2014

# Preparation and characterization of insulin loaded calcium alginate nanoparticles

Kush Patel
*University of Toledo*

A Thesis

entitled

# Preparation and Characterization of Insulin Loaded Calcium Alginate Nanoparticles

by

Kush Patel

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in

Pharmaceutical Sciences with Industrial Pharmacy Option

_____

Jerry Nesamony, PhD., Committee Chair

_____

Sai Hanuman Sagar Boddu, PhD., Committee Member

_____

Yousef Sari, PhD., Committee Member

_____

Patricia R. Komuniecki, PhD, Dean
College of Graduate Studies

The University of Toledo

August 2014

An Abstract of

Preparation and Characterization of Insulin Loaded Calcium Alginate Nanoparticles

by

Kush Patel

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the
Master of Science Degree in
Pharmaceutical Sciences with Industrial Pharmacy Option

The University of Toledo

August 2014

The purpose of this research work was to develop insulin loaded calcium alginate nanoparticles (CANp) for oral delivery of insulin. The preparation of Insulin-loaded calcium alginate nanoparticles was performed by a novel and efficient method, interfacial cross-linking in nanoemulsions (IFaCLiNE) developed in our research lab. The prepared nanoparticles were evaluated by particle characterization and surface charge measurements. Subsequently, human recombinant, insulin was loaded into CANp. Differential scanning calorimetry (DSC) was used to evaluate the thermal properties. The quantitative determination of insulin in CANp was done via an ELISA assay and *in vitro* drug release studies. A reversed phase C18 HPLC column with fixed compositions of 0.2 M sodium sulfate buffer and acetonitrile as mobile phases was used in the HPLC analysis of insulin. The insulin peak retention time was observed to be 7.2 minutes. To further identify the stability of insulin in CaNP the qualitative determination of insulin was made through LC-MS tandem mass spectrometry. The determination of precursor ions was

achieved through mass spectroscopy (MS) and the insulin peak detection using liquid chromatography.

# Acknowledgements

I would like to express the deepest appreciation to my committee chair Dr. Jerry Nesamony, for providing me a chance to be a part of his research group. He continually and convincingly conveyed a spirit of adventure in regard to research and scholarship, and an excitement in regard to teaching. Without his guidance and persistent help this thesis would not have been possible.

I would also like to thank my committee members, Dr. Sai Boddu and Dr. Youssef Sari for their support and guidance. I thank Dr. Kenneth Alexander for giving me valuable advices throughout my undergraduate and graduate coursework. I also thank Dr. Caren Steinmiller for agreeing to be graduate faculty representative. I thank Dr Joseph Lawrence for helping me with TEM and SEM. I thank Dr. Lidia Rodriguez for helping me with LC-MS studies. I also thank Dr Zahoor Shah for his help with optical plate reader for ELISA study. In addition, I thank Dr. Wayne Hoss, for providing me financial support throughout my course. I thank my classmates and friends for their support. I would also like to thank my labmates for their suggestions during my research project.

# Table of Contents

# List of Tables

# List of Figures

# Chapter 1

# Literature review

## 1.1 Introduction

The recombinant DNA technology has created a profound and rapid growth in the drug delivery therapies involving protein and peptide drugs. With new innovative technologies being adapted in protein chemistry, several challenges have to be solved in the context of patient compliance, product stability, and viable dosage forms to ensure commercialization of such molecules(George & Abraham, 2006). Currently over 30 different recombinant drugs have been approved by FDA (H.J.C Ericksson, 2002). An unanswered problem with regard to protein and peptide drugs is their limited shelf life (Manning, 1989). The desirable shelf life period for any drug is in between 1.5 to 2 years regardless of storage temperature (Carpenter, 1997). Currently commercially available lyophilized protein drugs have to be stored under refrigerated conditions in order to maintain safety and efficacy of the product. Another important challenge is the inability

to formulate protein and peptide drugs into solid state (Carpenter, 1997; Hageman, 1992; M. J. D. Pikal, K.M ; Roy, M.L; Riggin, R.M, 1991). Partly this is due to instability of protein drugs in classical solid dosage forms and hence the products are administered as an infusion through intravenous route than the oral route (W.Wang, 1999). Oral delivery is not possible since common types of oral dosage forms are unable to protect the drug against harsh acidic environment in stomach and proteolytic breakdown in GI tract (L. Shargel, 1999; Liu Xing, 2003).

Protein and polypeptide molecules have demonstrated potential biological activity when tested against numerous untreatable diseases such as diabetes, hemophilia, and cancer. In order to be incorporated into human body, a protein drug has to remain stable and should be able to produce its therapeutic effect at the target receptor. The protein drugs which are bioavailable tend to show high specificity and activity but demonstrate relatively low plasma concentrations.

The administration of a protein drug through the oral route generally leads to degradation by chemical reactions such as hydrolysis or proteolysis. Moreover proteins are metabolized by enzymes secreted into the GIT. One approach that can successfully deliver proteins orally is by protecting them from the acidic gastric environment (low pH) in the stomach. For example human insulin undergoes chemical degradation through the β-elimination reaction in the stomach. In order to circumvent the acidic stomach environment several pH sensitive hydro gels have been used to coat or entrap the protein drug facilitating its release into the small intestine which has alkaline pH. These bio-degradable polymers are available naturally or through chemical synthesis.

Solid dosage forms are considered to be the most preferable route for the safe storage and transportation. Currently commercially marketed protein drugs have to be maintained at recommended storage conditions throughout transportation until immediately before use. The instability of protein and peptides are generally classified into chemical and physical instabilities. The most common degradation reactions involve deamidation and aggregation reactions (Manning, 1989). Chemical instability is the process in which the covalent modification of protein occurs via a cleavage or bond formation. Common mechanisms of chemical degradation of proteins include Racemization reaction, deamidation and peptide bond cleavage, and β-elimination reaction. The physical instability is a change in the secondary (2°), tertiary (3°) and quaternary (4°) structure of protein and peptides a occurs during precipitation, denaturation, and aggregation (Costantino, 1994b).

The oral drug delivery is considered to be most efficient and painless route of administration (Lee, 2002; Russell-Jones, 1998; U.B. Kompella, 2001; W.Wang, 1999). Efforts are being made for the delivery of significantly higher molecular weights of protein drugs such as insulin. The overall bioavailability of proteins via the oral route is very low. Additionally protein moieties are susceptible to degradation by hydrolysis and chemical modification of acidic pH present in the stomach (B. Steffansen, 2004; Fasano, 1998; Fix, 1996; J.H. Hamman, 2005; J.P. Bai, 1995; Lehr, 1994; S.A. Galindo-Rodriguez, 2005; Sanders, 1990).

Parenteral delivery route avoid the biological barriers that prevent the entry of proteins through biological membranes. The local delivery of proteins has gained interest recently

due to the site-specific activity of the drug particularly in the mucosal tissue of gut, sinus, and lungs.

# Chapter 2

# Stability of proteins and peptides in calcium alginate nanoparticles: a review

Kush Patel[1] and Jerry Nesamony[1,*]

[1] Division of Industrial Pharmacy, Department of Pharmacy Practice, College of Pharmacy and Pharmaceutical Sciences, University of Toledo, Toledo, Ohio, USA

[*]**Corresponding author**:
Department of Pharmacy Practice
College of Pharmacy and Pharmaceutical Sciences
University of Toledo
3000 Arlington Avenue, MS1013
Toledo, OH 43614
419 383 1938 (Tel)
419 383 1950 (Fax)
jerry.nesamony@utoledo.edu

**Keywords:** alginates, physical factors, chemical factors, stability

## 2.1 Abstract

The stability of proteins and peptides is a very important consideration in the formulation development process involving such molecules. These molecules tend to undergo physical or chemical degradation under normal processing conditions. The effect of biological environment on stability and disposition of such molecules may also significantly reduce the bioavailability of such drugs. The purpose of the current review is to provide a basic understanding on the factors and mechanisms affecting stability of proteins and peptides in calcium alginate nanoparticles. Some of the common factors affecting the stability include changes in pH, temperature, humidity, aggregation, folding, hydrolysis etc.

## 2.2 Physical Instability of Proteins

### 2.2.1 Conformational Stability: Denaturation and Folding of proteins

The 3D folded structure of a protein is termed as the conformation of protein. Denatured proteins are referred to as proteins that are unfolded and inactive. Conformational stability refers to various forces that help to maintain the protein folded in the right way. There are four levels of forces related to protein structure. The primary structure of the protein refers to the actual sequence of amino acid in that protein and is determined by the peptide bonds. Secondary structures are referred to as backbone interactions held together by hydrogen bonds. Tertiary structure is the overall 3D structure of a single protein molecule with distant interactions between single proteins and is stabilized by van der waals interactions, hydrophobic bonding, disulfide bridges,

and hydrogen bonds. Quaternary structures describe different interactions between individual protein subunits. All the subunits are stabilized by the same bonds used to determine tertiary structure. This leads to the active conformation of protein.

### 2.2.2 Aggregation

Protein molecules undergo self- aggregation by physical or chemical forces to form dimers, trimmers, or oligomers. Self-association of the protein structure is a primary problem observed during its formulation development. Significant loss in biological activity occurs as a result of aggregation. However, many proteins show their biological effect as dimers or trimers and may lose some biological activity upon dissociation. As a result of aggregation, the effective molecular weight of the protein increases causing it to be more immunogenic in nature (B.S Kendrick, 1998; S. Krishnan, 2002). For example insulin undergoing physical instability due to formation of insoluble aggregates causes blockage in tubing and membranes of an infusion set.

The mechanism of aggregation involves a multi-step process. Initially, unfolding of protein takes place causing exposure of hydrophobic residues to the aqueous solvent which thereby undergoes association leading to protein aggregation. In order to prevent the colloidal stability, protein should be stabilized against attractive intermolecular forces by adjustment of pH, ionic strength, and buffer. The kinetics of protein aggregation requires energy transfer such as temperature increase, pH change, or salt concentration. Increase in entropy results in the aggregation process resulting from the release of water molecules from the protein. The formation of inclusion bodies within two protein moieties predominantly causes aggregation of two polypeptide chains with diameter as

large as 1 micron. The folding pathway mechanism of human insulin in monomer inducing solvent has been identified as due to the presence of equilibrium intermediates. Due to the effect of such intermediates on the stability of unfolded regions, insulin structure is prone to chemical degradation (Arkawa, 2009).

The process of aggregation can occur both in liquid and solid physical states of a protein. The commercially available protein drugs are composed of freeze-dried powders. These lyophilized powders tend to inhibit the physical and chemical instability processes of a protein drug.

### 2.2.3 Adsorption

Adsorption is the phenomena of attaching the hydrophobic part of the protein to a surface. The primary sequence of the polypeptide chain determines the process of adsorption. The factors affecting the rate of adsorption are size, shape, and structure. Size is considered as the important factor influencing the adsorption of proteins on to a surface. The surface activity of a protein is dependent on the primary sequence of its polypeptide chain. The unfolding of protein plays an important role in the surface activity of the proteins (Rechendorff, 2011). The disulfide bridges connecting polypeptide chains of two protein moieties show less surface activity due to less folding. The charge interactions between the protein and surface will be dependent on pH and ionic strength of the media. Protein adsorption occurs with most neutral molecules or slightly charged surfaces near its isoelectric point. The mixture of two protein moieties would have efficient adsorption as the surface charge layer becomes abundant with both negative and positive charges (Andrade, 1985). The adsorption mechanism consists of a rapid initial

phase followed by lag phase which is then suppressed by the steady state value. The loss from adsorption is very less in dilute solutions when compared to concentrated solutions (>5 IU/ml).

## 2.3 Chemical stability of Proteins

The most prominent chemical degradation pathways of peptide and protein drugs include deamidation, hydrolysis, oxidation, disulfide exchange, β-elimination, and racemization.

### 2.3.1 Deamidation reaction

Deamidation reactions are the common hydrolytic reactions that lead to degradation of peptides and proteins. The side chains of amino acids such as glutamine (Gln) and asparagine (Asn) undergo deamidation reactions involving non-enzymatic covalent modifications. The half-life and protein bioactivity of the molecule is significantly affected by deamidation reactions. The rate of elimination of deaminated protein is usually faster from the body as the primary sequence of the unfolded protein has been deaminated resulting in the failure of transforming into refolded state. These deaminated proteins are susceptible to irreversible aggregation. The clarification of the deamidation reactions in both *in vivo* and *in vitro* systems has been investigated. The deamidation reaction rate is mainly dependent upon primary, secondary and tertiary structures in addition to factors such as pH, temperature, ionic strength, and special intermolecular interactions (Patel, 1990). Usually the pH of 6 is considered as maximum pH for stability of most deamidation reactions. The shelf life of the protein or peptide

drug can be limited to less than a year if the formulation contains hydrolytically labile Asp-Pro bond. For example insulin contains 6 residues that are prone towards deamidation reaction. They are Gln, Gln, Asn, Asn, Asn, and Gln. The three Asn residues are considered as most labile ones which are present on C-terminal residue A 21. Hence, insulin undergoes quick hydrolytic degradation in acid solutions usually at very low pH (Pikal, 1997; Strickley, 1996). The formation of monodesamino-(A21)- insulin takes place due to extensive deamidation reactions occurring in the acidic medium. In neutral pH conditions, deamidation reactions take place at residue Asn B3 (Pikal, 1997).

### 2.3.2 Hydrolysis

In proteins, the peptide bonds of Asp-Pro are susceptible to hydrolysis breakdown in the formulation. The disruption in the primary structure of the adjacent peptide bond residues influences the hydrolytic reactions. The hydrolytic degradation of the Asp-Pro peptide bond can be attributed to the primary or tertiary folded structures of the Asp-Pro bonds (Carl W. Niekamp, 1969). The cleavage of peptide bond ThrA8-SerA9 occurs in crystalline suspensions of insulin.

### 2.3.3 Oxidation reaction

Oxidative reactions can occur in lyophilized and solution dosage formulations of protein drugs. Several amino acids that undergo oxidation include histidine, tyrosine, cystine, and methionine (Dubost, 1996). There is a partial or little loss in biological activity of a protein undergoing oxidative degradation. It has been found that oxidation generally decreases as the protein formulations are freeze dried or lyophilized (Fransson.

J; Florin-Robertsson.E ; Axelsson, 1996). However, it does not hold true in all the cases. This is because the formation of exclusion of protein from ice matrix increases the cystine concentration residue which will increase the oxidation process. The use of antioxidants such as phenolic and hydroxyanisole compounds can reduce the oxidation process.

### 2.3.4 β-Elimination and disulfide exchange

The cleavage of disulfide bonds by β-elimination reaction from cystine residues results in the formation of thiols (Costantino, 1994). The catalytic disulfide exchange occurs from the free thiols undergoing β-elimination reaction. Generally unpaired cysteine residues are responsible for the disulfide exchange. The formation of new disulfide bridges occurs through the formation of cysteine residues at different sites (Jason Kerr 2013).

### 2.3.5 Racemization

Racemization reactions lead to loss of biological activity with the formation of D-enantiomers. The D-enantiomers are more resistant towards the proteolytic enzymes thereby improving the stability of the resulting peptide (Clarket, 1967).

## 2.4 Factors affecting protein stability

The folding chemistry of proteins plays an important role in the globular confirmation which is essential for its biological activity. Only about 5-20 kcal/mol of the free energy separates the stable folded form from the unfolded confirmations under the physiological conditions (C.N. Pace, 1996; Creightons(ed.), 1989; Dill., 1990; Jaenicke, 1991; R.

Jaenicke. In R. Huber, 1988). The equilibrium between large stabilizing and destabilizing forces helps to maintain the net conformational stability of the protein (Israelachvili, 1992). The molecular hydrophobic interactions, hydrogen bonding, and van der waals interactions lead to free energy of folding in protein structure. Protein conformational entropy drives an opposing force towards protein folding process. Unfolding of protein results in the local and nonlocal entropy processes. Small changes in the physical parameters such as temperature change and pH change can induce protein unfolding and can further destabilize the structure and configuration of the proteins (Dill., 1990).

### 2.4.1 Temperature

The free energy of unfolding ($\Delta G_{unf}$) shows a negative value when accounting for both higher and lower temperature values that results in unfolding of the protein (G. Graziano, 1997; N.T. Southall, 2002; P.L. Privalov 1988; Privalov, 1990). Physical degradation of proteins results when proteins are subjected to high temperatures. In such case proteins are prone towards irreversible denaturation because of aggregation (Pace, 2001). Protein aggregation is observed during heating below the equilibrium melting temperature of the protein (A.C. Dong, 1995; J.F. Carpenter, 1999). The kinetic energy of the reaction is affected by the change in temperature because of the increase in the rate constants of the activated reactions. This results in the increase in the inter-molecular collision energy of molecules of reactants leading to high activation energies. Increase in temperature also induces diffusion controlled reactions and thereby increasing the rate of diffusion (Atkins, 1994).

### 2.4.2 pH changes

pH change is an another important factor that produces physical instability of protein conformational structure. The rate of aggregation is strongly affected by the change in pH. Studies have shown that protein structures tend to be fairly stable at narrow pH ranges. However, outside the narrow range, they might aggregate together to form clusters. Examples include urokinase (M. Vrkljan, 1994), relaxin (T.H. Nguyen, 1996), and insulin (L. Nielsen, 2001). The presence of positive or negative charges on the amino acids of protein molecules results in electrostatic interactions between charged particles present in solution at either acidic or basic pH (Dill., 1990). An excessive charge density is observed between the charges present on the surface of the proteins and the charged particles of the acidic or basic media. This results in the destabilization of the folded protein confirmation. In such case, pH-induced unfolding results in a state of lower electrostatic free energy (Dill., 1990). When the iso-electric point (pI) of a protein is closer to any pH value, then anisotropic charge distribution takes place leading to the formation of dipoles from the surface of proteins (G.R Grimsley, 1999; K. Takano, 2000).

## 2.5 Alginates

Alginate is a bio-degradable and naturally occurring polymer widely used in pharmaceutical and biotechnology industries. It is anionic in nature possessing low toxicity and undergoes gelation upon addition of divalent cations such as calcium ions (Gombotz Wayne R, 1998). The release of drug molecules from alginate matrix is dependent on the degree of cross-linking of the polymer. The currently available

marketed formulations of sodium alginates have molecular weights ranging from 32,000 to 40,000 g/mol. The viscosity of alginate solution increases as the molecular weight of polymer is increased (LeRoux MA, 1999).

## 2.6 Sources

The alginates which are available commercially are obtained from three species of brown algae such as *Ascophyllum nodosum, Laminaria hyperborean, and Macrocystis pyrifera* (Clark DE, 1936; M., 2008). Another source is through isolation of bacterial alginates from *Azotobacter vinelandii* and *Pseudomonas* species (G.Skjak-Braek, 1986).

## 2.7 Chemical Structure

The chemical entity of alginates is comprised of alternating blocks of 1-4 linked α-L-guluronic acid and β-D-mannuronic acid residues. Figure 2.1 shows the pictorial representation of the binding of mannuronic and guluronic residues commonly seen in alginates.

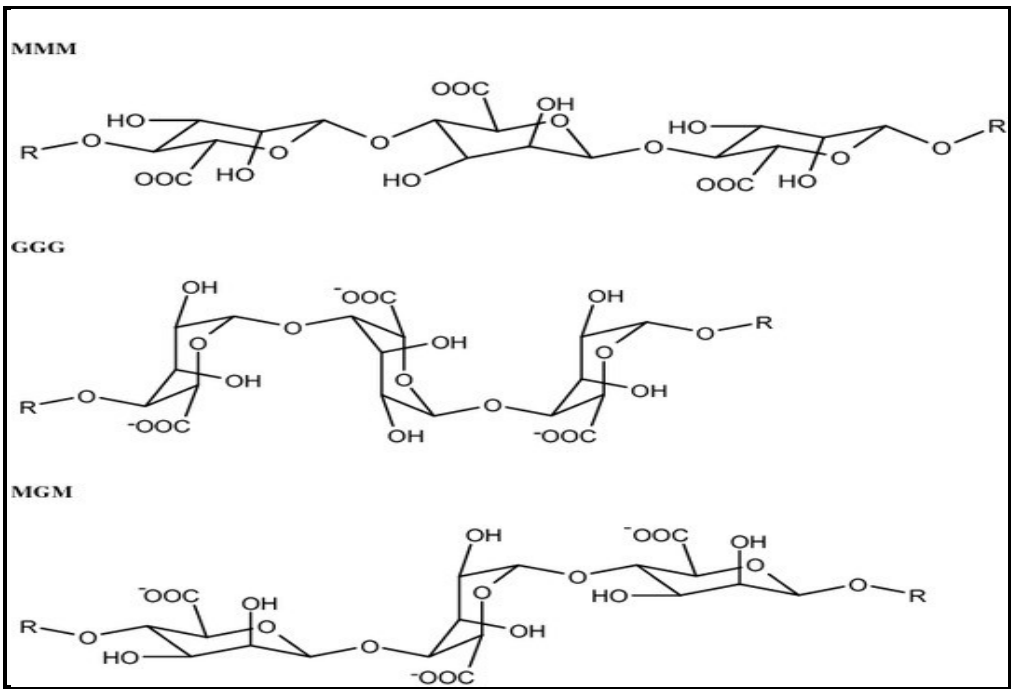


**Figure 2.1**: Chemical structures of G-block, M-block and alternating block in alginate.

The geometrical alignment of the G-block and M-block regions is dependent upon the shape and the mode of the linkage of the attached monomers. The G-block regions are inverted vertically while the M-block regions are aligned horizontally. The binding of the $Ca^{+2}$ ions takes place in the vicinity between two G-block regions (B. l. A.Haug, 1962; B. l. A.Haug, O.Smidsrod, 1967).

