA-300 system of Seiko Instruments, Inc. Japan. A Si_3N_4 tip on the cantilever with a length of 100nm and a depth of 400 nm (SN-AF01-A, Olympus Optical Co.) was used with tapping mode. The imaging was recorded in non-contact mode under ambient condition. Solutions of PEI and carrageenan on the mica disk were observed as control.

Biomolecular interaction analysis

This method was previously published in International Proceedings of Chemical, Biological, Environmental Engineering Journal [21]. Analysis of biomolecular binding of carrageenan and PEI was done using BIACORE X, Pharmacia Biosensor AB, Uppsala, Sweden equipped with sensor chip C1 BIACORE Lot no. 10020781. Samples of κ, ι, λ-carrageenan solution and PEI solution at 1 mg/mL concentration was injected at 120 uL with a flowrate of 10 uL/min.

Surface composition of the multi-layer by X-ray photoelectron spectroscopy (XPS)

The surface composition of the multi-layer was measured using X-ray photoelectron spectroscopy (XPS), model JPS-9000MX (JEOL) using Mg K_α radiation (10 kV-10 mA). Peak positions were calibrated C_{1s} position at 284 eV.

Bacterial growth inhibition

Antibacterial activity was determined using the methodology of S.G. Hu et al [22], with some modification. The bacteria used in this study were: *E. cloaceace, S.*

aureaus and *E. faecalis.* Frozen preserved stock was thawed at room temperature, and then 0.1 mL was pipetted and streaked into a quadrant on a sheep-blood agar plate (Difco Laboratories, Detroit, MI) and cultured at 37°C overnight. Afterward, a single colony was scraped with a loop and swabbed onto a 15°-slant medium (10 mL of nutrient agar) and was incubated at 37 ^{o}C. After 18–24 h of culturing, 20 mL of PBS was added (72 mL of 0.2M Na_2HPO_4 was mixed with 28 mL of 0.2*M* NaH_2PO_4, and 0.5 g of NaCl and 2 g/L Tween 80 were added; this brought the total volume to 1000 mL). After mixing, 1 mL of the solution was moved into 9 mL of nutrient broth (concentration = 8 g/L) and mixed with a vortex mixer. The solution was then diluted with PBS to 1. 5 ± 0.3 x 10^5 cells/mL and was placed in flasks (three samples, with PEI/Carrageenan coated mica disk per sample for each group). After incubation at 37 ^{o}C for 0–18 h, 20 mL of PBS was added, and the mixture was stirred for 30 s. Consecutive dilute solutions were prepared by 1 mL of the previous solution being mixed with 9 mL of PBS. From this solution, 1 mL was transferred to a 50-mL centrifugal tube, mixed with 15 mL of nutrient agar (at 45 ^{o}C), poured into a 9-cm plate, allowed to cool, and incubated at 37 °C for 18–24 h. The number of surviving bacteria was then counted.

Paper strip test for combined action

The nutrient agar was prepared by dissolving 2.3 g in 100 mL of water and autoclaved for 15 minutes at 121 ^{o}C. After sterilization, 10 mL was plated and allowed to solidify. The sterilized agar medium have been seeded with the test organism in the same manner as in the agar-diffusion test. One of the strip was coated with PEI and placed vertically on the plate while the other strip coated with

carrageenan and multilayers of PEI/Carrageenan was placed horizontally.

Zone of inhibition

The nutrient agar was prepared as of the preparation of the paper strip test. Test organisms were streaked singly and continuously in the sterilized agar media parallel to each other. Test strips coated with PEI/Carrageenan were placed on top of the media crossing the streaked line.

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CHAPTER 9

SUMMARY AND CONCLUSION

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SUMMARY AND CONCLUSION

1. Evaluation of pDNA-polyethylenimine complex coated with *iota*-carrageenan for *in vitro* gene delivery

In this study, the use of carrageenan for the formation of an interpolyelectrolyte ternary complex through electrostatic interaction for *in vitro* gene delivery is viable. Carrageenan attached to the cationic surface of the pDNA/PEI complex. It increased the stability of the pDNA/PEI complex, decreased its cytotoxic effects and protected the DNA from DNase degradation and other glycosaminoglycans without losing its efficiency for gene expression in CHO-K1, HUH-7 and COS-7 cells. The use of carrageenan as a protective coating for pDNA/PEI complex in gene delivery is promising.

2. Encapsulation of Glucose Oxidase (GOD) in polyelectrolyte complexes of chitosan–carrageenan

This study has demonstrated that chitosan/carrageenan complexes at charge ratios of 3 and 5 can allow entrap GOD and control its release. Electrostatic interaction is the main mechanism involved in the incorporation of the protein into the complex. The controlled release of GOD from the complex is due to the swelling mechanism of the chitosan/carrageenan complex. The chitosan/carrageenan complex can be a promising agent for protein delivery. The ease of preparation and the ability to protect the protein integrity make chitosan/carrageenan complexes a promising drug delivery system for oral administration of peptides and proteins.

3. Ability of chitosan/carrageenan complex to encapsulate bovine serum albumin (BSA) for potential use in protein delivery

This study has demonstrated that chitosan/carrageenan complex at charge ratios of 3 & 5 can allow entrapment of BSA and control its release. Electrostatic interaction is the main mechanism involved in the incorporation of protein and its release from the complex. It has found importance in the application of chitosan/carrageenan complex for protein delivery. The ease of preparation and the ability of protecting protein integrity, make chitosan/carrageenan complex a promising drug delivery system for oral administration of peptides and proteins.

4. Structural studies on ι-carrageenan derived oligosaccharides and its application

The study was able to isolate and characterize two oligo-saccharides from *iota* carrageenan using mild acid hy-drolysis. The oligosaccharides were of be sulfated tetra-saccharide of the structure: β-D-galactopyranose(Gal*p*) 4S-(1→4)-α-D-AnGal*p*2S-(1→3)-β-D-galactopyranose (Gal*p*)4S-(1→4)-)-α-D-AnGal*p*2S-(1→3). The oligo-saccharides have the ability to form a complex with chi-tosan to encapsulate Bovine Serum Albumin (BSA) and have the potential to be used in protein delivery system.

5. Formation of polyethylenimine/carrageenan bi-layer as monitored by atomic force microscopy and biomolecular interaction analysis

The study showed that through AFM imaging and biosensor analysis, bi-layer formation of PEI and carrageenan can be monitored and evaluated.

6. Antibacterial activity of polyethylenimine/carrageenan multi-layer against pathogenic bacteria

The study showed that multi-layers of carrageenan/polyethyleimine were formed using the layer-by-layer assembly absorption technique. The formation was monitored by atomic force microscopy (AFM) and biomolecular interaction analysis. PEI interacted molecularly with carrageenan to form the multi-layer. The multi-layer showed antibacterial activity against *Enterobacter cloaceae*, *Staphylococcus aureus* and *Enterococcus faecalis.* PEI/*Iota*-Carrageenan is effective in inhibiting the growth of *S. aureus and E. faecalis* while PEI/*Lambda*-Carrageenan is most effective in inhibiting the growth of *E. cloaceae*. PEI/*Kappa*-Carrageenan is effective in inhibiting the growth of *E. faecalis.* All multi-layers have contact-killing effect to the test organisms. Synergism was also observed between PEI and carrageeenan for its combined action on antibacterial growth.

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