### 2.8 Biomedical applications

Alginates are used in the pharmaceutical industry due to their thickening, gelling, and stabilizing properties. They are widely used in tissue engineering and regeneration procedures. Alginates are also used as controlled release agents in various modified release dosage forms.

## 2.9 Physical Properties

Three physical properties have been found to influence the nature and degree of cross-linking of alginates by cations. These include (i) molecular size of polymers (ii) Composition, and (iii) sequential structure (A.Martinsen, 1991; O.Smidsrod, 1973). The greater the flexibility of alginate polymers better the degree of cross-linking of alginates. The descending order for the flexibility of alginate polymers in solution is as follows MG > MM > GG (G= α-L-guluronic acid; M=β-D-mannuronic acid) (O.Smidsrod, 1973).

## 2.10 Chemical Reactivity

Chemical interaction between alginates and positively charged proteins occurs with the calcium ions competing with the available carboxylic acid sites on alginate. This leads to the process of coacervation with cationic proteins which facilitates high loading capacity for the active agent within the alginate polymer matrix (R.J Mumper, 1994).

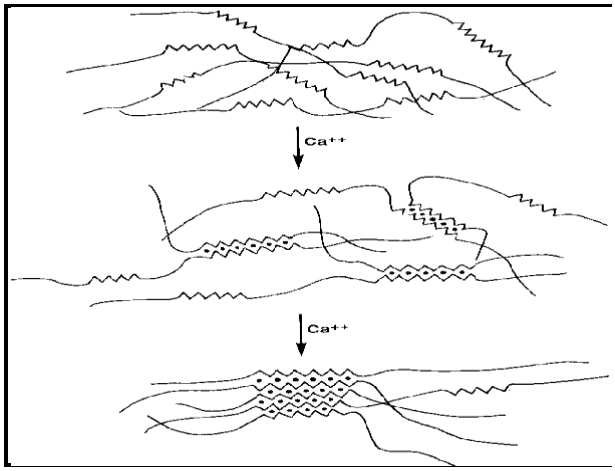


**Figure 2.2**: Schematic representation of cross-linking of calcium ions in alginate matrix

### 2.11 Alginates for oral delivery of drugs

#### 2.11.1 Bioadhesiveness

The adhesive nature of alginates can be advantageous for delivery of drugs to the GI tract (W.R. Gombotz, 1998). Mucoadhesive based drug delivery systems increase the residence time at the site of action. Several naturally occurring polymers with high charge densities possess excellent mucoadhesive properties (K.K Kwok, 1991a; W.R. Gombotz, 1998). Studies have indicated that polyanionic polymers show good mucoadhesive properties than polycationic and nonionic polymers (D.E. Chickering, 1995). The mucoadhesive properties of alginates makes it feasible for use in the delivery of proteins incorporated into its matrix for sustained drug release (H.Chang, 1985; K.K Kwok, 1991b). Due to this property, the drug bioavailability has also been shown to improve.

#### 2.11.2 pH sensitivity

The pH of the environment plays an important role in the release of drugs from alginate matrices. Alginate matrices without encapsulated drug tend to shrivel at acidic pH microenvironment (S.C Chen, 2004). However, encapsulated drugs are not released from the alginate matrix when exposed to acidic environment. The variable behavior of alginates in the gastric and intestinal environment plays a significant role in determining the drug release profiles. The hydrated sodium alginate is converted into porous and insoluble alginic acid when passing through acidic pH. In the intestinal pH, a soluble viscous layer of alginate is formed.

### 2.11.3 Gelation conditions

The process of gelation can be carried out under mild environmental conditions with the use of non-toxic reagents. Divalent cations such as $Mg^{2+}$, $Ca^{2+}$ easily form gels when mixed in aqueous alginate dispersions into which the protein can be entrapped within the reticulate polymer structure formed due to cross-linking by divalent cations.

## 2.12 Alginates in delivery of proteins

Alginates serve as excellent carriers for the delivery of protein drugs which can be incorporated into alginates under ambient conditions. The release rate of drugs from the alginate matrices have been found to vary with nature of cation used to cross-link as well as presence of other compatible polymers. An immediate or burst release of the entrapped drugs from alginate matrices have been observed due to the hydrophilic and porous nature of the alginates. However, several protein growth factors exhibited sustain release (Lee KY, 2003; Silva EA, 2010). The physical cross-linking of sodium alginate with the high isoelectric point proteins such as chymotrypsin and lysozyme have been shown to be responsible for the sustained or prolonged release (Wells LA, 2007).

## 2.13 Entrapment of protein into alginate

The effective degree of cross-linking determines the encapsulation efficiency of alginates to protein drugs and is mainly responsible for the sustained release of drugs from the polymer matrix (George M, 2006). For example insulin loaded alginate nanoparticles prepared by the cross-linking of microemulsion mixtures of aqueous

calcium chloride and sodium alginate showed sustain release *in vitro* in simulated intestinal fluid (pH=7.4) (Silva CM, 2006).

### 2.14 Release of proteins from alginates

There are two mechanisms by which protein release from alginate matrix takes place. They are: (i) degradation of polymeric network and (ii) protein diffusion through pores of polymeric network. The degradation mechanism is most suitable for controlled delivery of protein drugs. However protein release from alginate matrices occurs primarily via diffusion of drug through the pores (I.L Andresen, 1977). The rate of diffusion of large protein molecules is less than smaller molecules because of increase in molecular weight of the protein (H. Tanaka, 1984). The changes release mechanism of drug from alginate matrices appear to be related to the molecular weight of entrapped drug and ionic interaction between drug and negatively charged alginates (Mi, 2002; Sriamornsak, 2007). Additionally, the rate of protein diffusion from alginate matrix is also dependent on charge of protein moiety. When positively charged proteins interact with negatively charged alginate drug release is inhibited and process of diffusion will be reduced (G. S.-B. O.Smidsrod, 1990; T. Espevik, 1993). However, proteins possessing net negative charge will have a rapid or burst release from the alginate matrix due to the repulsion between like charges.

The surface drying method and porosity are two contributing factors that have significant impact on the drug release profiles. The porosity of the nanoparticles or beads can be modulated by partially drying the polymer matrix (O.Smidsrod, 1973; Smidsroed, 1973). When nanoparticles or beads are exposed to complete dehydration, surface

cracking or surface erosion can occur which leads to an unstable protein formulation (C.K. Kim, 1992). Fourier transform infrared (FTIR) spectroscopy evaluation of alginate matrices demonstrated that alginates with high mannuronic acid content bind more strongly to poly-L-lysine layers in multiple coating experiments which lead to stable sustained release matrices. Several studies have also revealed that at low pH, a decrease in pore size of alginates is observed which leads to the reduction in the release of macromolecules from the matrix (C.K. Kim, 1992; S. Sugawara, 1994; T. Espevik, 1993; T.Yotsuyanagi, 1987).

### 2.15 Drug release from alginate matrices

Alginates are useful in the entrapment of bioactive compounds and drugs. Sustained or controlled delivery system at desired rate is obtained when drug is released from encapsulated alginate matrices (Mi, 2002; Sriamornsak, 2007). The factors governing the movement of encapsulated drug from alginate matrix are property of drug itself and chemical composition of alginate polymer. The functional groups in alginates also have a significant effect on rate of drug release from nanoparticles. It has been shown that the steric effect of bulkier groups of alginates may result in weaker bonding (De Vos, 1996; Klock, 1997).

### 2.16 Chemical stability and degradation

Calcium alginate gel matrix degrades through the mechanism of removal of calcium ions. Such process can be induced using chelating agents or by incorporating high concentrations of mono or divalent cations such as $Na^{+}$ or $Mg^{2+}$ (I.W. Sutherland, 1991).

Due to the removal of calcium ions from alginates, the cross-linking unravels which results in destabilization of the matrix. The entrapped proteins from within the matrix will start to leak leading to solubilization of the higher molecular weight alginate polymers. The encapsulated proteins of lower molecular weight alginates and low α-L-guluronic acid are released at much faster rates then the higher molecular weight alginate polymers (Y. Murata, 1993).

The most prominent hydrolysis reaction undergone by alginates is proton catalyzed hydrolysis reaction which is dependent upon physical factors including pH, temperature and time (B. l. A.Haug, 1962, 1963a, 1963b; B. l. A.Haug, O.Smidsrod, 1967). At low pH, the release rate of drugs from alginates becomes rapid which leads to the decrease in its molecular weight (R.J Mumper, 1994). Moreover, alginates form strong complexes with polycations such as chitosan, albumin and synthetic polymers like polyethyleneimine (O.Smidsrod, 1973). These polyion complexes stabilize the matrix and do not dissolve in the presence of calcium ion chelating agents.

## 2.17 Limitations

There have been several limitations in the preparation of calcium alginate loaded nanoparticles or beads. Several studies have been performed to modify alginates for drug delivery purposes. The most common problem encountered in the preparation of alginate loaded nanoparticles is leaching of drug through pores (M.L Torre, 1998; P. Liu, 1999). Use of an effective cross-linking agent for the preparation of alginate matrix has proved to be successful to address this problem. The cross-linking of alginates within

microemulsion mixtures of cyclohexane (CH) and DOSS have been investigated for various applications (A.R. Kulkarni, 2000; L.W. Chan, 2002).

## 2.18 References


1. George M, Abraham TE. Polyionic hydrocolloids for the intestinal delivery of protein drugs: Alginate and chitosan — a review. Journal of Controlled Release. 2006;114(1):p.1-14.

2. H.J.C Ericksson WLJH, B.van Veen, G.W Somsen, G.J de Jong, H.W Frijlink. Investigations into the stabilisation of drugs by sugar glasses: I. Tablets prepared from stabilised alkaline phosphatatse. Int JPharm. 2002(249):p.59-70.

3. Manning MCP, K. ; Borchardt, R. T. Stability of protein pharmaceuticals. Pharm Res 1989;6:p. 903-18.

4. Carpenter JFP, M.J. ; Chang, B.S; Randolph, T.W. Rational design of stable lyophilized protein formulations: Some practical advice. Pharm Res. 1997;14:p. 969-75.

5. Hageman MJA, T. J, Manning, M. C. Water sorption and solid-state stability of proteins. In *Stability of protein pharmaceuticals, Part A: Chemical and Physical pathways of protein degradation*. Plenum press: New York1992.

6. Pikal MJD, K.M ; Roy, M.L; Riggin, R.M. The effects of formulation variables on the stability of freeze-dried human growth hormone. Pharm Res. 1991;8:p. 427-36.

7. W.Wang. Instability, stabilization and formulation of liquid protein pharmaceuticals. Int JPharm. 1999;185:p. 129-88.

8. Liu Xing CD, Xie Liping, Zhang Rongquing. Oral colon-specific drug delivery for bee venom peptide: development of a coated calcium alginate gel beads-entrapped liposome. J Control Release. 2003;93:p.293-300.

9. L. Shargel AY, editor. Applied biopharmaceutics and Pharmacokinetics. New York: McGraw-Hill; 1999.

10. Costantino HRL, R. ; Klibanov, A. M. Solid phase aggregation of proteins under pharmaceutically relevant consitions. JPharm Sci. 1994;83:p.1662-9.

11. Lee HJ. Protein drug oral delivery: the recent progress. Arch Pharm Res 25. 2002:p.572-84.

12. U.B. Kompella VHL. Delivery systems for penetration enhancement of peptide and protein drugs: design considerations. Advanced Drug Delivery Reviews. 2001;46:p.211-45.

13. Russell-Jones GJ. Use of vitamin B12 conjugates to deliver protein drugs by the oral route. Crit Rev Ther Drug Carr Syst. 1998;15:p.557-86.

14. B. Steffansen CUN, B. Brodin, A.H. Eriksson, R. Andersen, S. Frokjaer. Intestinal solute carriers: an overview of trends and strategies for improving oral drug absorption. Eur J Pharm Sci 21. 2004:p.3-16.

15. Fasano A. Modulation of intestinal permeability: an innovative method of oral delivery for the treatment of inherited and acquired human diseases. Mol Genet Metab. 1998;64:p.12-8.

16. J.P. Bai LLC, J.H. Guo. Targeting of peptide and protein drugs to specific sites in the oral route. Crit Rev Ther Drug Carr Syst. 1995;12:p.339-71.

17. Fix JA. Oral controlled release technology for peptides: status and future prospects. Pharm Res. 1996;13:p.1760-4.

18. Sanders LM. Drug delivery systems and routes of administration of peptides and protein drugs. Eur J Drug Metab Pharmacokinet 1990;15:p.95-102.

19. J.H. Hamman GME, A.F. Kotze. Oral delivery of peptide drugs:barriers and developments. BioDrugs 19. 2005: p.165-77.

20. Lehr CM. Bioadhesion technologies for the delivery of peptide and protein drugs to the gastrointestinal tract. Crit Rev Ther Drug Carr Syst 11. 1994: p.119-60.

21. S.A. Galindo-Rodriguez EA, H. Fessi, E. Doelker. Polymeric nanoparticles for oral delivery of drugs and vaccines: a critical evaluation of in vivo studies. Crit Rev Ther Drug Carr Syst 22. 2005: p.419-64.

22. S. Krishnan EYC, J.N. Webb, B.S. Chang, D.Shan, M.Goldenberg, M.C Manning, T.W Randolph, J.F. Carpenter. Aggregation of granulocyte colony stimulating factor under physiological conditions: Characterization and thermodynamic inhibition. Biochem. 2002;41: p.6422-31.

23. B.S Kendrick JLC, X. Lam, T. Nguyen, T.W Randolph, M.C Manning, J.F carpenter. Aggregation of recombinant human interferon gamma: Kinetics and structural transitions. JPharm Sci. 1998;87: p.1069-76.

24. Arkawa JSPaT. Mechanism of protein aggregation. Pharmaceutical Biotechnology. 2009;10: p.348-51.

25. Rechendorff K. "The influence of surface roughness on protein adsorption". Denmark: University of Aarhus; 2011.

26. Andrade JD. Surface and interfacial Aspects of Biomedical Polymers. New York and London. 1985. p. 10-21.

27. Patel KB, R.T. Chemical pathways of peptide degradation II. Kinetics of deamidation of an asparaginyl residue in a model hexapeptide. PharmRes. 1990;7: p.703-11.

28. Strickley RGA, B.D. Solid state stability of human insulin I. Mechamisn and effect of water on kinetics on degradation in lyophiles from pH 2-5 solutions. PharmRes. 1996;13: p.1142-53.

29. Pikal MJR, D.R. The stability of insulin in cystalline and amorphous solids; observation of greater stability for amorphous form. PharmRes. 1997;14: p.1379-87.

30. Carl W. Niekamp HFHJMlJ. Peptide-bond hydrolysis equilibria in native proteins. Conversion of virgin into modified soybean trypsin inhibitor. ACS publications. 1969;8(1): p.16-22.

31. Dubost DCK, M.J; Zimmerman, J.A; Bogusky, M.J; Coddington, A.B; Pitzenberger, S.M. Characterization of solid state reaction product from a lyophilized formulation of a cyclic heptapeptide. Novel example of an excipient -induced oxidation. PharmRes. 1996;13: p.1811-4.

32. Fransson. J; Florin-Robertsson.E ; Axelsson KN, C. Oxidation of human insulin like growth factor I in formulation studies: Kinetics of methionine oxidation in aqueous solutions and solid states. PharmRes. 1996;13: p.1252-7.

33. Costantino HRL, R. ; Klibanov, A. M. Moisture induced aggregation of lyophilized insulin. PharmRes. 1994;11: p.21-9.

34. Jason Kerr JLS, Donald R. Griffin , Darice Y. Wong , and Andrea M. Kasko *. Steric Effects in Peptide and Protein Exchange with Activated Disulfides. Biomacromolecules. 2013;14(8): p.2822-9.

35. Clarket TGaS. Deamidation, Isomerization and Racemization at Asparaginyl and Aspartyl Residues in Peptides. The Hournal of Biological Chemistry. 1967;262(2): p.785-94.

36. Dill. KA. Dominant forces in protein folding. Biochemistry. 1990;29: p.7133-55.

37. C.N. Pace BAS, M. Mcnutt, K. Gajiwala. Forces contributing to confirmational stability of proteins. FASEB J 1996;10: p.75-83.

38. R. Jaenicke. In R. Huber ELWe. Protein structure and protein engineering. Berlin1988. 16-36 p.

39. Protein structure: A Practical approach [press release]. Oxford: IRL Press1989.

40. Jaenicke R. Protein folding: Local structures, domains, sub-units and assemblies. Biochemistry. 1991;30: p.3147-61.

41. Intermolecular and surface forces [press release]. San Diego, California: Academic Press1992.

42. G. Graziano FC, A. Riccio, G. Barone. A reassessment of the molecular origin of cold denaturation. J Biochem (Tokyo). 1997;122: p.395-401.

43. N.T. Southall KAD, A.D.J. Haymet. A view of the hydrophobic effect. J Phys Chem B. 2002;106: p. 521-533.

44. Privalov PL. Cold denaturation of proteins. *Crit Rev Bio-chem Mol Biol*. 1990;25: p.281-305.

45. P.L. Privalov SJG. Stability of protein structure and hydrophobic interaction. *Adv Protein Chem*. 1988;39: p.191-234.

46. Pace CN. Polar group burial contributes more to protein stability than non-polar group burial. *Biochem*. 2001;40: p.310-313.

47. J.F. Carpenter BSK, B.S. Chang, M.C. Manning, T.W. Randolph. Inhibition of stress-induced aggregation of protein therapeutics. *Methods Enzymol*. 1999;309: p.236-55.

48. A.C. Dong SJP, S.D Allison, J.F Carpenter. Infrared spectroscopic studies of lyophilization-induced and temperature-induced protein aggregation. JPharm Sci. 1995;84: p.415-24.

49. Atkins P. Physical Chemistry. New York: W.H. Freeman and Company; 1994.

50. M. Vrkljan TMF, M.E. Powers, J. Henkin, W.R. Porter, H. Staack, J.F. Carpenter, M.C Manning. Thermal-stability of low molecular weight urokinase during heat treatment. 2. Effect of polymer additives. *Pharm Res*. 1994;11: p.1004-8.

51. Stability and characterization of recombinant human relaxin [press release]. Plenum Press1996.

52. L. Nielsen RK, A.Coats, S. Frokjaer, J. Brange, S. Vyas, V.N. Uversky, A.L. Fink. Effect of environmental factors on the kinetics of insulin fibril formation: Elucidation of the molecular mechanism. *Biochem*. 2001;40: p.6036-6046.

53. K. Takano KT, Y. Yamagata, K. Yutani. Contribution of salt bridges near the surface of a protein to the confirmational stability. *Biochemistry*. 2000;39: p.12375-81.

54. G.R Grimsley KLS, L.R Fee, R.W. Alston, B.M.P Huyghues-Despointes, R.L. Thurlkill, J.M. Scholtz and C.N. Pace. Increasing protein stability by altering long-range columbic interactions. *Protein Sci*. 1999;8: p.1843-9.

55. Gombotz Wayne R WS. Protein release from alginate matrices. Advanced Drug Delivery Reviews. 1998;31: p.267-85.

56. LeRoux MA G, Setton LA. Compressive and shear properties of alginate gels: effects of sodium ions and alginate concentration. JBiomed Mater Res. 1999;47: p.46-53.

57. Clark DE GH, inventorAlginic acid and process of making same1936.

58. M. R. Main properties and current applications of some polysaccharides as biomaterials. Polym Int. 2008;57: p.397-430.

59. G.Skjak-Braek HG, B. larsen. Monomer sequence and acetylation pattern in some bacterial alginates. Carbohydrate Res. 1986;154: p.239-50.

60. A.Haug Bl, O.Smidsrod. Studies on the sequence of uronic acid residues in alginic acid. Acta Chem Scand. 1967;21: p.691-704.

61. A.Haug Bl. Quantitative determination of the uronic acid composition of alginates. Acta Chem Scand. 1962;16: p.1908-18.

62. O.Smidsrod. The relative extension of alginates having different chemical composition. Carbohyr Res. 1973;27: p.107-18.

63. A.Martinsen GS-B, O.Smidsrod, F.Zanetti, S.paoletti. Comparison of different methods for determination of molecular weight and molecular weight distribution of alginates. Carbohydrate Polymers. 1991;15: p.171-93.

64. R.J Mumper ASH, P. Puolakkainen, L.S Bouchard, W.R. Gombotz. Calcium-alginate beads for the oral delivery of transforming growth factor-B1: Stabilization of TGF-B1 by the addition of polyacrylic acid within acid-treated beads. J Control Release. 1994;30: p.241-51.

65. W.R. Gombotz SFW. Protein release from alginate matrices. Advanced Drug Delivery Reviews. 1998;31: p.267-85.

66. K.K Kwok MJG, D.J Burgess. Production of 5-15 um diameter alginate-polylysine microcapsules by an air atomization technique. Pharm Res. 1991;8: p.341-4.

67. D.E. Chickering EM. Bioadhesive microspheres: I. A novel electrobalance-based method to study adhesive interactions between individual microspheres and intestinal mucosa. J Control Release. 1995(34): p.251-61.

68. H.Chang HP, P. Kelly, J. Robinson. Bioadhesive polymers as platforms for oral controlled drug delivery. Synthesis and evaluation of some swelling, water-insoluble bioadhesive polymers. J Pharm Sci. 1985(74): p.399-405.

69. K.K Kwok MJG, D.J Burgess. Sterile microencapsulation of BCG in alginate-poly-L-lysine by an air spraying technique. Proc Int Symp Control Release Bioact Mater 16. 1991: p.170-1.

70. S.C Chen YCW, F.L. Mi, Y.H. Lin, L.C. Yu, H.W. Sung. A novel pH-sensitive hydrogel composed of *N,O*-carboxymethyl chitosan and alginate cross-linked by genipin for protein drug delivery. J Control Release 96. 2004: p.285-300.

71. Lee KY PM, Mooney DJ. Comparison of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in SCID mice. J Control Release. 2003(87): p.49-56.

72. Silva EA MD. Effects of VEGF temporal and spatial presentation on angiogenesis. Biomaterials. 2010(31): p.1235-41.

73. Wells LA SH. Extended release of high pI proteins from alginate micrspheres via a novel encapsulation technique. Eur J Pharm Biopharm. 2007(65): p.329-35.

74. George M AT. Polyionic hydrocolloids for intestinal delivery of proteins drugs. J Control Release. 2006(114): p.1-14.

75. Silva CM RA, Ferreira D, Veiga F. Insulin encapsulation in reinforced alginate microspheres prepared by internal gelation. Eur J Pharm Sci. 2006(29): p.148-59.

76. I.L Andresen OS, O.Smidsrod, K. Ostgaard, P.C Hemmer. Some biological functions of matrix components in benthic algae in relation to their chemistry and the composition of seawater. ACS Symp Ser 48. 1977: p.361-81.

77. H. Tanaka MM, I.A Veliky. Diffusion characteristics of substrates in Ca-alginate gel beads. Biotechnol Bioeng 26. 1984: p.53-8.

78. Mi F-L, Sung,H.W & Shyu, S.S. Drug release from chitosan-alginate complex beads reinforced by an naturally occuring cross-linking agents. Carbohydrate Polymers, 48(1). 2002: p.61-72.

79. Sriamornsak PT, N & Korkerd, K. Swelling, erosion and release behavior of alginate-based matrix tablets. European Journal of Pharmaceutics and Biopharmaceutics, 66(3). 2007: p.435-50.

80. O.Smidsrod GS-B. Alginate as immobilization matrix for cells. TIBTECH 8. 1990: p.71-8.

81. T. Espevik MO, G. Skjak-Braek, L.Ryan, S.D Wright, A.Sundan. The involvement of CD14 in stimulation of cytokine production by uronic acid polymers. EurJImmunol23. 1993: p.255-61.

82. Smidsroed OG, R.M & Whittington, S. G. Relative extension of alginates having different chemical composition. Carbohydrate Research,27(1). 1973: p.107-18.

83. C.K. Kim EJL. The controlled release of blue dextran from alginate beads. Int JPharm79. 1992: p.11-9.

84. T.Yotsuyanagi TO, T.Ohhashi, K.Ikeda. Calcium induced gelation of alginic acid and pH-sensitive reswelling of dried gels. Chem Pharm Bull35. 1987: p.1555-63.

85. S. Sugawara TI, M. Otagiri. The controlled release of prednisolone using alginate gel. Pharm Res11. 1994: p.272-7.

86. De Vos PDH, B. Wolters, G.H & Van Schilfgaarde, R. Factors influencing the adequacy of microencapsulation of rat pancreatic islets. Transplantation 62(7). 1996: p.888-93.

87. Klock G, Pfeffermann, A. Ryser, C. Grohn, P.Kuttler, B. Hahn, H.J,etal. Biocompatibility of mannuronic acid-rich alginates. Biomaterials, 18(10). 1997: p.707-13.

88. I.W. Sutherland DBE. Alginates, . Biomaterials: Novel Materials from biological sources. 1991: p.309-31.

89. Y. Murata KN, E.Miyamoto, S.Kawashima, S.H Seo. Influence of erosion of calcium-induced alginate gel matrix on the release of Brilliant Blue. J Control Release 23. 1993: p.21-6.

90. A.Haug Bl. The solubility of alginate at low pH. Acta Chem Scand 17. 1963: p.1653-62.

91. A.Haug Bl. The degradation of alginates at different pH values. Acta Chem Scand 17. 1963: p.1466-8.

92. P. Liu TRK. Alginate-pectin-poly-L-Lysine particulate as a potential controlled release formulation. JPharm Pharmacol 51. 1999: p.141-9.

93. M.L Torre PG, L.Maggi, R. Stefli, E. Ochoa, Machiste, U.Conte. Formulation and characterization of calcium alginate beads containing ampicillin. Pharm Dev Technol3. 1998: p.193-8.

94. L.W. Chan PWSH. Effects of aldehydes and methods of cross-linking on properties of calcium alginate microspheres prepared by emulsifiction. Biomaterials 23. 2002: p.1319-26.

95. A.R. Kulkarni KSS, T.M. Aminabhavi, A.M. Dave, M.H. Mehta. Glutareldehyde crosslinked sodium alginate beads containing liquid pesticide for soil application. J Control Release 63. 2000: p.97-105.

# Chapter 3

# Development and validation of a rapid RP-HPLC method for quantification of human recombinant Insulin

Kush Patel[1] and Jerry Nesamony[1,*]

[1] Division of Industrial Pharmacy, Department of Pharmacy Practice, College of Pharmacy and Pharmaceutical Sciences, University of Toledo, Toledo, Ohio, USA

[*]**Corresponding author**:
Department of Pharmacy Practice
College of Pharmacy and Pharmaceutical Sciences
University of Toledo
3000 Arlington Avenue, MS1013
Toledo, OH 43614
419 383 1938 (Tel)
419 383 1950 (Fax)
jerry.nesamony@utoledo.edu

**Keywords:** Method validation, quantification, insulin, standard deviation, accuracy, precision, LOD, LOQ

## 3.1 Abstract

A reversed phase HPLC system was developed and validated for human recombinant insulin according to International Conference of Harmonization (ICH) guidelines. A reversed phase C18 column was used with an isocratic mobile phase of 0.2M sodium sulfate buffer adjusted to pH=2.4 with ortho phosphoric acid and acetonitrile enabled efficient separation between 7 to 8 minutes. The flow rate was set to 1.5ml/min with column temperature set to 40 °C. The protein identification was made by PDA detection at 214 nm. The quantification of insulin was carried out by performing analyses in a series of concentrations ranging from 0.3 IU/ml to 5.5 IU/ml. The method was validated to demonstrate accuracy, specificity, inter and intra- day precision, limit of quantitation (LOQ), limit of detection (LOD), and percent recovery. The calibration curves plotted showed significant linear correlation coefficients ($R^2$=0.9978) within the tested ranges. In conclusion, a successful method was developed for the rapid quantification of human insulin. The inter-day and intra-day precision was calculated to be 99.32±0.745. The RP-HPLC method is uncomplicated, precise, and accurate and can potentially be used for quantitative analysis of insulin containing formulations.

## 3.2 Introduction

Insulin is a protein hormone; secreted by the β-cells present in the Islets of Langerhans in the pancreas. Insulin is comprised of 51 amino acids sequence arranged into two chains A and B with molecular weight of 5800 Daltons(Moussa, 2010). Chain A contains 30 amino acids and chain B contains 21 amino acids(Moussa, 2010). It regulates the storage of glycogen in the liver and accelerates the oxidation of sugar in cells. When the body

does not produce enough insulin or when the body does not respond to insulin in the regular manner Diabetes is produced(EP Kroef, 1989). About 26% of all diabetic patients rely on routine injections of exogenous insulin alone or in combination with oral agents to control their blood sugar levels(Lebovitz, 2011). Originally insulin was available from animal sources only such as porcine and bovine(H, 1998). But with the advent of biotechnology derived products recombinant human insulin that is identical to human insulin was made from recombinant DNA technology and is used in the treatment of Diabetes Mellitus. The human insulin, recombinant is produced by the linkage of A and B chains of human insulin by the separate strains of E.Coli K12(Johnson, 1982). We used Recombinant Human Insulin for the development of the HPLC method.

Analytical techniques like spectroscopy (Gordon Roberts, 2011), gel electrophoresis(Kryndushkin DS, 2003), thermal analysis(W. D. Laws 1949) have been employed for the protein quantification studies. HPLC have been widely used in the determination of high molecular weight protein chemistry as well. Several immune and non-immune techniques have been produced for the identification of insulin. HPLC and capillary electrophoresis (CE) are considered to be non-immune methods for insulin determination(Khaksa G., 1998). The main disadvantage associated with the non-immune methods for protein determination is the interference from the sample matrix. However, HPLC is considered to be fast and rapid technique for the quantification of protein and peptide drugs in the solution form(Khaksa G., 1998). The determination of insulin using HPLC provides simple and convenient procedure for the quantification process.

The revered phase HPLC method reported in this manuscript was developed according to International conference of Harmonization (ICH) guidelines. The determination of Insulin peak was done quantitatively by trying various combinations of mobile phases with a stationary phase column. In this manuscript, the combination of 0.2M sodium sulfate buffer with pH 2.3 and Acetonitrile were used as mobile phases. The HPLC-diode array detection is considered to be highly effective screening method. The prime advantage observed with this method is the identification of analyte both by retention time and UV spectrum. The peak resolution and band broadening are the important parameters in the validation of HPLC method for any analyte. According to the USP standards, the retention time of Insulin peak is around 29 minutes but in the method reported here the retention time was reduced to approximately 7 minutes.

**3.3 Experimental**

Materials

Insulin, Human Recombinant was obtained from Sigma Aldrich (St. Louis Missouri USA). Acetonitrile, HPLC grade sub-micron filtered was obtained from Fisher scientific (New Jersey USA). Sodium sulfate anhydrous, USP and o-Phosphoric acid, NF were supplied by Fisher Scientific (New Jersey USA). HPLC water was prepared in our laboratory using Reverse osmosis water filtered through 0.45µm Nylon membrane Filters. The prepared water was degassed for 30 minutes.

Equipment

Waters e2695 installed with Empower software for isocratic solvent delivery system equipped with Water 2998 PDA detector was used to perform all chromatographic separations. A reverse phase Symmetry Shield$^{TM}$ C-18 column (5 µm, 250 mm X 4.6 mm, Waters, USA) was used.

Chromatographic conditions

An isocratic separation method was employed with mobile phase composition of 73:27(v/v) of 0.2 M sodium sulfate buffer (pH=2.4 adjusted with o-phosphoric Acid) and acetonitrile respectively. The isocratic run was made for 10 minutes. The eluent was pumped at a flow rate of 1.5 ml/min with an injection volume of 50µl. The detection wavelength was set to 214 nm and the column temperature was maintained at 40 °C.

Preparation of Standard Insulin solutions

Five standard insulin solutions with concentrations ranging between 5.6 and 0.4 IU/ml were prepared from an insulin stock solution of 275 IU/ml. The stock solution was prepared by dissolving insulin in 0.2 M sodium sulfate buffer. This buffer was chosen in order to suppress the process of adsorption due to the presence of salts(K.Makino, 1979). The peak resolution is also high and an effective protein separation is obtained.

Method Validation

The calibration curves were plotted for human insulin by employing area response versus the concentration of the standard samples. The five standard solutions of insulin were

injected into HPLC column and the area response versus insulin concentration was evaluated using waters e2695 Empower software. The method validation parameters such as linearity, precision, accuracy, range, LOD and LOQ were determined according to the ICH guidelines(*Proceedings of the International Conference on Harmonization (ICH)*, 1996). The peak area and linear concentration range from 0.4 to 5.6 IU/ml were plotted and the line equation thus obtained was used to measure concentrations. The precision of the analysis was evaluated for inter-day and intra-day time periods. The peak areas of the chromatograms were obtained and the relative standard deviation (RSD) was reported as well. The percent recovery was determined by the calibration graphs obtained by direct injections of standard solutions. The LOD and LOQ values were determined by injecting low concentrations of standard solution under chromatographic conditions(Boardman, 1959). LOD was calculated using signal-to-noise of 3:1 and LOQ with signal-to-noise ratio of 10:1. The signal corresponds to standard deviation of the intercept and noise corresponds to the slope of calibration curve.

### 3.4 Results and Discussion

A representative chromatogram that shows the insulin peak is shown in Figure 3.1. The distinct peak at 7.220 min was observed to possess good peak shape and peak resolution. The peaks eluting around 2 minutes represented buffer peaks.

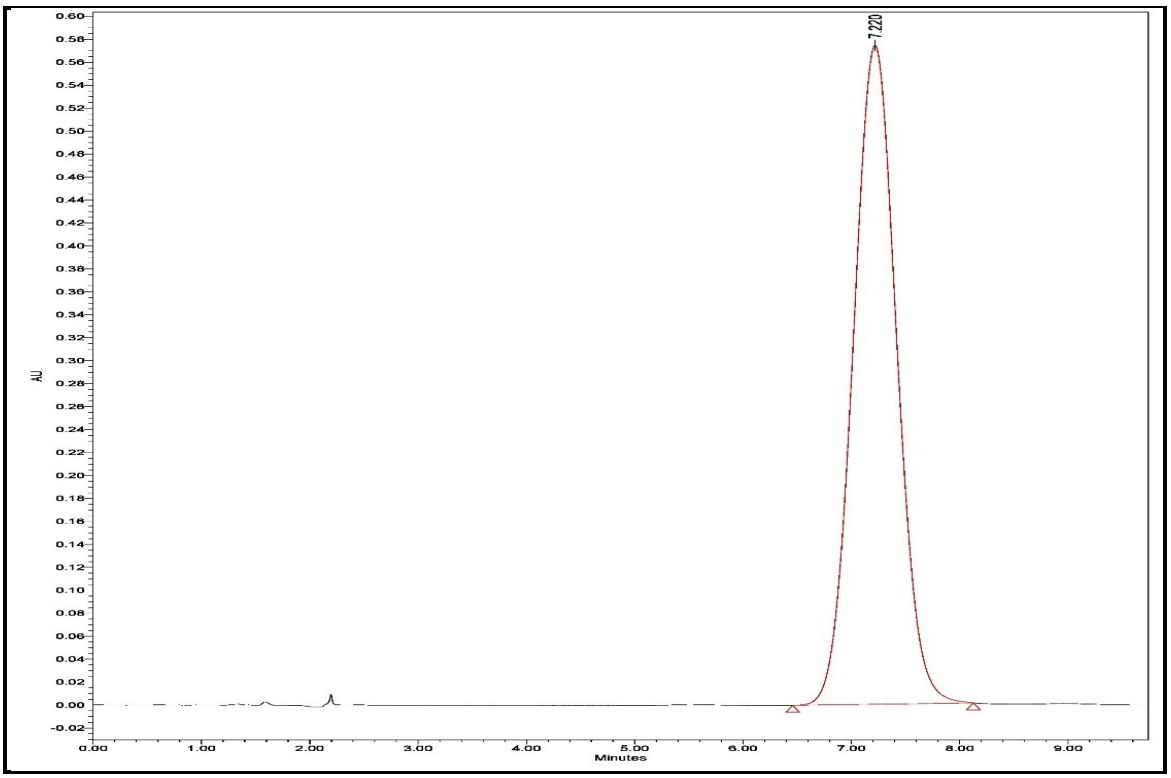


**Figure 3.1**: RP-HPLC chromatogram for Human Recombinant Insulin

The retention time at 7.220 minutes indicated that Recombinant Insulin is separating from the sample dilution mixture.

**Table 3.1**: Specificity results for Human Insulin

| Sample | Average retention time |
|---|---|
| Buffer peak | 2.2 min±0.072 |
| Human Insulin | 7.220 min±0.026 |

After the Insulin peak elution at 7.220 min, the sample was still made to run for 60 minutes in order to specify the existence and identity of other peaks that can correlate to the insulin peak. No such distinct and identical peaks were observed during the 60 minute run. The specificity test results were positive in terms of peak identification for human recombinant insulin.

The accuracy and percent recovery of various insulin concentrations are reported in the table 3.2. The area under the curve response to Insulin concentrations of 5.6 IU/ml, 2.8 IU/ml, 1.4 IU/ml and 0.7 IU/ml were used to obtain the percent recovery. The overall percent recovery obtained was 99.3±0.7.

**Table 3.2**: Accuracy results (n=3)±S.D

| Concentration(IU/ml) | Average peak area ±S.D | Calculated amount(IU) ±S.D | % recovery ±S.D | % RSD |
|---|---|---|---|---|
| 5.6 | $4717866.33 \pm 9.5 * 10^3$ | 202.56±0.38 | 101.32±0.19 | 0.19 |
| 2.8 | $2032337.67 \pm 3.5 * 10^4$ | 96.43±1.39 | 95.25±1.39 | 1.45 |
| 1.4 | $776814.67 \pm 2.4 * 10^4$ | 46.85±0.96 | 98.21±1.92 | 1.27 |
| 0.7 | $253008.33 \pm 2.99 * 10^3$ | 25.78±0.12 | 102.50±0.47 | 0.48 |

Average % recovery = 99.32±0.99

Average % RSD = 0.745

This data indicated that the percent recovery and accuracy for insulin were in between the optimum concentration range of 95-105%. The statistical analysis was done through one way ANOVA test with p value of less than 0.05. The peak areas did not vary significantly for all triplicate runs cycle for each concentration. The peak area increased linearly with increase in insulin concentration. The relative standard deviation and the percent recovery were calculated using the standard deviation of each run and the mean peak area values(McLeod AN, 1990). The measured and calculated data signifies that the accuracy of the assay method was good enough to quantify the protein concentration ranges tested.

The linearity of the assay method was evaluated by plotting the peak area as a function of analyte concentration. A linear relationship was observed over the range of 0.35-5.6 IU/ml using the analytical procedure. The correlation coefficient was determined to be 0.9978. The linearity parameters are shown in table 3.3.

**Table 3.3**: Table showing validation parameters

| Parameters | Value |
|---|---|
| Linearity range | 0.35-5.6 IU/ml |
| Slope of regression | 25262 |
| Intercept | 396637 |
| SD of Intercept | 11001.8 |
| Correlation Coefficient ($R^2$) | 0.9978 |

The precision of the analytical procedure was determined from inter and intra day results as shown in table 3.4.

**Table 3.4**: Data showing precision results (n=3)±S.D

| Concentration (IU/ml) | Day 1 | % RSD | Day 2 | % RSD |
|---|---|---|---|---|
| 5.6 | $4844588\pm4.4*10^3$ | 0.16 | $4825136\pm4.6*10^3$ | 0.23 |
| 2.8 | $2180247.6\pm2.6*10^3$ | 1.25 | $2168541\pm2.7*10^3$ | 0.89 |
| 1.4 | $828626.6\pm3.8*10^3$ | 1.96 | $834021\pm3.7*10^3$ | 1.56 |
| 0.7 | $285718.3\pm4.5*10^2$ | 0.45 | $279632\pm4.7*10^2$ | 1.39 |
| 0.35 | $56652.3\pm3.8*10^2$ | 2.3 | $56207\pm3.5*10^2$ | 0.35 |

The values corresponding to the insulin concentrations didn't vary from day 1 to day 2. One way ANOVA test results showed no significant change in the precision values. This indicates that there was no significant difference in the peak values ($p < 0.05$).

LOD and LOQ

The limit of detection and limit of quantitation were determined using the standard deviation value of the y-intercept and the slope of the calibration curve(D Senthil Rajan, 2006). The slope of the regression line and the intercept were obtained from the graph. The theoretical concentration of insulin was calculated from the line equation.

The limit of detection value was calculated to be 0.04 IU/ml and limit of quantitation value was determined as 0.12 IU/ml. LOD ($DL = 3.3\sigma/S$) and LOQ ($QL=10\sigma/S$) are

calculated using the y-intercept and the slope of the analyte calibration curve(D Senthil Rajan, 2006).

### 3.5 Conclusions

The quantification of human recombinant insulin was performed using an RP-HPLC method. The analytical procedure was validated and parameters such as accuracy, precision, LOD, LOQ, linearity, and specificity determined. The retention time for human insulin was found to be 7.220 min. The analytical conditions did not significantly affect the accuracy of the HPLC method. The specificity and linearity of the insulin samples lied within the specified limits. The method demonstrated good precision and reproducibility. The RP-HPLC method may be suitable for rapid quantification of recombinant human insulin and can be applied during routine analysis in similar conditions.

### 3.6 References

1. Moussa BA. Validated RP-HPLC method for determination of Recombinant Insulin in bulk dosage form. E-Journal of Chemistry. 2010;7: p.449-57.

2. EP Kroef RO, EL Campbell, RD Johnson. Production scale of biosynthetic human insulin by reverse phase liquid chromatography. Journal of Chromatography A. 1989.

3. Lebovitz HE. Insulin: Potential Negative Consequences of Early Routine Use in Patients With Type 2 Diabetes. American Diabetes Association. 2011;34: p.225-30.

4. H K, editor Insulin: availability, affordability, and harmonization. WHO Drug; 1998.

5. Johnson IS. Authencity and Purity of Human Insulin (recombinant DNA)1982. 4-12 p.

6. Gordon Roberts L-YL. Protein NMR Spectroscopy: Practical Techniques and Applications. Sons JW, editor. Hoboken: ChemBioChem/Wiley-VCH; 2011.

7. Kryndushkin DS AI, Ter-Avanesyan MD, Kushnirov VV. Yeast [PSI+] prion aggregates are formed by small Sup35 polymers fragmented by Hsp104. Journal of Biological Chemistry. 2003;278(49): p.49636-43.

8. W. D. Laws WGF. Differential Thermal Analysis of Proteins. Anal Chem. 1949;21(9): p.1058-9.

9. Khaksa G. NK, Bhat M., Udupa N. High-performance liquid chromatographic determination of insulin in rat and human plasma. Analytical Biochemistry. 1998;260: p.92-5.

10. K.Makino NS, F. Moriya, S. Rokushika and H. Hatano. Liquid Chromatography detectors. Chem Lett. 1979: p.675.

11. Proceedings of the International Conference on Harmonization (ICH)1996.

12. Boardman NK. Chromatography of insulin in ion-exchange resin. Journal of Chromatography A. 1959;2: p.398-405.

13. McLeod AN AdMA, Wood SP. High-performance liquid chromatography of insulin. Accessibility and flexibility. Journal of Chromatography A. 1990;502: p.325-36.

14. D Senthil Rajan KVG, U Mandal, M Ganesan, A Bose, AK Sarkar, TK Pal. Development of RP-HPLC for analysis of human insulin. Indian Journal of Pharmaceutical Sciences. 2006;68(5): p.662-5.

# Chapter 4

# Preparation and Characterization of Insulin loaded Calcium Alginate Nanoparticles

Kush Patel[1] and Jerry Nesamony[1,*]

[1] Division of Industrial Pharmacy, Department of Pharmacy Practice, College of Pharmacy and Pharmaceutical Sciences, University of Toledo, Toledo, Ohio, USA

[*]**Corresponding author**:
Department of Pharmacy Practice
College of Pharmacy and Pharmaceutical Sciences
University of Toledo
3000 Arlington Avenue, MS1013
Toledo, OH 43614
419 383 1938 (Tel)
419 383 1950 (Fax)
jerry.nesamony@utoledo.edu

**Keywords:** Insulin, calcium alginate nanoparticles, diabetes, characterization, *invitro*, quantification

## 4.1 Abstract

The purpose of this research work was to develop insulin loaded calcium alginate nanoparticles (CANp) for oral delivery of insulin. The preparation of Insulin-loaded calcium alginate nanoparticles was performed by a novel and efficient method, interfacial cross-linking in nanoemulsions (IFaCLiNE) developed in our research lab. The prepared nanoparticles were evaluated by particle characterization and surface charge measurements. Subsequently, human recombinant, insulin was loaded into CANp. Differential scanning calorimetry (DSC) was used to evaluate the thermal properties. The quantitative determination of insulin in CaNP was done via an ELISA assay and *in vitro* drug release studies. A reversed phase C18 HPLC column with fixed compositions of 0.2 M sodium sulfate buffer and acetonitrile as mobile phases was used in the HPLC analysis of insulin. The insulin peak retention time was observed to be 7.2 minutes. To further identify the stability of insulin in CaNP the qualitative determination of insulin was made through LC-MS tandem mass spectrometry. The determination of precursor ions was achieved through mass spectroscopy (MS) and the insulin peak detection using liquid chromatography.

## 4.2 Introduction

Diabetes affects over 8% of the US population, and its prevalence in US residents aged 65 and older is an astounding 27%. About 26% of diabetic patients take insulin with an oral agent or by itself as part of regular therapy. Daily insulin injections are still the only available method to give insulin to diabetic patients and carry potential health risks such as local hypertrophy, fat deposition at injection site, and peripheral insulinemia (Eva

Tudurí, 2012). Moreover, the patient adherence is low with parenteral route of administration. There is a need for the development of an alternative drug delivery technique for insulin with minimal side effects and better adherence.

Insulin is a peptide hormone produced by the β-cells of pancreas and helps regulate the blood sugar levels in the body. It is comprised of 51 amino acids sequence (A chain=30 amino acids and B chain = 21 amino acids) with molecular weight of ~5800 Daltons (EP Kroef, 1989). It regulates the storage of glycogen in the liver and accelerates the oxidation of sugar in cells. The recombinant human insulin is a form of insulin made from recombinant DNA technology that is identical to human insulin and is used in the treatment of Diabetes Mellitus (Kohlmann, 2008).

In the recent years biopolymers have been investigated extensively and used in the biomedical and pharmaceutical industries. Biopolymers are in general nontoxic and easily degraded and eliminated from the body thereby making them safe for human use. A variety of compounds including antifungal, antiviral, and aminoglycosides have been successfully formulated into natural biopolymer carrier systems(Adam Friedman M.D 2013). The present study involves the use of alginates with the aim of developing a natural polymer-based drug delivery system for insulin.

Alginate is a naturally occurring copolymer that is extracted from brown seaweeds *Phaeophyceae*, mainly *Laminaria*. It is composed of two monomeric units namely 1,4-linked β-D-Mannuronic acid (M) and α-guluronic acid (G). These monomers are distributed as homogenous blocks (GG or MM) or as alternating sequences (GM). The

resultant cavity formed when two G-blocks are positioned opposite to each other, fits in a divalent cation (Calcium, has the right size) leading to the formation of calcium alginate beads (Draget KI, 2006). The resultant structure of linked chains in called an egg-box model (figure 4.1). Owing to the flat shape of the M-chains, they cannot chelate the calcium ions. Alginate is particularly useful in the encapsulation of unstable water-soluble molecules, such as proteins because it creates a compatible hydrophilic environment that protects proteins from denaturation (Augst AD, 2006).

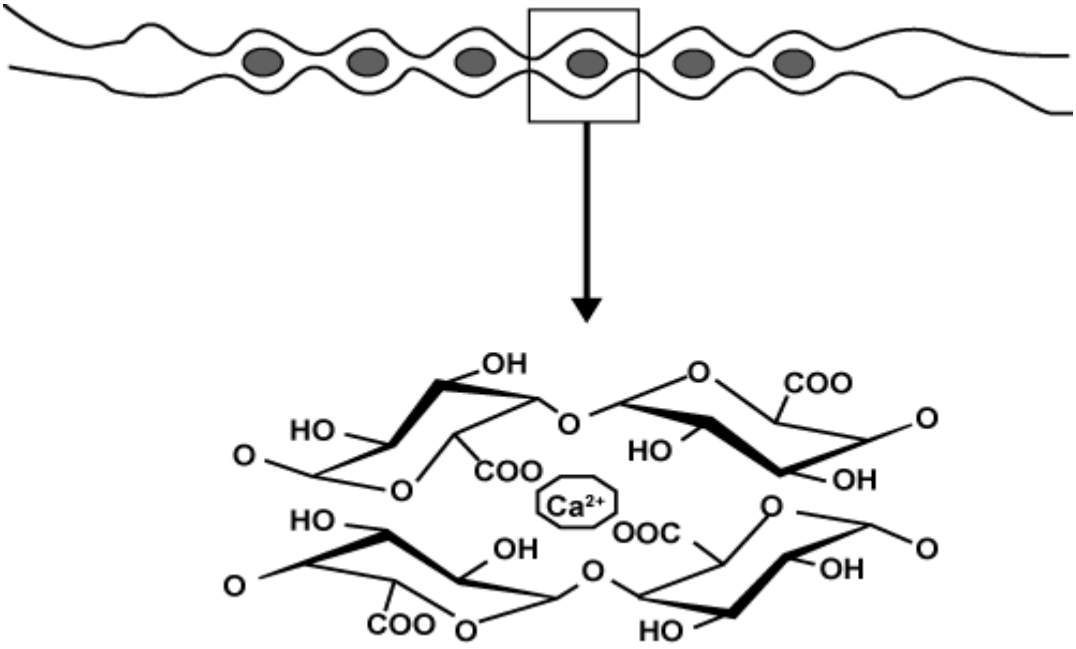


**Figure 4.1**: Egg-box model

Oral drug delivery is considered to be the most favorable route of drug administration for systemic effects. Majority of patients prefer to take drugs orally rather than through other routes. One requirement for successfully delivering proteins orally is by protecting the drug from degradation in the acidic environment (low pH) of the stomach (L.Shargel, 1999). Human insulin is prone to the β-elimination reaction thereby undergoing chemical

degradation in the stomach when given orally. One solution to the problem is formulation of pH sensitive hydrogels using appropriate polymers (Y. Kimura, 1993). The pH sensitive polymers can facilitate drug release into the small intestine by remaining intact in the stomach but swelling or degrading in the intestine which has alkaline pH.

Thus one of the key features of an oral delivery system for insulin should be the ability to transit through stomach with minimal impact from the adverse gastric environment. In this research we used calcium alginate which is a biopolymer to incorporate insulin. Calcium alginate is an enteric polymer that can transit through the stomach without being degraded by the gastric acid and enzymes. This leads to preservation of biological activity and stability of insulin and similar substrates before being presented to the site from where it can be absorbed.

The use of microemulsion as templates for formation of calcium alginate nanoparticles arises from the idea that, by appropriate control of synthesis parameters, one can use microemulsions as nanodispersions in order to produce nanoscale particles. These templates are thermodynamically stable dispersions, and can be considered as nonreactors (Nesamony J, 2005b).They can be used to carry out chemical reactions eventually leading to the formation of particles less than 1 micron in diameter. The use of DOSS as a surfactant has been approved as an oral, topical and intramuscular excipient (US Food and Drug Administration's Interactive Ingredients Database) (Nesamony J, 2005a). In this study two water-in-oil (w/o) microemulsions using DOSS as surfactant and different water components were prepared, which upon mixing eventually lead to the formation of calcium alginate nanospheres in 200-550 nm range. The process utilizes

mild experimental conditions thereby minimizing the physical and chemical stresses upon the biopharmaceuticals during preparation.

## 4.3 Materials and Methods

Materials

Sodium alginate (low viscosity, 2%) was purchased from Fisher Scientific, Pittsburgh, PA; Lot No: 89H0178; CAS No-9005-38-3. Calcium Chloride was purchased from Fisher Scientific Mfg. Corp, Lot No. 124 H06086; CAS # 10035-04-8. Dioctyl Sodium Sulphosuccinate (DOSS) was obtained from Sigma-Aldrich, St Louis, MO. Lot # 092162; CAS # 577-11-7. Human Insulin, Recombinant was acquired from Cat no. 91077C-1G and lot no. 12A213-D, (size 1g and storage -10 to -40C) was supplied by Sigma Aldrich FC St. Louis Missouri USA. Cyclohexane, USP and Ethanol, USP were obtained from Fisher Scientific, Pittsburgh, PA; Lot # 096744; CAS# 110-82-7. The other chemicals used for HPLC and LC-MS were of ACS grade.

Methods

### 4.3.1 Formulation and preparation of blank Nanoparticles (CANp)

The calcium alginate nanoparticles prepared in this research project were synthesized via a new method developed in our laboratory. The method involved use of micro-emulsified reactors in which the water phase solutions were prepared containing 0.5% (w/v) sodium alginate solution and the other containing 0.5% (w/v) calcium chloride solution. Subsequently, an aliquot of the surfactant DOSS was added to 100 ml cyclohexane (CH) to form a solution. The mixture was stirred using a magnetic stirring bar until a clear

solution of DOSS and CH was obtained. The aqueous sodium alginate solution and aqueous calcium chloride solution were emulsified into two separate formulations with an aliquot of DOSS-CH mixture by vortexing for 3 min followed by homogenization for 15 min. The prepared template microemulsions were allowed to stabilize thermodynamically for 12-24 hrs in order to obtain stable microemulsion formulation. After equilibration, calcium chloride nanoemulsion reactor was poured into the sodium alginate nanoemulsion reactor. Simultaneously, the process of interfacial cross linking between the reactor nanoemulsions of sodium alginate and calcium chloride lead to the formation of calcium alginate nanoparticles. The excess of surfactant was then removed by using ethanol as a separating solvent. The nanoparticles were then centrifuged for 30 minutes under 21,000 rpm at 23 °C. After centrifugation, the supernatant CH was siphoned off and ethanol, USP was added. The nanoparticles were gradually washed with ethanol solution followed by centrifugation for 30 minutes under 21,000 rpm at 23 °C. The washing process of nanoparticles was done 3- 4 times. The nanoparticles were eventually harvested by evaporating the excess ethanol in a vacuum dry oven operated at ambient room temperature.

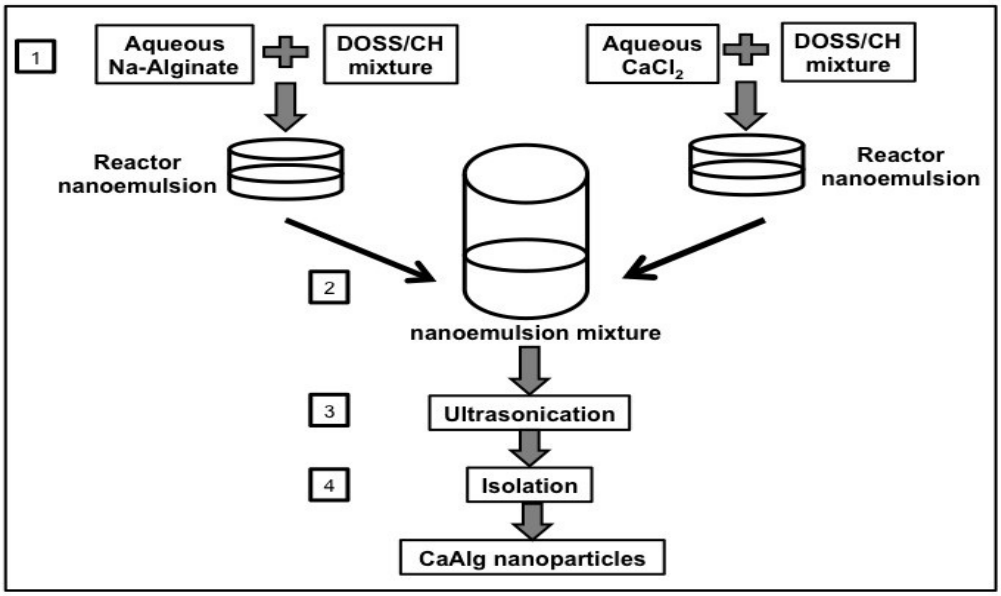


**Figure 4.2**: Schematic diagram of process used to prepare CANs

### 4.3.2 Incorporation of insulin into nanoparticles

Human insulin, recombinant was added into the aqueous sodium alginate dispersion. This was then emulsified into a nanoemulsion and then equilibrated for 24 hours. Thereafter, the process shown in figure 4.2 was followed for the preparation of drug loaded calcium alginate nanoparticles.

### 4.3.3 Characterization of Nanoparticles

a. Particle size determination

The particle size was measured through dynamic light scattering (DLS). Both aqueous 0.5%Na-Alginate/ DOSS/cyclohexane and aqueous 0.5%$CaCl_2$/ DOSS/cyclohexane microemulsions were placed in 6X50 mm culture tubes. Dynamic light scattering

apparatus (PSS Nicomp ZLS 300, California, USA) equipped with a He-Ne laser (100 mW, 658 nm) was used to perform the measurements. The scattered light was collected at 90° and analyzed using proprietary software supplied by the instrument manufacturer. The samples were run in triplicates and the mean particle size with standard deviation was reported.

b. Zeta Potential Determination

The DLS instrument was used to measure the surface charge present on a nanoparticle which is commonly referred to as zeta potential. The DLS instrument operated in the electrophoretic light scattering (ELS) mode allows measuring the zeta potential with scattering angle of -14.06° and temperature of 23°C. The measurements were performed on both the blank nanoparticles and insulin-loaded nanoparticles.

### 4.3.4 Thermal Analysis

Blank CANp and insulin-loaded nanoparticles were characterized by thermal analysis in a Perkin Elmer Diamond DSC. Samples were hermetically sealed in aluminum pans and subjected to a heating cycle from 10 ˚C-200 ˚C at a rate of 10 ˚C/min against an empty pan under a nitrogen atmosphere. DSC data was analyzed using Pyris software.

### 4.3.5 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to characterize the size, shape and surface of the particles (FEI Quanta 3D FEG Dual Beam Electron Microscope). The samples were prepared by placing dried nanoparticles powder on a double sided adhesive tape fixed on an aluminum stub. The samples were made electrically conductive by sputtering

with gold in vacuum for 55 sec at 20 mA. The sample was then visualized in the electron microscope.

### 4.3.6. Transmission Electron Microscopy

One drop of the prepared sample was pipetted onto a Formvar/Carbon 400 mesh copper grid (Ted Pella, CA). The particles were suspended in 95% ethanol and placed on to the TEM grid. After 10 minutes, excess sample was removed using a lint free wipe and the sample air dried overnight at room temperature prior to imaging using a scanning transmission electron microscope (Hitachi HD-2300A, Hitachi High Technologies America, IL, USA) operating at an acceleration voltage of 200 kV.

### 4.3.7. HPLC analysis of Insulin

The quantitative determination of insulin was done in an HPLC system comprising of Waters e2695 separations module for isocratic solvent delivery equipped with Waters 2998 PDA detector. The HPLC system was operated using Waters Symmetry Shield$^{TM}$ RP18 5µm particle size with column diameter of 4.6 mm and length of 250 mm. An isocratic separation method was employed with mobile phase composition of 73:27 (v/v) of 0.2 M sodium sulfate buffer (pH 2.4 with o-phosphoric acid) and acetonitrile (ACN) respectively. The isocratic run was made for 10 minutes. The eluent was pumped at a flow rate of 1.5 ml/min with a simultaneous injection volume of 50µl. The detection wavelength was set to 214 nm and the column temperature was maintained at 40 °C. The method was validated and was found linear in the range of 0.35 IU/ml-5.6 IU/ml ($R^2$ = 0.9978).

### 4.3.8. *In vitro* drug release studies

An aliquot of the insulin loaded nanoparticles were placed in 30 ml of phosphate buffer saline (pH 7.4) kept in a reciprocating shaker water bath maintained at 37 °C. Appropriate amount of samples were withdrawn and replaced by fresh buffer medium. The loading capacity and entrapment efficiency of insulin were determined. The released insulin was quantitated by HPLC.

### 4.3.9. ELISA assay

An in vitro release was performed by placing insulin loaded CANp in PBS (pH 7.4) kept in a shaking water bath maintained at 37 °C. Aliquots of the buffer were withdrawn at regular time intervals and replenished with fresh buffer. The samples containing released insulin were then analyzed via an enzyme linked immuno sorbent assay (ELISA) using a commercial insulin ELISA kit (Invitrogen Corp, CA, catalog # KAQ1251). The optical density values were determined based on the readings obtained from plate reader.

### 4.3.10. LC-MS detection for Insulin

The analysis of insulin was performed using a Triple Quadrupole mass spectrometer (Varian 320-MS auto sampler) with an electrospray ionization source interfaced with a multi-dimensional liquid chromatography. The revered phase Symmetry Shield$^{TM}$ RP18 5µm particle size with column diameter of 4.6 mm and length of 250 mm (Waters, USA) was used for the analysis. The mobile phase was composed of 0.1% formic acid in a mixture of $H_2O$: ACN (98:2, v/v) (solvent A) and 0.1% formic acid in a mixture of $H_2O$: ACN (2:98, v/v) (solvent B). The linear gradient method was then run with 80% solvent

A at the start (t=0 min), held for 5 min, decreased to 60% solvent A at t=10min, and to 40% solvent A at t=14 min and held there for 2 minutes (until 16 min). The gradient was returned to the initial mobile phase composition (80% solvent A) at t=22 min before starting the next injection. The sample loop volume was 300µl each and the injection volume was 20µl for all the runs.

### 4.3.11. LC-MS *invitro* study

The drug *invitro* studies was performed for insulin loaded CANp. Accurately weighed 5mg of CANp were dispersed into 20ml of ammonium hydroxide buffer solution adjusted to pH 7.4. Appropriate amount of samples were withdrawn at specific intervals followed by replacement with buffer solution. Samples were collected at 0.5, 1, 1.5, 2 and 4h time intervals. The analysis of insulin was made through Triple Quadrupole mass spectrometer (Varian 320-MS auto sampler) with an electrospray ionization source interfaced with a multi-dimensional liquid chromatography.

## 4.4 Results and Discussion

### 4.4.1 Formulation and Preparation of blank nanoparticles

The aim of this study was to produce calcium alginate nanoparticles by interfacial cross-linking method and to study the influence of polymeric properties and formulation parameters on the final nanoparticle size, drug-polymer interactions, and *in vitro* drug release studies. The idea underlying development of this method is based on the fact that alginates in presence of a divalent metal ion form a gel like structure. In solutions,

alginates behave like flexible oils. However, upon interaction with divalent metal ions like calcium, they form an ordered structure.

The preparation of w/o microemulsion served as the media for the formation of nanoparticles. Two separate microemulsions containing sodium alginate and calcium chloride were prepared. Upon mixing of two, a third microemulsion containing calcium alginate nanoparticles formed.

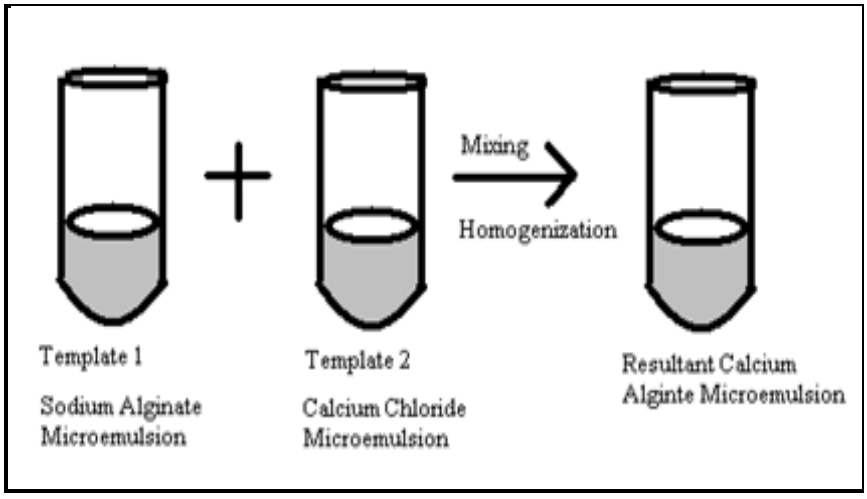


**Figure 4.3**: Schematic representation of preparation of calcium alginate nanoparticles by interfacial cross linking method

Ternary phase diagram

Ternary phase diagrams can be three dimensional, but may also be represented in two dimensions for the ease of drawing and reading. Different concentrations of DOSS and Cyclohexane were prepared and are shown in the Table 4.1.

| **Table 4.1** |
|---|
| a) 0.1M DOSS/Cyclohexane |
| b) 0.2M DOSS/Cyclohexane |
| c) 0.3M DOSS/Cyclohexane; |
| d) 0.4M DOSS/Cyclohexane |

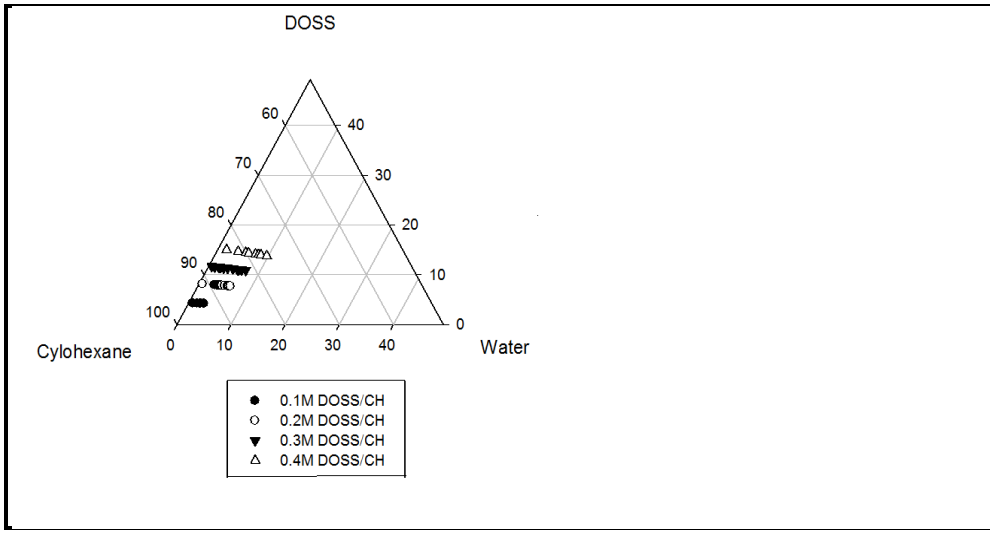


**Figure 4.4**: Ternary phase diagram showing incorporation of water by various concentrations of DOSS/cyclohexane

To each of the DOSS-CH mixture, water was added drop wise using titration method and visually inspected. The resultant emulsions were transparent, slightly turbid or a milky emulsion depending on the composition. The trial compositions were plotted on a ternary phase diagram. The area bounded by the points in the phase diagram corresponds to the concentration range of component mixtures that produced visually clear and transparent microemulsions. About 0.25-1% aqueous sodium alginate dispersions were used in place of water to test the efficiency of the organic phases to form stable microemulsions with them. A similar procedure was repeated for calcium chloride microemulsion formulation.

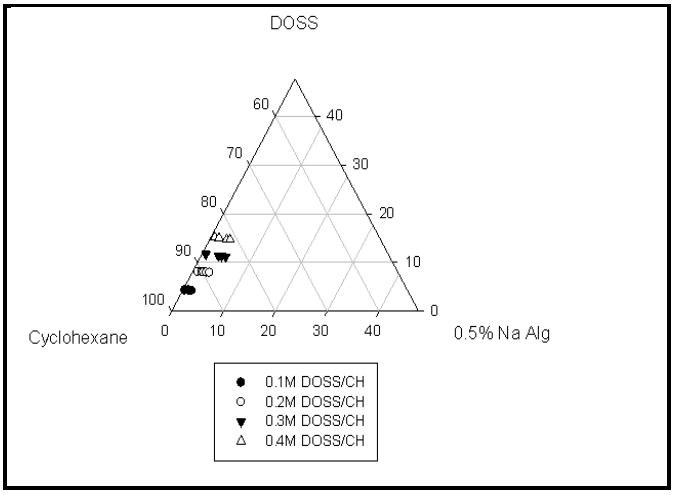


**Figure 4.5**: 0.5% sodium alginate by various concentrations of DOSS-CH.

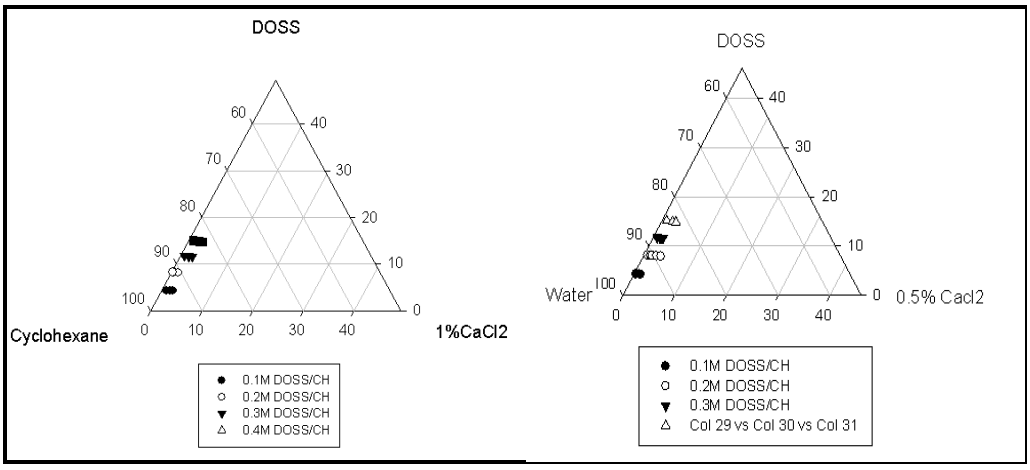


**Figure 4.6**(a)-left: Ternary phase diagrams depicting incorporation of 1% calcium chloride; **Figure 4.6**(b)-right: 0.5% calcium chloride by various concentrations of DOSS-CH

Cyclohexane demonstrated formation of clear microemulsions over a wide range of compositions of DOSS, aqueous sodium alginate, and aqueous calcium chloride. Based on the stability of the formulations, quantity of DOSS present, and ability to emulsify aqueous components, 0.2M DOSS-CH solution was used for further characterization. Small amounts of DOSS were desirable in the final product to facilitate ease of recovery of nanoparticles.

A comparison of all ternary phase diagrams indicated that DOSS-CH mixtures emulsified approximately similar amounts of calcium chloride and sodium alginate into clear microemulsions. An identical method was used to evaluate the presence of insulin in aqueous sodium alginate on microemulsion formation. It was determined that up to 2 % w/w insulin in sodium alginate formed clear and stable microemulsion in 0.2 M DOSS-CH solution.

### 4.4.2 Physicochemical properties of Calcium Alginate nanoparticles

a. Particle size determination

The particle size distribution influences the in vivo distribution, biological fate, toxicity, and targeting ability of nanoparticles. The particle size was measured using dynamic light scattering (DLS). The particle size of reactor emulsions was found to be less than 10 nm. The mean particle size of calcium alginate nanoparticles was found to be 456 ± 48 nm.

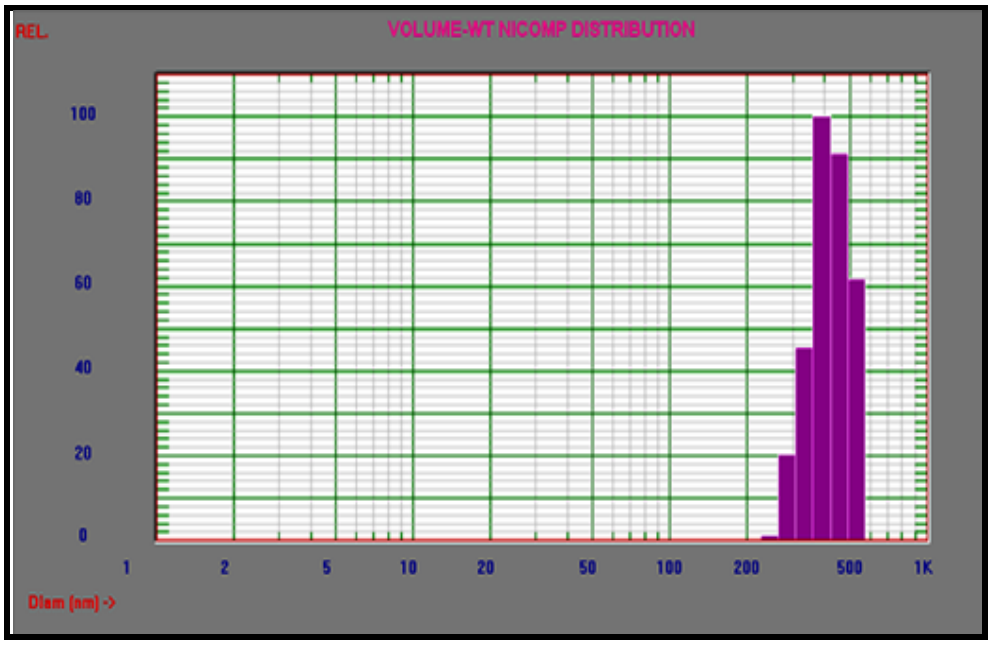


**Figure 4.7**: Particle size distribution of insulin loaded calcium alginate nanoparticles after mixing of reactor microemulsions.

b. Zeta potential determination

The DLS instrument was used in its electrophoretic light scattering mode to measure zeta potential. An average zeta potential of - 21 ± 1 mv was obtained. The negative charge could be the contribution of the sulphonate group of residual DOSS and carboxyl groups

of alginic acid (Chavanpatil, 2007). Nanoparticles are generally taken up by M cells and absorptive endocytes. It is likely that these negatively charged particles can be rapidly opsonized and massively cleared by the fixed macrophages when exposed to these cells. Sometimes surface modifications of these nanoparticulate systems with hydrophilic polymers are the most common way to control the opsonization process and to improve the biological half-life of the system (Pinto Reis, 2006).

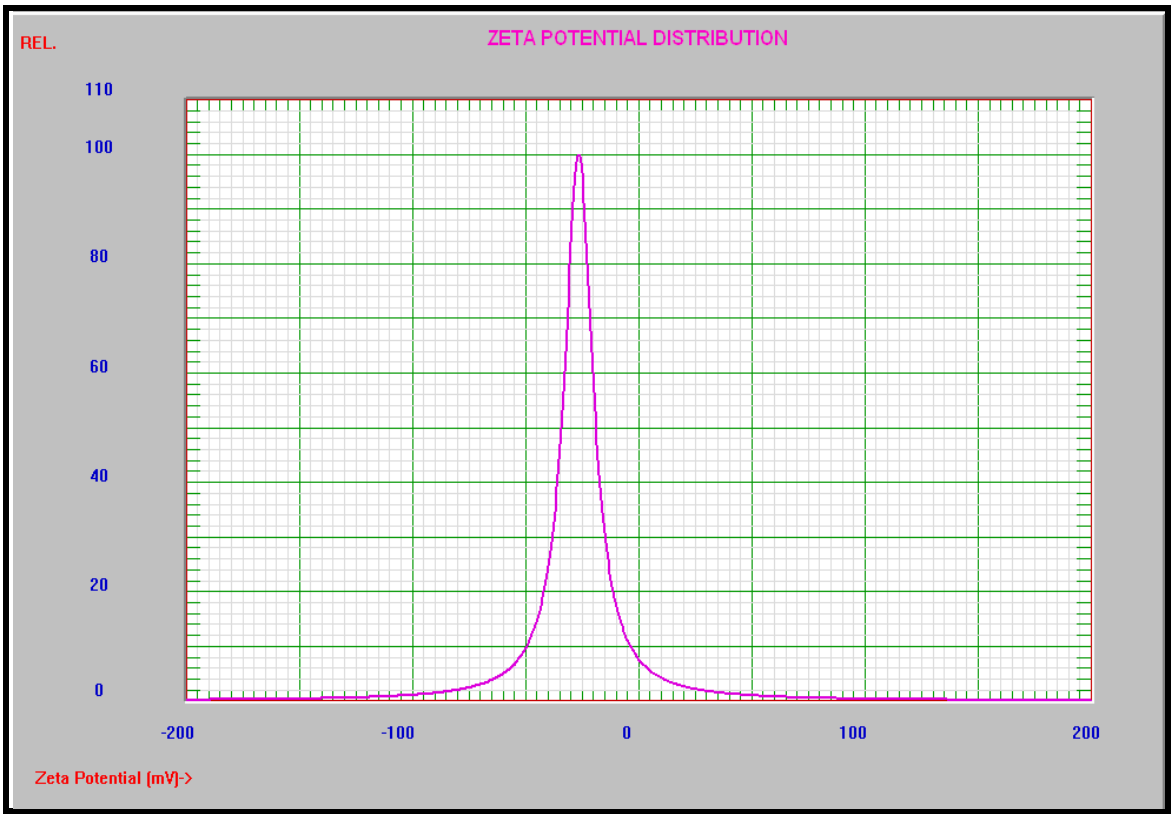


**Figure 4.8**: Zeta potential for insulin loaded calcium alginate nanoparticles

### 4.4.3 Thermal analysis

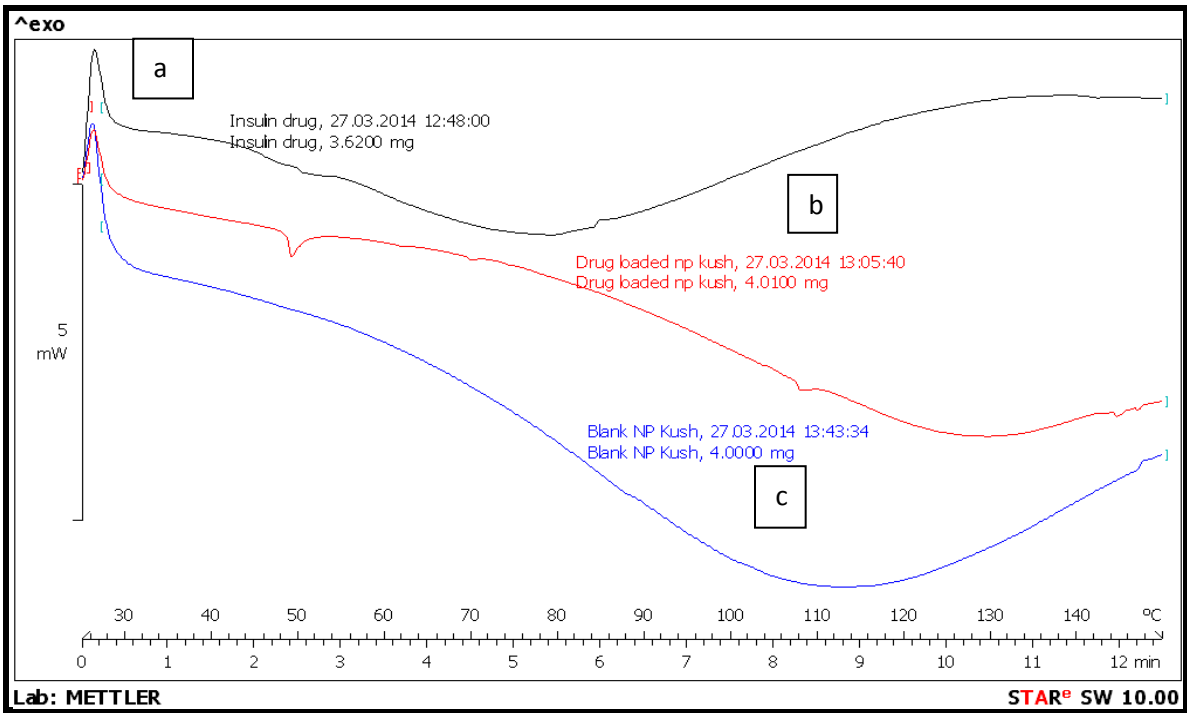


**Figure 4.9**: DSC thermograms representing a) insulin b) insulin loaded CANp c) blank CANp.

In figure 4.9, the thermograms of blank and drug loaded calcium alginate nanoparticles shows a broad endothermic event at around 110 °C and 130 °C respectively. The endothermic peaks represent loss of water moiety associated with hydrophilic groups of polymers (Mimmo, 2005). The insulin thermogram (labeled (a)) is characterized by a broad endothermic event at ~80 °C. This event corresponds to an enthalpic relaxation seen in crystalline insulin(Rigsbee, 1997). An endothermic event observed at 48 °C in thermogram (b) is the shift in the insulin enthalpic relaxation. The peak appears sharp and shifted to lower temperature owing to higher degree of hydration of insulin molecules within the nanoparticles(Sarmento, Ferreira, Veiga, & Ribeiro, 2006).

### 4.4.4 Scanning electron microscopy

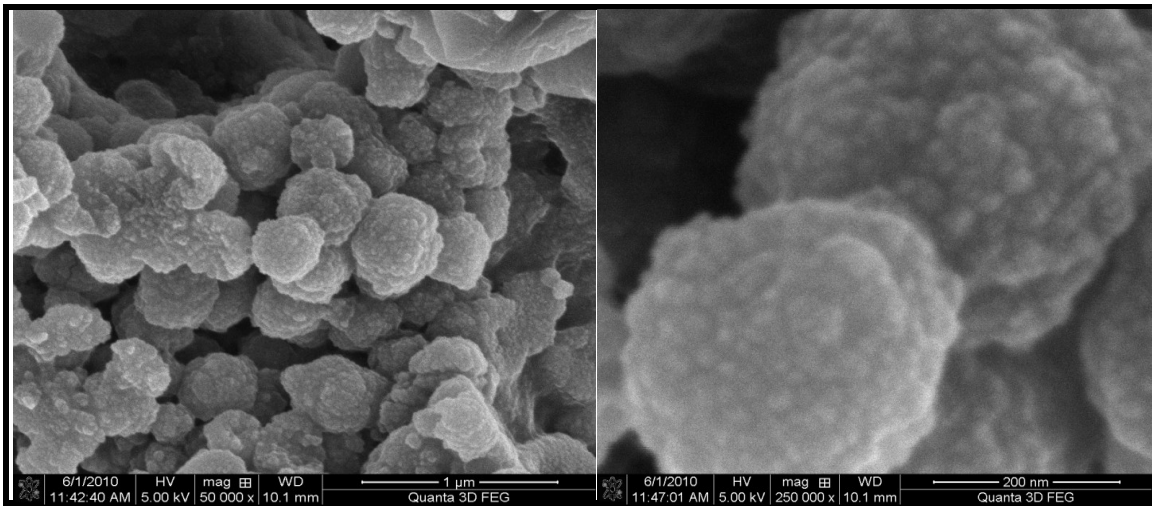


**Figure 4.10**(a) and **4.10**(b): SEM images of calcium alginate nanoparticles

The SEM images show nanoparticles that are more or less spherical in shape. The higher magnification image (figure 4.10 (b)) shows details of surface morphology. The surface of CANp appears to be irregular perhaps due to gradual drying of the nanoparticles during isolation and recovery process.

### 4.4.5 Transmission electron microscopy

The TEM images in figure 4.11(a) show that the matrix has a dense spherical shape with smooth periphery.

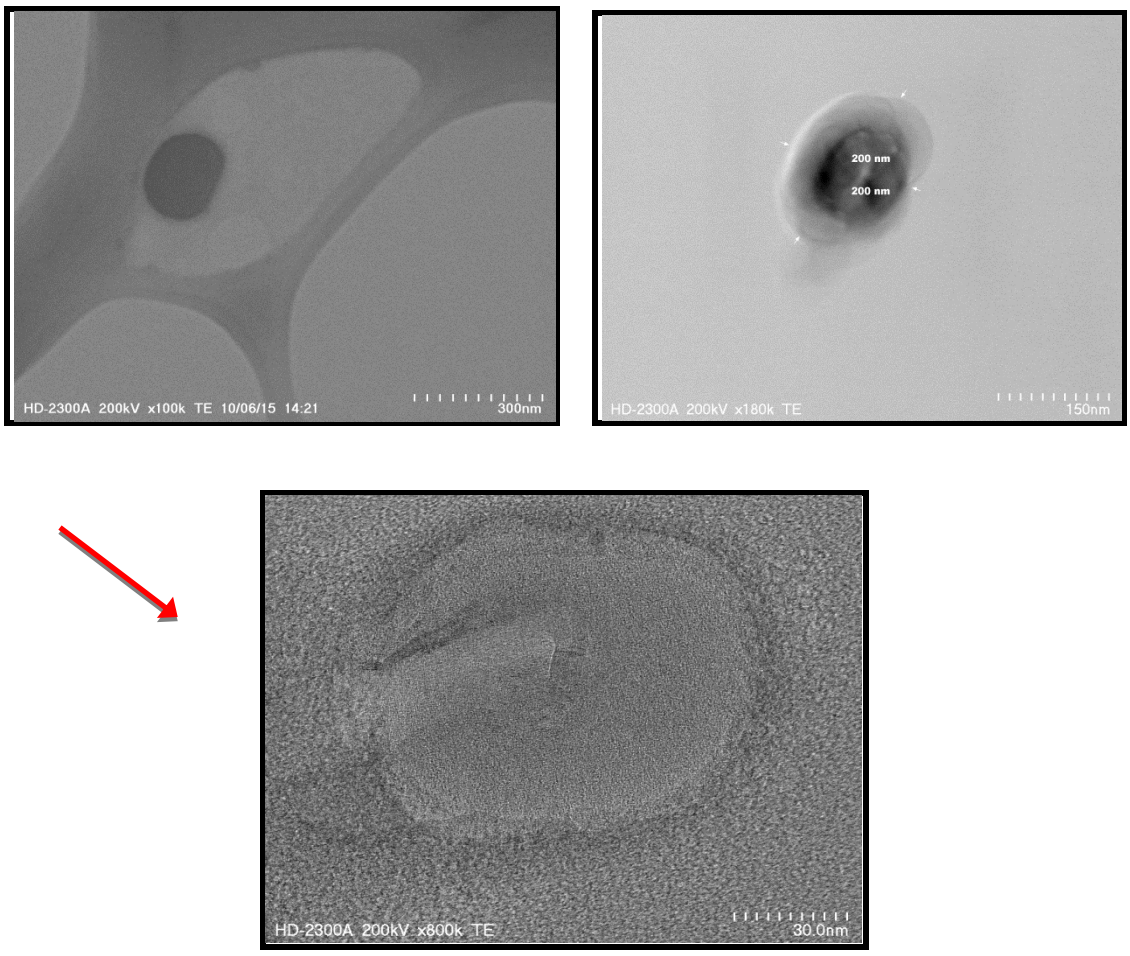


**Figure 4.11**: TEM of CANp (a) low magnification (b) higher magnification (c) highest magnification

In figure 4.11 (a) one CANp can be seen surrounded by residual DOSS. Figure 4.11 (b) offers more details of the internal matrix of the CANp. The TEM image shows a CANp of ~ 100 nm that is more or less spherical in shape. The dark spots visible within the particle represents calcium ions that cross link the alginate chains. Alginate being composed of carbohydrate units does not scatter electrons and hence does not appear as a dark matrix in the TEM image. Additionally the area marked by the arrow demonstrates the egg-box structure formed by layers of alginate units cross-linked by calcium ions.

### 4.4.6 HPLC analysis of Insulin

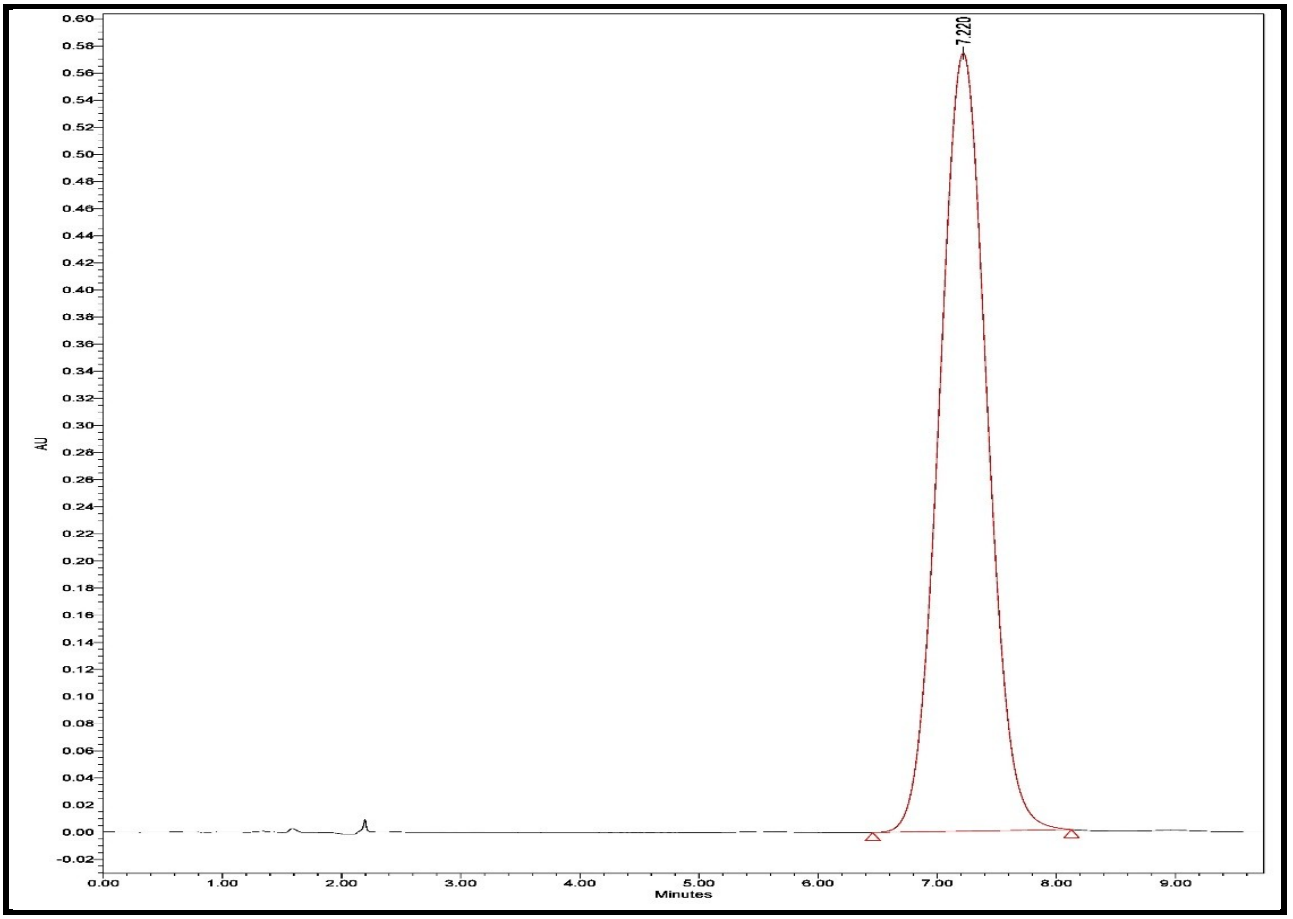


**Figure 4.12**: HPLC peak of Insulin

It was observed from the chromatograms that the identity of the insulin peak was clearly indicative from the retention time of the peaks eluted. In figure 4.12 the peak with a retention time at 7.220 minutes indicated that insulin is separating from the sample.

After the insulin peak elution at 7.220 min, the sample was still made to run up to 60 minutes to identify any peaks that may elute later during the run. No other peaks were observed during the entire run. The area under the curve response to Insulin

concentrations of 5.6 IU/ml, 2.8 IU/ml, 1.4 IU/ml and 0.7 IU/ml were used to obtain the percent recovery. The overall percent recovery obtained was 99.32±0.745.

### 4.4.7 *In vitro* drug release

The *in vitro* release studies for insulin loaded CANp was performed by placing aliquots of drug loaded nanoparticles in phosphate buffer saline of pH 7.4 maintained at 37 °C. The calibration curve obtained for human insulin standard solutions is shown in figure 4.13. The equation of the regression line was y= 25262x – 396637; $R^2$ = 0.9978, where "y" is peak area and "x" is insulin concentration in µg/ml.

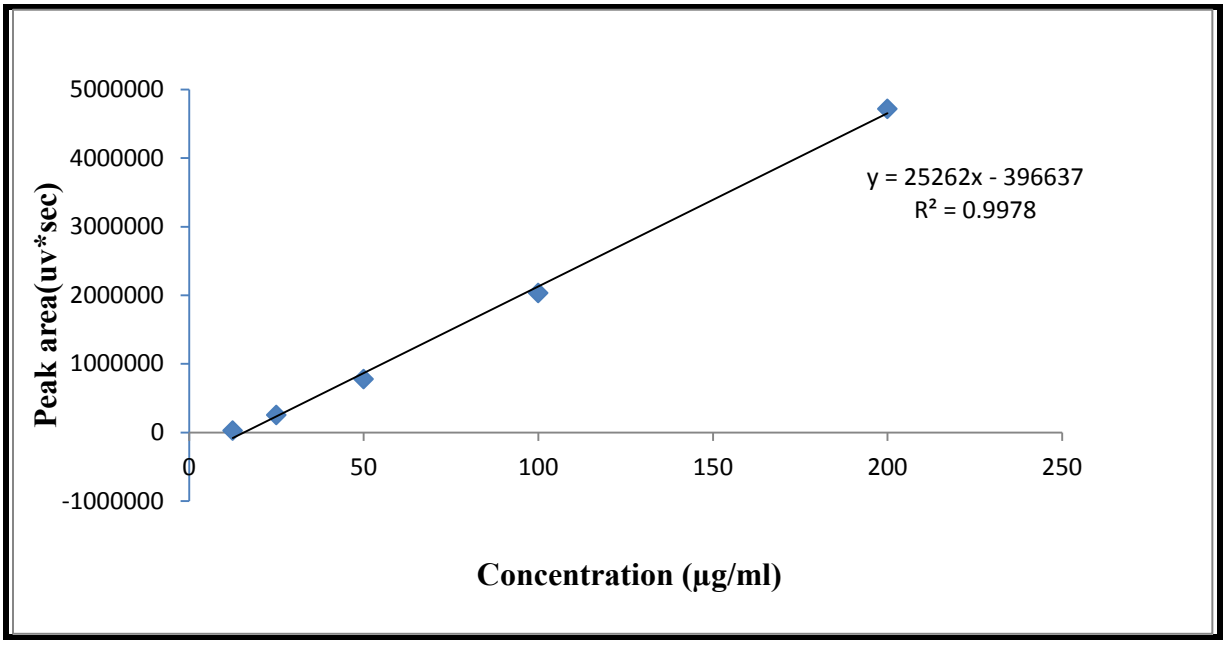


**Figure 4.13**: Calibration plot for insulin

The peak areas of insulin from *in vitro* release study were substituted in the equation obtained from calibration curve to determine the release profile. Aliquots of the release medium were taken at 0, 0.25, 0.5, 0.75, 1, 2 and 4h during the study and were analyzed.

The insulin drug release profile was characterized by an initial burst effect followed by a sustained flow pattern. The loading capacity of insulin in CANp was 13.1% and the entrapment efficiency calculated was 9.8%. Approximately >50% of the entrapped drug was released within1 h followed by a continuous slow release giving rise to a plateau. It can be concluded that the presence of protein adsorbed on the nanoparticle surface causes the initial burst release to occur, which is followed by slower release of the entrapped insulin within the core.

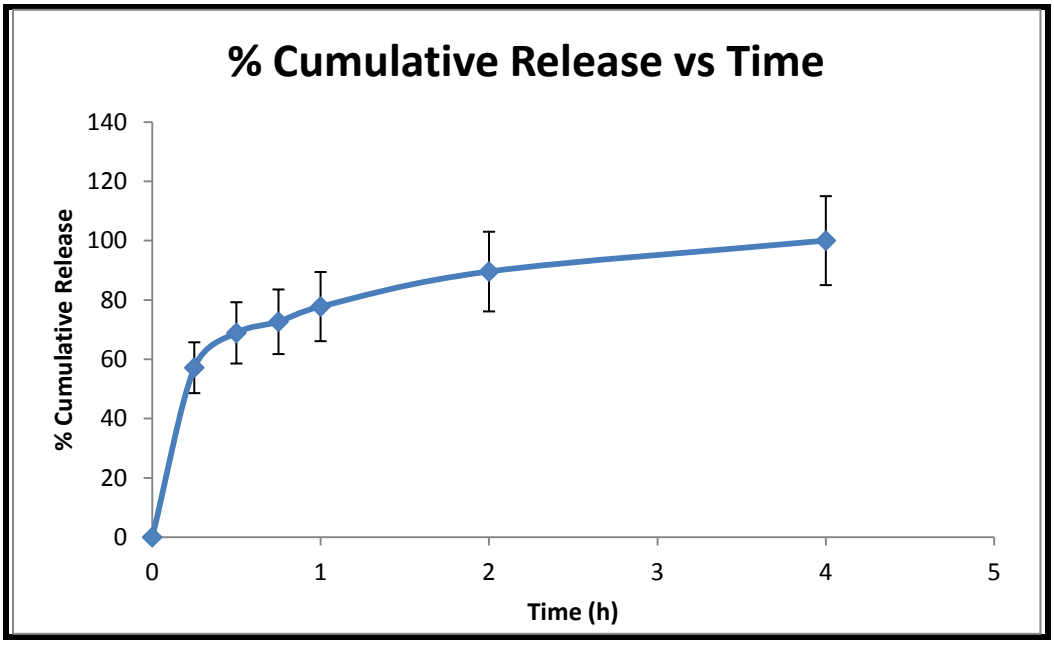


**Figure 4.14**: In vitro release profile of insulin from CANp(n=3)±S.D

### 4.4.8 Insulin ELISA assay

The structural integrity of entrapped insulin from CANp was evaluated using ELISA assay. It can be seen that about 300-400 µIU/ml of insulin was released within first 2h. The integrity of the insulin in the alginate matrix is evident from the ELISA study since

only insulin molecules that retain their structural integrity reacts with insulin specific antibodies. The process of nanoparticle preparation did not produce considerable loss of biological activity. The labile bonds and reactive side chains of proteins makes them fragile molecules which can lead to loss of biological activity.

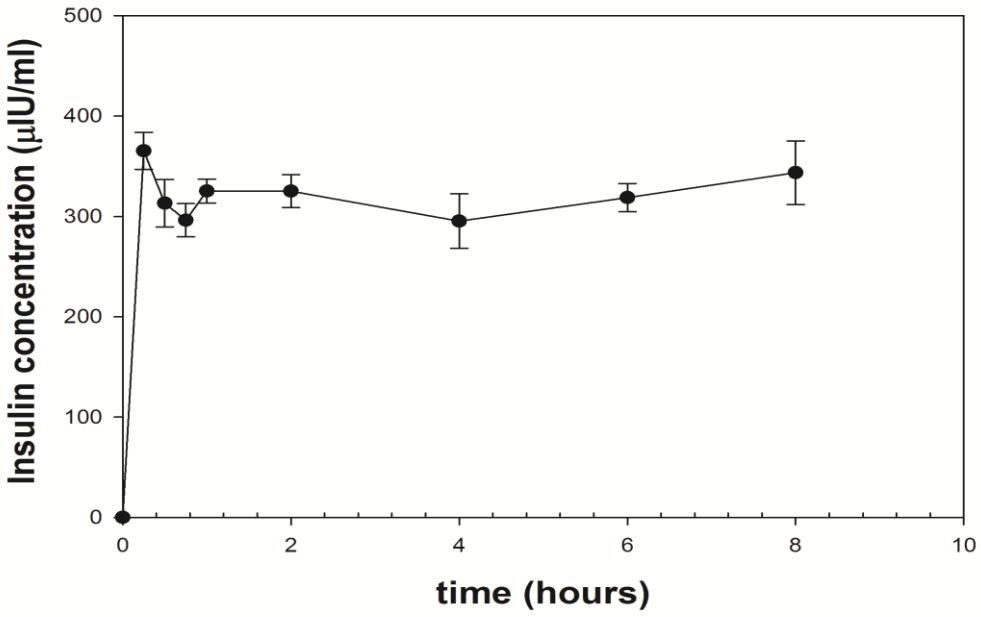


**Figure 4.15**: Quantitative analysis of insulin using ELISA assay(n=3)±S.D

The ELISA human insulin assay showed a succesful measurement of biologically active insulin with high degree of specificity using monoclonal antibodies (Touitou E, 1986). The results suggest that insulin loaded CANp can preserve the biological activity of associated insulin in the presence of several hydrophobic components as well as polar organic solvents.

### 4.4.9 LC-MS detection of Insulin

The positive ion ESI of insulin formed a five-fold protonated molecule $[M+5H]^{5+}$ at m/z 1161.9, corresponding to the average molecular weight of 5809.5 Da(Moussa, 2010). The higher MS background is generally observed when monitoring fragments below 300 ~m/z ratio for peptides(Erin E. Chambers, 2014). A single low molecular weight fragment yielding human insulin was seen at m/z of 226 may be from the final 2 amino acids in the B chain. The chromatographic peak elution for insulin was obtained around 11.7 minutes. The calibration curve with varying concentration ranges from 40-80 ppm of insulin was plotted and is shown in figure 4.16.

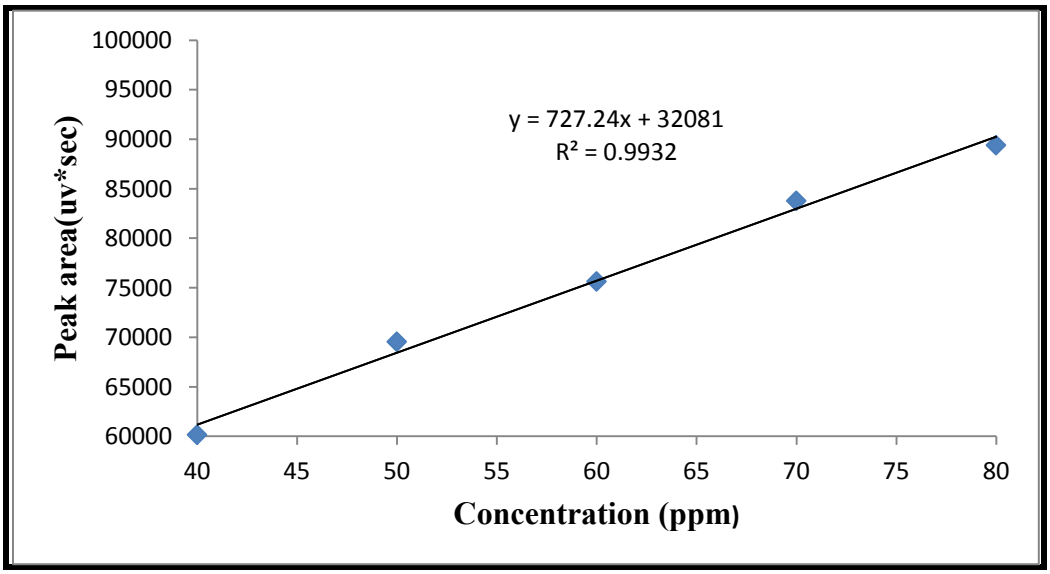


**Figure 4.16**: Calibration plot of insulin using LC-MS

Figure 4.17 shows the peak identification of insulin at specific precursor ion mass of 1161.5 m/z. The relative abundance of precursors formed were affected by the flow rate into the mass spectrum. Subsequently, gradual fragmentation at the utilized flow rate

was performed. The single reaction monitoring (SRM) transitions of insulin were predicted by making more than three runs. The intensity was then optimized.

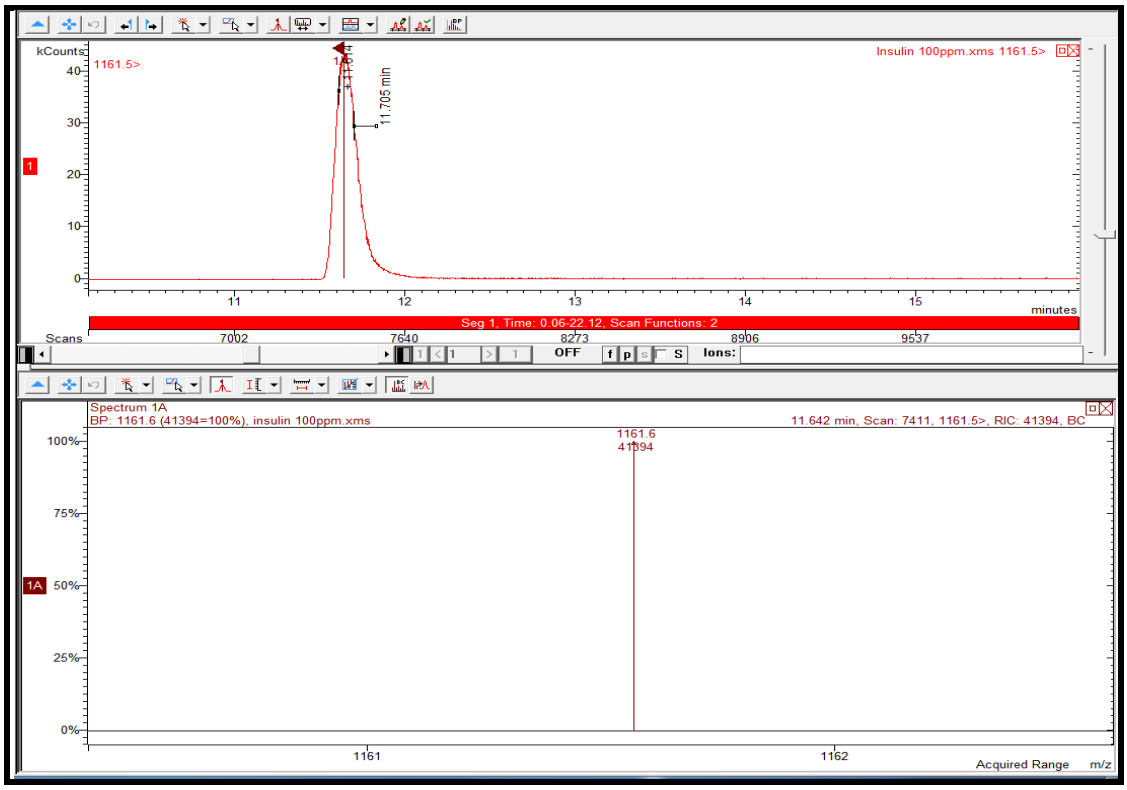


**Figure 4.17**: LC/MS detection of insulin at SRM

### 4.4.10 LC-MS *invitro* study

The *invitro* studies show an immediate burst release of insulin from CANp. Approximately about 80% of the entrapped drug is released within 2h. This indicates that most of the drug present on the surface of the nanoparticles is getting released which is follwed by sustained release or plateau phase. Moreover, the chemical integrity of insulin inside CANp stays intact and hence there is less significant loss in the biological activity of insulin. Figure 4.18 shows the percent cumulative release of insulin with respect to time.

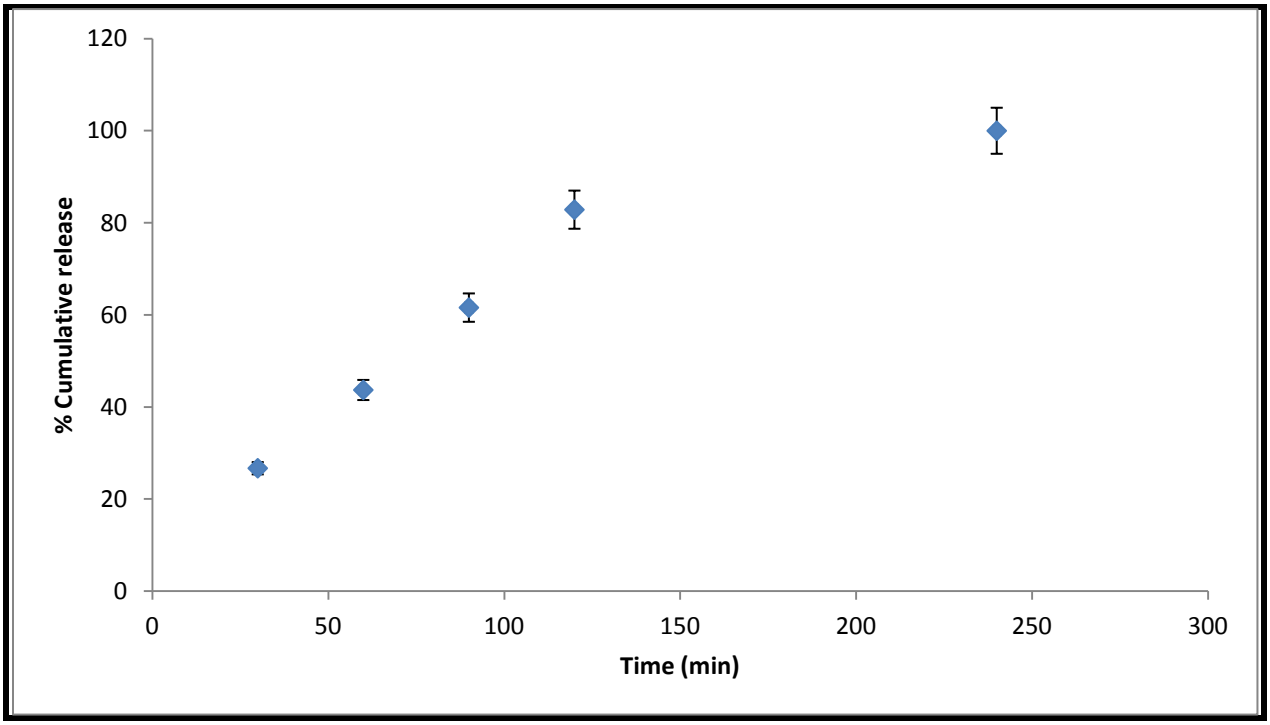


**Figure 4.18:** LC-MS *invitro* release profile (n=3)±S.D

### 4.4.11 Comparison of drug release profiles from HPLC, ELISA assay and LC-MS

The drug release studies from HPLC showed an immediate or burst release of insulin from CANp. It is a powerful technique used for quantification of proteins with higher molecular weights. From the drug release profile, more than 70% of drug is getting released within first hour. This might represent dose dumping of insulin from CANp which show hypoglycemic effect. ELISA is the most common method employed for protein quantification. However, the development of ELISA technique faced challenges including matrix interferences, cross reactivity and high quality reagents(Kristine de Dios, 2013). The LC-MS based approach for the qualitative determination of protein showed good selectivity, multiplexing capability and minimal matrix effects. Although, the implementation of mass spectrometric assays in protein identification is much less since mass spectrometric assays have not provided a clear advantage over ELISA assays. Certain cases have shown the quantification of proteins which are very difficult or impossible to measure by immunological methods(M. Bantscheff, 2007). This includes the assaying of the protein isoforms, measurement and quantification of the panels of protein in biological samples. The LC peak at single chain reaction monitoring (SRM) showed good peak shape and resolution than HPLC(Jessica M. Faupel-Badger, 2010). Overall, the quantification analysis was done using HPLC technique, the biological integrity of protein was preserved using ELISA assay and the qualitative determination was done through LC-MS analysis.

## 4.5 Conclusions

Insulin loaded calcium alginate nanoparticles were successfully prepared by the new interfacial cross linking technique developed in our laboratory. The inherent tendency of alginates to form gels in presence of a divalent ion, enables preparation of nanosized particles using emulsions as templates. Careful screening of excipients and preparation conditions led to formation of stabilized microemulsions which were then further processed to form solid nanoparticles. Spherical calcium alginate nanoparticles of sizes between 200-500nm were successfully obtained. DLS, zeta potential, SEM, and TEM studies of the nanoparticles confirmed physico-chemical properties that are desirable in drug delivery applications. *In vitro* release studies demonstrated burst release of insulin from the CANp and this indicates drug adsorption onto the surface along with encapsulation. ELISA assay demonstrated that the entrapped insulin maintained its structural and biologic integrity during the preparation, isolation, and recovery process. The HPLC and LC-MS techniques enabled quantitative and qualitative determination of insulin released from CANp in *in vitro* conditions. Future in vivo studies in diabetic rats will help to assess the biological effect of insulin loaded CANp.

## 4.6 References

1. Eva Tudurí, J. E. B. a. T. J. K. (2012). Restoring insulin production for type 1 diabetes. *Journal of Diabetes, 4*(4), p. 319-331.

2. EP Kroef, R. O., EL Campbell, RD Johnson. (1989). Production scale of biosynthetic human insulin by reverse phase liquid chromatography. *Journal of Chromatography A*.

3. Kohlmann, M. R. L. a. K. L. (2008). Recombinant Human Insulin. *Biotechnology Progress, 8*(6), p. 469-478.

4. Adam Friedman M.D , K. B. M. D. (2013). Nanotechnology in the Treatment of Infectious Diseases. *Nanotechnology in Dermatology*, p. 187-200.

5. Alginates. [press release]. Boca Raton, FL, USA: CRC Press 2006

6. Augst AD, K. H., Mooney DJ. (2006). Alginate Hydrogels as Biomaterials. *Macromolecular Bioscience, 8*(6), p.623-633.

7. L. Shargel, A. Y. (Ed.). (1999). *Applied biopharmaceutics and Pharmacokinetics*. New York: McGraw-Hill

8. Biomedical Applications of Polymeric Materials [press release]. Boca Raton, FL: CRC Press Inc 1993.

9. Nesamony J, K. W. (2005b). IPM/DOSS/water microemulsions as reactors for silver sulfadiazine nanocrystals synthesis. *J.Pharm Sci, 94*(6), p. 1310-1320.

10. Mohanraj, V. a. Y. C. (2007). Nanoparticles-a review. *Tropical Journal of Pharmaceutical Research, 5*(1), p. 561-573.

11. Muller, R. a. K. W. (1993). Surface modification of iv injectable biodegradable nanoparticles with poloxamer polymers and poloxamine 908. *International journal of pharmaceutics, 89*(1), p. 25-31.

12. Chavanpatil, M. D. (2007). Polymer-surfactant nanoparticles for sustained release of water-soluble drugs. *Journal of pharmaceutical sciences, 96*(12), p. 3379-3389

13. Pinto Reis, C. (2006). Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. *Nanomedicine: Nanotechnology, Biology and Medicine, 2*(1), p.8-21.

14. Mimmo, T., Marzadori, C., Montecchio,D. and Gessa, C. (2005). Characterization of Ca and Al pectate gels by thermal analysis and FT-IR spectroscopy. *Carbohydrate Research,27(1), 340*(16), p. 2510-2519.

15. Rigsbee, M. J. P. a. D. R. (1997). The Stability of Insulin in Crystalline and Amorphous Solids: Observation of Greater Stability for the Amorphous Form. *Pharmaceutical Research, 14*, p. 1379-1387.

16. Sarmento, B., Ferreira, D., Veiga, F., & Ribeiro, A. (2006). Characterization of insulin-loaded alginate nanoparticles produced by ionotropic pre-gelation through DSC and FTIR studies. *Carbohydrate Polymers, 66*(1), p. 1-7.

17. Touitou E, R. A. (1986). Targeted enteral delivery of insulin to rats. *Int J Pharm, 30*, p. 95-99.

18. Moussa, B. A. (2010). Validated RP-HPLC method for determination of Recombinant Insulin in bulk dosage form. *E-Journal of Chemistry, 7*, p. 449-457.

19. Erin E. Chambers, K. J. F., Norman Smith, Leah Ashraf, Janaka Karalliedde, David Cowan and Cristina Legido-Quigley. (2014). Multidimensional LC-MS/MS Enables Simultaneous Quantification of Intact Human Insulin and Five Recombinant Analogs in Human Plasma. *Anal. Chem, 86*(1), p. 694-702.

20. Kristine de Dios, A. M., Robin Marsden, Jason Pinkstaff. (2013). Comparison of Bioanalytical methods for the quantification of PEGylated human insulin. *Journal of Immunological Methods, 396*, p. 1-7.

21. M. Bantscheff, M. S., G. Sweetman, J. Rick, and B. Kuster. (2007). Quantitative mass spectrometry in proteomics: a critical review. *Analytical and Bioanalytical Chemistry, 389*(4), p. 1017-1031.

22. Jessica M. Faupel-Badger, B. J. F., Xia Xu, Roni T. Falk, Larry K. Keefer, Timothy D. Veenstra , Robert N. Hoover and Regina G. Ziegler. (2010). Comparison of Liquid Chromatography-Tandem Mass Spectrometry, RIA, and ELISA Methods for Measurement of Urinary Estrogens. *Cancer Epidemiology Biomarkers Prev, 19*, 292.

# Chapter 5

# Discussion

In this study, calcium alginate nanoparticles were prepared using aqueous calcium chloride and aqueous sodium alginate mixtures with the addition of surfactant DOSS and Cyclohexane as an oil particle. This interfacial cross linking in nanoemulsion (iFacLine) method merges into two steps, namely the formation of reactor nanoemulsion from both aqueous calcium chloride and aqueous sodium alginate microemulsion mixture, by the regulation at the ambient room temperature of the surrounding environment. The CANp are formed when ultracentrifugation is applied at higher rounds per minute (rpm) speed. The idea underlying development of this method is based on the fact that alginates in presence of a divalent metal ion form a gel like structure. In solutions, alginates behave like flexible oils. However, upon interaction with divalent metal ions like calcium, they form an ordered structure. The excessive surfactant surrounding the CANp was removed using 95% ethanol solution, USP. The washing was performed 3-4 times in order to remove maximum amount of surfactant possible. This can be clearly demonstrated from the transmission electron microscopy (TEM) image of CANp in figure 3.11(b). Some of

the common methods involved in the preparation of CANp include phase inversion temperature (PIT) technique(Machado AH1), reverse microemulsion template(You), dropping aqueous alginate solution into solution of calcium salt(You).

A comparison of all ternary phase diagrams indicated that DOSS-CH mixtures emulsified approximately similar amounts of calcium chloride and sodium alginate into clear microemulsions. An identical method was used to evaluate the presence of insulin in aqueous sodium alginate on microemulsion formation. It was determined that up to 2 % w/w insulin in sodium alginate formed clear and stable microemulsion in 0.2 M DOSS-CH solution. The average particle size obtained was 456 ±48nm. The particle size distribution influences the in vivo distribution, biological fate, toxicity, and targeting ability of nanoparticles. The zeta potential values were reported to be negative. When nanoparticles are administered intravenously their surface charge determines its capability to interact with cell surface and other biological components. This is turn affects the in vivo fate of the nanoparticles (Mohanraj; Muller). Zeta potential can also be used to determine whether a charged active material is encapsulated within the system or adsorbed onto the surface or both. The negative surface charge indicates that the particles may be coated with an oppositely charged polymer such as chitosan or a similar biopolymer.

The surface morphology of CANp can be witnessed from the SEM images which showed spherical shape particles. The SEM images revealed the formation of non-aggregated and confirmed the size of the particles to be in nanometer range. The TEM images were in the agreement with the dynamic light scattering data which showed the particle sizing of the

CANp. The presence of surfactant DOSS on the surface of CANp might have been possible for the low entrapment efficiency of insulin loaded CANp.

The thermal behavior of CANp was determined using Differential scanning Calorimetry. The melting point of insulin is ~80 $^{0}$C. This confers that an exothermic peak should be observed between 75-85 $^{0}$C. The endothermic peaks represent loss of water moiety associated with hydrophilic groups of polymers (Mimmo). The insulin thermogram (labeled (a)) is characterized by a broad endothermic event at ~80 °C. This event corresponds to an enthalpic relaxation seen in crystalline insulin(Rigsbee).

The reversed phase HPLC system was used for the identification of the insulin peak. Simultaneously, quantification of the peaks was done through insulin standards and the validation parameters were identified. The peak was eluted at 7.220 min and the sample was still made to run up to 60 minutes to identify any peaks that may elute later during the run. No other peaks were observed during the entire run. The area under the curve response to Insulin concentrations of 5.6 IU/ml, 2.8 IU/ml, 1.4 IU/ml and 0.7 IU/ml were used to obtain the percent recovery. The overall percent recovery obtained was 99.32±0.745.

Subsequently, the *invitro* release study of insulin was performed underlying the fact that burst or immediate release of insulin could be obtained from CANp. The loading capacity of insulin in CANp was 13.1% and the entrapment efficiency calculated was 9.8%. Approximately >50% of the entrapped drug was released within1 h followed by a continuous slow release giving rise to a plateau. The biological integrity/assay of insulin in CANp was preserved from the study performed from enzyme ELISA assay. ELISA

assay is considered to be one of the most reliable assays for the antigen/antibody complex forming proteins. The ELISA human insulin assay showed a succesful measurement of biologically active insulin with high degree of specificity using monoclonal antibodies (Touitou E). The chemical integrity of insulin and the qualitative determination was performed using LC-MS technique.

**References**


1.Machado AH1, Lundberg D, Ribeiro AJ, Veiga FJ, Lindman B, Miguel MG, Olsson U. "Preparation of Calcium Alginate Nanoparticles Using Water-in-Oil (W/O) Nanoemulsions." *Pub Med* 28.9 (2012): 4131-41. Print.

2. Mimmo, T. , Marzadori, C., Montecchio,D. and Gessa, C. "Characterization of Ca and Al Pectate Gels by Thermal Analysis and Ft-Ir Spectroscopy." *Carbohydrate Research,27(1)* 340.16 (2005): 2510-19. Print.

3. Mohanraj, V. and Y. Chen. "Nanoparticles-a Review." *Tropical Journal of Pharmaceutical Research* 5.1 (2007): 561-73. Print.

4. Muller, R. and K. Wallis. "Surface Modification of Iv Injectable Biodegradable Nanoparticles with Poloxamer Polymers and Poloxamine 908." *International journal of pharmaceutics* 89.1 (1993): 25-31. Print.

5. Rigsbee, Michael J. Pikal and Daniel R. "The Stability of Insulin in Crystalline and Amorphous Solids: Observation of Greater Stability for the Amorphous Form." *Pharmaceutical Research* 14 (1997): 1379-87. Print.

6. Touitou E, Rubinstein A. "Targeted Enteral Delivery of Insulin to Rats." *Int J Pharm* 30 (1986): 95-99. Print.

7. You, Jin-Oh. "Efficient Gene Delivery with Calcium -Alginate Nanoparticles." New york: McGraw-Hill, 2006. Print.

# References

1. George M, Abraham TE. Polyionic hydrocolloids for the intestinal delivery of protein drugs: Alginate and chitosan — a review. Journal of Controlled Release. 2006;114(1):p.1-14.

2. H.J.C Ericksson WLJH, B.van Veen, G.W Somsen, G.J de Jong, H.W Frijlink. Investigations into the stabilisation of drugs by sugar glasses: I. Tablets prepared from stabilised alkaline phosphatatse. Int JPharm. 2002(249):p.59-70.

3. Manning MCP, K. ; Borchardt, R. T. Stability of protein pharmaceuticals. Pharm Res 1989;6:p. 903-18.

4. Carpenter JFP, M.J. ; Chang, B.S; Randolph, T.W. Rational design of stable lyophilized protein formulations: Some practical advice. Pharm Res. 1997;14:p. 969-75.

5. Hageman MJA, T. J, Manning, M. C. Water sorption and solid-state stability of proteins. In *Stability of protein pharmaceuticals, Part A: Chemical and Physical pathways of protein degradation*. Plenum press: New York1992.

6. Pikal MJD, K.M ; Roy, M.L; Riggin, R.M. The effects of formulation variables on the stability of freeze-dried human growth hormone. Pharm Res. 1991;8:p. 427-36.

7. W.Wang. Instability, stabilization and formulation of liquid protein pharmaceuticals. Int JPharm. 1999;185:p. 129-88.

8. Liu Xing CD, Xie Liping, Zhang Rongquing. Oral colon-specific drug delivery for bee venom peptide: development of a coated calcium alginate gel beads-entrapped liposome. J Control Release. 2003;93:p.293-300.

9. L. Shargel AY, editor. Applied biopharmaceutics and Pharmacokinetics. New York: McGraw-Hill; 1999.

10. Costantino HRL, R. ; Klibanov, A. M. Solid phase aggregation of proteins under pharmaceutically relevant consitions. JPharm Sci. 1994;83:p.1662-9.

11. Lee HJ. Protein drug oral delivery: the recent progress. Arch Pharm Res 25. 2002:p.572-84.

12. U.B. Kompella VHL. Delivery systems for penetration enhancement of peptide and protein drugs: design considerations. Advanced Drug Delivery Reviews. 2001;46:p.211-45.

13. Russell-Jones GJ. Use of vitamin B12 conjugates to deliver protein drugs by the oral route. Crit Rev Ther Drug Carr Syst. 1998;15:p.557-86.

14. B. Steffansen CUN, B. Brodin, A.H. Eriksson, R. Andersen, S. Frokjaer. Intestinal solute carriers: an overview of trends and strategies for improving oral drug absorption. Eur J Pharm Sci 21. 2004:p.3-16.

15. Fasano A. Modulation of intestinal permeability: an innovative method of oral delivery for the treatment of inherited and acquired human diseases. Mol Genet Metab. 1998;64:p.12-8.

16. J.P. Bai LLC, J.H. Guo. Targeting of peptide and protein drugs to specific sites in the oral route. Crit Rev Ther Drug Carr Syst. 1995;12:p.339-71.

17. Fix JA. Oral controlled release technology for peptides: status and future prospects. Pharm Res. 1996;13:p.1760-4.

18. Sanders LM. Drug delivery systems and routes of administration of peptides and protein drugs. Eur J Drug Metab Pharmacokinet 1990;15:p.95-102.

19. J.H. Hamman GME, A.F. Kotze. Oral delivery of peptide drugs:barriers and developments. BioDrugs 19. 2005: p.165-77.

20. Lehr CM. Bioadhesion technologies for the delivery of peptide and protein drugs to the gastrointestinal tract. Crit Rev Ther Drug Carr Syst 11. 1994: p.119-60.

21. S.A. Galindo-Rodriguez EA, H. Fessi, E. Doelker. Polymeric nanoparticles for oral delivery of drugs and vaccines: a critical evaluation of in vivo studies. Crit Rev Ther Drug Carr Syst 22. 2005: p.419-64.

22. S. Krishnan EYC, J.N. Webb, B.S. Chang, D.Shan, M.Goldenberg, M.C Manning, T.W Randolph, J.F. Carpenter. Aggregation of granulocyte colony stimulating factor under physiological conditions: Characterization and thermodynamic inhibition. Biochem. 2002;41: p.6422-31.

23. B.S Kendrick JLC, X. Lam, T. Nguyen, T.W Randolph, M.C Manning, J.F carpenter. Aggregation of recombinant human interferon gamma: Kinetics and structural transitions. JPharm Sci. 1998;87: p.1069-76.

24. Arkawa JSPaT. Mechanism of protein aggregation. Pharmaceutical Biotechnology. 2009;10: p.348-51.

25. Rechendorff K. "The influence of surface roughness on protein adsorption". Denmark: University of Aarhus; 2011.

26. Andrade JD. Surface and interfacial Aspects of Biomedical Polymers. New York and London. 1985. p. 10-21.

27. Patel KB, R.T. Chemical pathways of peptide degradation II. Kinetics of deamidation of an asparaginyl residue in a model hexapeptide. PharmRes. 1990;7: p.703-11.

28. Strickley RGA, B.D. Solid state stability of human insulin I. Mechamisn and effect of water on kinetics on degradation in lyophiles from pH 2-5 solutions. PharmRes. 1996;13: p.1142-53.

29. Pikal MJR, D.R. The stability of insulin in cystalline and amorphous solids; observation of greater stability for amorphous form. PharmRes. 1997;14: p.1379-87.

30. Carl W. Niekamp HFHJMlJ. Peptide-bond hydrolysis equilibria in native proteins. Conversion of virgin into modified soybean trypsin inhibitor. ACS publications. 1969;8(1): p.16-22.

31. Dubost DCK, M.J; Zimmerman, J.A; Bogusky, M.J; Coddington, A.B; Pitzenberger, S.M. Characterization of solid state reaction product from a lyophilized formulation of a cyclic heptapeptide. Novel example of an excipient -induced oxidation. PharmRes. 1996;13: p.1811-4.

32. Fransson. J; Florin-Robertsson.E ; Axelsson KN, C. Oxidation of human insulin like growth factor I in formulation studies: Kinetics of methionine oxidation in aqueous solutions and solid states. PharmRes. 1996;13: p.1252-7.

33. Costantino HRL, R. ; Klibanov, A. M. Moisture induced aggregation of lyophilized insulin. PharmRes. 1994;11: p.21-9.

34. Jason Kerr JLS, Donald R. Griffin , Darice Y. Wong , and Andrea M. Kasko *. Steric Effects in Peptide and Protein Exchange with Activated Disulfides. Biomacromolecules. 2013;14(8): p.2822-9.

35. Clarket TGaS. Deamidation, Isomerization and Racemization at Asparaginyl and Aspartyl Residues in Peptides. The Hournal of Biological Chemistry. 1967;262(2): p.785-94.

36. Dill. KA. Dominant forces in protein folding. Biochemistry. 1990;29: p.7133-55.

37. C.N. Pace BAS, M. Mcnutt, K. Gajiwala. Forces contributing to confirmational stability of proteins. FASEB J 1996;10: p.75-83.

38. R. Jaenicke. In R. Huber ELWe. Protein structure and protein engineering. Berlin1988. 16-36 p.

39. Protein structure: A Practical approach [press release]. Oxford: IRL Press1989.

40. Jaenicke R. Protein folding: Local structures, domains, sub-units and assemblies. Biochemistry. 1991;30: p.3147-61.

41. Intermolecular and surface forces [press release]. San Diego, California: Academic Press1992.

42. G. Graziano FC, A. Riccio, G. Barone. A reassessment of the molecular origin of cold denaturation. J Biochem (Tokyo). 1997;122: p.395-401.

43. N.T. Southall KAD, A.D.J. Haymet. A view of the hydrophobic effect. J Phys Chem B. 2002;106: p. 521-533.

44. Privalov PL. Cold denaturation of proteins. *Crit Rev Bio-chem Mol Biol*. 1990;25: p.281-305.

45. P.L. Privalov SJG. Stability of protein structure and hydrophobic interaction. *Adv Protein Chem*. 1988;39: p.191-234.

46. Pace CN. Polar group burial contributes more to protein stability than non-polar group burial. *Biochem*. 2001;40: p.310-313.

47. J.F. Carpenter BSK, B.S. Chang, M.C. Manning, T.W. Randolph. Inhibition of stress-induced aggregation of protein therapeutics. *Methods Enzymol*. 1999;309: p.236-55.

48. A.C. Dong SJP, S.D Allison, J.F Carpenter. Infrared spectroscopic studies of lyophilization-induced and temperature-induced protein aggregation. JPharm Sci. 1995;84: p.415-24.

49. Atkins P. Physical Chemistry. New York: W.H. Freeman and Company; 1994.

50. M. Vrkljan TMF, M.E. Powers, J. Henkin, W.R. Porter, H. Staack, J.F. Carpenter, M.C Manning. Thermal-stability of low molecular weight urokinase during heat treatment. 2. Effect of polymer additives. *Pharm Res*. 1994;11: p.1004-8.

51. Stability and characterization of recombinant human relaxin [press release]. Plenum Press1996.

52. L. Nielsen RK, A.Coats, S. Frokjaer, J. Brange, S. Vyas, V.N. Uversky, A.L. Fink. Effect of environmental factors on the kinetics of insulin fibril formation: Elucidation of the molecular mechanism. *Biochem*. 2001;40: p.6036-6046.

53. K. Takano KT, Y. Yamagata, K. Yutani. Contribution of salt bridges near the surface of a protein to the confirmational stability. *Biochemistry*. 2000;39: p.12375-81.

54. G.R Grimsley KLS, L.R Fee, R.W. Alston, B.M.P Huyghues-Despointes, R.L. Thurlkill, J.M. Scholtz and C.N. Pace. Increasing protein stability by altering long-range columbic interactions. *Protein Sci*. 1999;8: p.1843-9.

55. Gombotz Wayne R WS. Protein release from alginate matrices. Advanced Drug Delivery Reviews. 1998;31: p.267-85.

56. LeRoux MA G, Setton LA. Compressive and shear properties of alginate gels: effects of sodium ions and alginate concentration. JBiomed Mater Res. 1999;47: p.46-53.

57. Clark DE GH, inventorAlginic acid and process of making same1936.

58. M. R. Main properties and current applications of some polysaccharides as biomaterials. Polym Int. 2008;57: p.397-430.

59. G.Skjak-Braek HG, B. larsen. Monomer sequence and acetylation pattern in some bacterial alginates. Carbohydrate Res. 1986;154: p.239-50.

60. A.Haug Bl, O.Smidsrod. Studies on the sequence of uronic acid residues in alginic acid. Acta Chem Scand. 1967;21: p.691-704.

61. A.Haug Bl. Quantitative determination of the uronic acid composition of alginates. Acta Chem Scand. 1962;16: p.1908-18.

62. O.Smidsrod. The relative extension of alginates having different chemical composition. Carbohyr Res. 1973;27: p.107-18.

63. A.Martinsen GS-B, O.Smidsrod, F.Zanetti, S.paoletti. Comparison of different methods for determination of molecular weight and molecular weight distribution of alginates. Carbohydrate Polymers. 1991;15: p.171-93.

64. R.J Mumper ASH, P. Puolakkainen, L.S Bouchard, W.R. Gombotz. Calcium-alginate beads for the oral delivery of transforming growth factor-B1: Stabilization of

TGF-B1 by the addition of polyacrylic acid within acid-treated beads. J Control Release. 1994;30: p.241-51.

65. W.R. Gombotz SFW. Protein release from alginate matrices. Advanced Drug Delivery Reviews. 1998;31: p.267-85.

66. K.K Kwok MJG, D.J Burgess. Production of 5-15 um diameter alginate-polylysine microcapsules by an air atomization technique. Pharm Res. 1991;8: p.341-4.

67. D.E. Chickering EM. Bioadhesive microspheres: I. A novel electrobalance-based method to study adhesive interactions between individual microspheres and intestinal mucosa. J Control Release. 1995(34): p.251-61.

68. H.Chang HP, P. Kelly, J. Robinson. Bioadhesive polymers as platforms for oral controlled drug delivery. Synthesis and evaluation of some swelling, water-insoluble bioadhesive polymers. J Pharm Sci. 1985(74): p.399-405.

69. K.K Kwok MJG, D.J Burgess. Sterile microencapsulation of BCG in alginate-poly-L-lysine by an air spraying technique. Proc Int Symp Control Release Bioact Mater 16. 1991: p.170-1.

70. S.C Chen YCW, F.L. Mi, Y.H. Lin, L.C. Yu, H.W. Sung. A novel pH-sensitive hydrogel composed of *N,O*-carboxymethyl chitosan and alginate cross-linked by genipin for protein drug delivery. J Control Release 96. 2004: p.285-300.

71. Lee KY PM, Mooney DJ. Comparison of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in SCID mice. J Control Release. 2003(87): p.49-56.

72. Silva EA MD. Effects of VEGF temporal and spatial presentation on angiogenesis. Biomaterials. 2010(31): p.1235-41.

73. Wells LA SH. Extended release of high pI proteins from alginate micrspheres via a novel encapsulation technique. Eur J Pharm Biopharm. 2007(65): p.329-35.

74. George M AT. Polyionic hydrocolloids for intestinal delivery of proteins drugs. J Control Release. 2006(114): p.1-14.

75. Silva CM RA, Ferreira D, Veiga F. Insulin encapsulation in reinforced alginate microspheres prepared by internal gelation. Eur J Pharm Sci. 2006(29): p.148-59.

76. I.L Andresen OS, O.Smidsrod, K. Ostgaard, P.C Hemmer. Some biological functions of matrix components in benthic algae in relation to their chemistry and the composition of seawater. ACS Symp Ser 48. 1977: p.361-81.

77. H. Tanaka MM, I.A Veliky. Diffusion characteristics of substrates in Ca-alginate gel beads. Biotechnol Bioeng 26. 1984: p.53-8.

78. Mi F-L, Sung,H.W & Shyu, S.S. Drug release from chitosan-alginate complex beads reinforced by an naturally occuring cross-linking agents. Carbohydrate Polymers, 48(1). 2002: p.61-72.

79. Sriamornsak PT, N & Korkerd, K. Swelling, erosion and release behavior of alginate-based matrix tablets. European Journal of Pharmaceutics and Biopharmaceutics, 66(3). 2007: p.435-50.

80. O.Smidsrod GS-B. Alginate as immobilization matrix for cells. TIBTECH 8. 1990: p.71-8.

81. T. Espevik MO, G. Skjak-Braek, L.Ryan, S.D Wright, A.Sundan. The involvement of CD14 in stimulation of cytokine production by uronic acid polymers. EurJImmunol23. 1993: p.255-61.

82. Smidsroed OG, R.M & Whittington, S. G. Relative extension of alginates having different chemical composition. Carbohydrate Research,27(1). 1973: p.107-18.

83. C.K. Kim EJL. The controlled release of blue dextran from alginate beads. Int JPharm79. 1992: p.11-9.

84. T.Yotsuyanagi TO, T.Ohhashi, K.Ikeda. Calcium induced gelation of alginic acid and pH-sensitive reswelling of dried gels. Chem Pharm Bull35. 1987: p.1555-63.

85. S. Sugawara TI, M. Otagiri. The controlled release of prednisolone using alginate gel. Pharm Res11. 1994: p.272-7.

86. De Vos PDH, B. Wolters, G.H & Van Schilfgaarde, R. Factors influencing the adequacy of microencapsulation of rat pancreatic islets. Transplantation 62(7). 1996: p.888-93.

87. Klock G, Pfeffermann, A. Ryser, C. Grohn, P.Kuttler, B. Hahn, H.J,etal. Biocompatibility of mannuronic acid-rich alginates. Biomaterials, 18(10). 1997: p.707-13.

88. I.W. Sutherland DBE. Alginates, . Biomaterials: Novel Materials from biological sources. 1991: p.309-31.

89. Y. Murata KN, E.Miyamoto, S.Kawashima, S.H Seo. Influence of erosion of calcium-induced alginate gel matrix on the release of Brilliant Blue. J Control Release 23. 1993: p.21-6.

90. A.Haug Bl. The solubility of alginate at low pH. Acta Chem Scand 17. 1963: p.1653-62.

91. A.Haug Bl. The degradation of alginates at different pH values. Acta Chem Scand 17. 1963: p.1466-8.

92. P. Liu TRK. Alginate-pectin-poly-L-Lysine particulate as a potential controlled release formulation. JPharm Pharmacol 51. 1999: p.141-9.

93. M.L Torre PG, L.Maggi, R. Stefli, E. Ochoa, Machiste, U.Conte. Formulation and characterization of calcium alginate beads containing ampicillin. Pharm Dev Technol3. 1998: p.193-8.

94. L.W. Chan PWSH. Effects of aldehydes and methods of cross-linking on properties of calcium alginate microspheres prepared by emulsifiction. Biomaterials 23. 2002: p.1319-26.

95. A.R. Kulkarni KSS, T.M. Aminabhavi, A.M. Dave, M.H. Mehta. Glutareldehyde crosslinked sodium alginate beads containing liquid pesticide for soil application. J Control Release 63. 2000: p.97-105.

96. Moussa BA. Validated RP-HPLC method for determination of Recombinant Insulin in bulk dosage form. E-Journal of Chemistry. 2010;7: p.449-57.

97. EP Kroef RO, EL Campbell, RD Johnson. Production scale of biosynthetic human insulin by reverse phase liquid chromatography. Journal of Chromatography A. 1989.

98. Lebovitz HE. Insulin: Potential Negative Consequences of Early Routine Use in Patients With Type 2 Diabetes. American Diabetes Association. 2011;34: p.225-30.

99. H K, editor Insulin: availability, affordability, and harmonization. WHO Drug; 1998.

100. Johnson IS. Authencity and Purity of Human Insulin (recombinant DNA)1982. 4-12 p.

101. Gordon Roberts L-YL. Protein NMR Spectroscopy: Practical Techniques and Applications. Sons JW, editor. Hoboken: ChemBioChem/Wiley-VCH; 2011.

102. Kryndushkin DS AI, Ter-Avanesyan MD, Kushnirov VV. Yeast [PSI+] prion aggregates are formed by small Sup35 polymers fragmented by Hsp104. Journal of Biological Chemistry. 2003;278(49): p.49636-43.

103. W. D. Laws WGF. Differential Thermal Analysis of Proteins. Anal Chem. 1949;21(9): p.1058-9.

104. Khaksa G. NK, Bhat M., Udupa N. High-performance liquid chromatographic determination of insulin in rat and human plasma. Analytical Biochemistry. 1998;260: p.92-5.

105. K.Makino NS, F. Moriya, S. Rokushika and H. Hatano. Liquid Chromatography detectors. Chem Lett. 1979: p.675.

106. Proceedings of the International Conference on Harmonization (ICH)1996.

107. Boardman NK. Chromatography of insulin in ion-exchange resin. Journal of Chromatography A. 1959;2: p.398-405.

108. McLeod AN AdMA, Wood SP. High-performance liquid chromatography of insulin. Accessibility and flexibility. Journal of Chromatography A. 1990;502: p.325-36.

109. D Senthil Rajan KVG, U Mandal, M Ganesan, A Bose, AK Sarkar, TK Pal. Development of RP-HPLC for analysis of human insulin. Indian Journal of Pharmaceutical Sciences. 2006;68(5): p.662-5.

110. Eva Tudurí, J. E. B. a. T. J. K. (2012). Restoring insulin production for type 1 diabetes. *Journal of Diabetes, 4*(4), p. 319-331.

111. EP Kroef, R. O., EL Campbell, RD Johnson. (1989). Production scale of biosynthetic human insulin by reverse phase liquid chromatography. *Journal of Chromatography A*.

112. Kohlmann, M. R. L. a. K. L. (2008). Recombinant Human Insulin. *Biotechnology Progress, 8*(6), p. 469-478.

113. Adam Friedman M.D , K. B. M. D. (2013). Nanotechnology in the Treatment of Infectious Diseases. *Nanotechnology in Dermatology*, p. 187-200.

114. Alginates. [press release]. Boca Raton, FL, USA: CRC Press 2006

115. Augst AD, K. H., Mooney DJ. (2006). Alginate Hydrogels as Biomaterials. *Macromolecular Bioscience, 8*(6), p.623-633.

116. L. Shargel, A. Y. (Ed.). (1999). *Applied biopharmaceutics and Pharmacokinetics*. New York: McGraw-Hill

117. Biomedical Applications of Polymeric Materials [press release]. Boca Raton, FL: CRC Press Inc 1993.

118. Nesamony J, K. W. (2005b). IPM/DOSS/water microemulsions as reactors for silver sulfadiazine nanocrystals synthesis. *J.Pharm Sci, 94*(6), p. 1310-1320.

119. Mohanraj, V. a. Y. C. (2007). Nanoparticles-a review. *Tropical Journal of Pharmaceutical Research, 5*(1), p. 561-573.

120. Muller, R. a. K. W. (1993). Surface modification of iv injectable biodegradable nanoparticles with poloxamer polymers and poloxamine 908. *International journal of pharmaceutics, 89*(1), p. 25-31.

121. Chavanpatil, M. D. (2007). Polymer-surfactant nanoparticles for sustained release of water-soluble drugs. *Journal of pharmaceutical sciences, 96*(12), p. 3379-3389

122. Pinto Reis, C. (2006). Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. *Nanomedicine: Nanotechnology, Biology and Medicine, 2*(1), p.8-21.

123. Mimmo, T., Marzadori, C., Montecchio,D. and Gessa, C. (2005). Characterization of Ca and Al pectate gels by thermal analysis and FT-IR spectroscopy. *Carbohydrate Research,27(1), 340*(16), p. 2510-2519.

124. Rigsbee, M. J. P. a. D. R. (1997). The Stability of Insulin in Crystalline and Amorphous Solids: Observation of Greater Stability for the Amorphous Form. *Pharmaceutical Research, 14*, p. 1379-1387.

125. Sarmento, B., Ferreira, D., Veiga, F., & Ribeiro, A. (2006). Characterization of insulin-loaded alginate nanoparticles produced by ionotropic pre-gelation through DSC and FTIR studies. *Carbohydrate Polymers, 66*(1), p. 1-7.

126. Touitou E, R. A. (1986). Targeted enteral delivery of insulin to rats. *Int J Pharm, 30*, p. 95-99.

127. Moussa, B. A. (2010). Validated RP-HPLC method for determination of Recombinant Insulin in bulk dosage form. *E-Journal of Chemistry, 7*, p. 449-457.

128. Erin E. Chambers, K. J. F., Norman Smith, Leah Ashraf, Janaka Karalliedde, David Cowan and Cristina Legido-Quigley. (2014). Multidimensional LC-MS/MS Enables Simultaneous Quantification of Intact Human Insulin and Five Recombinant Analogs in Human Plasma. *Anal. Chem, 86*(1), p. 694-702.

129. Kristine de Dios, A. M., Robin Marsden, Jason Pinkstaff. (2013). Comparison of Bioanalytical methods for the quantification of PEGylated human insulin. *Journal of Immunological Methods, 396*, p. 1-7.

130. M. Bantscheff, M. S., G. Sweetman, J. Rick, and B. Kuster. (2007). Quantitative mass spectrometry in proteomics: a critical review. *Analytical and Bioanalytical Chemistry, 389*(4), p. 1017-1031.

131. Jessica M. Faupel-Badger, B. J. F., Xia Xu, Roni T. Falk, Larry K. Keefer, Timothy D. Veenstra , Robert N. Hoover and Regina G. Ziegler. (2010). Comparison of Liquid Chromatography-Tandem Mass Spectrometry, RIA, and ELISA Methods for Measurement of Urinary Estrogens. *Cancer Epidemiology Biomarkers Prev, 19*, 292.

132. Machado AH1, Lundberg D, Ribeiro AJ, Veiga FJ, Lindman B, Miguel MG, Olsson U. "Preparation of Calcium Alginate Nanoparticles Using Water-in-Oil (W/O) Nanoemulsions." *Pub Med* 28.9 (2012): 4131-41. Print.

133. Mimmo, T. , Marzadori, C., Montecchio,D. and Gessa, C. "Characterization of Ca and Al Pectate Gels by Thermal Analysis and Ft-Ir Spectroscopy." *Carbohydrate Research,27(1)* 340.16 (2005): 2510-19. Print.

134. Mohanraj, V. and Y. Chen. "Nanoparticles-a Review." *Tropical Journal of Pharmaceutical Research* 5.1 (2007): 561-73. Print.

135. Muller, R. and K. Wallis. "Surface Modification of Iv Injectable Biodegradable Nanoparticles with Poloxamer Polymers and Poloxamine 908." *International journal of pharmaceutics* 89.1 (1993): 25-31. Print.

136. Rigsbee, Michael J. Pikal and Daniel R. "The Stability of Insulin in Crystalline and Amorphous Solids: Observation of Greater Stability for the Amorphous Form." *Pharmaceutical Research* 14 (1997): 1379-87. Print.

137. Touitou E, Rubinstein A. "Targeted Enteral Delivery of Insulin to Rats." *Int J Pharm* 30 (1986): 95-99. Print.

138. You, Jin-Oh. "Efficient Gene Delivery with Calcium -Alginate Nanoparticles." New york: McGraw-Hill, 2006. Print.