DEVELOPMENT OF ALGINATE NANOSPHERES AS A PROTEIN DELIVERY DEVICE FOR CARTILAGE TISSUE ENGINEERING

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ABSTRACT

Delivery of bioactive proteins is a valuable strategy in cartilage tissue engineering (CTE) because of their ability to regulate the gene expression and extracellular matrix (ECM) production of engineered cartilage. This, however, has been challenged by the nature of bioactive proteins including their instability, poor tissue penetration ability, short half-life and a relatively high price. Development of nanospheres as a protein delivery device should solve these issues by promoting the temporal and spatial presentation of such bioactive proteins in a defined target for the enhanced half-life time and effectiveness. Among various polymer-based micro/nanospheres, alginate micro/nanospheres have been widely used as a protein delivery device because of their mild and easy protein encapsulation process, inert nature, non-toxicity and biocompatibility. However, one of the major limitations of using alginate as a protein delivery device is its high initial burst release due to its high porosity and instability if exposed in a higher pH release media. To address these issues, this study aimed to develop the protein loaded alginate nanospheres as a delivery device with a reduced initial burst release. The hypothesis was, "*Increasing the alginate concentration, cross-linking time or drying time reduces the initial burst release independently of associated changes to the size and number of nanospheres.*" Bovine Serum Albumin (BSA) was used as a model protein in this study to evaluate the performance of alginate nanospheres as a protein delivery device, while protein loaded alginate nanospheres were prepared via a combination of water-in-oil emulsification and external gelation method. The process parameters tested to reduce the initial burst release include, alginate concentration, cross-linking time and drying time. The effects of these process parameters on the nanosphere size and distribution pattern, relative number of microspheres, initial burst release, protein release kinetics and encapsulation efficiency (EE%) were investigated. Also, if the change in size and relative number of nanospheres by varying these process parameters affected the initial burst release was investigated. It has been illustrated that by properly increasing the alginate concentration, cross-linking time and drying time it was possible to reduce initial burst release by 13%, and among various process parameters only the alginate concentration showed a significant effect on the initial burst release, when considered alone. Also, it was confirmed that during determination of the effect of various process parameters the relative number of nanospheres significantly affected the initial burst release. Taken together, this study demonstrates that regulating various process parameters is a mean to reduce initial burst release of

alginate nanospheres, urging more studies on alginate nanospheres for their potential application in CTE.

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LIST OF ABBREVIATIONS

AC	Articular Cartilage
Alg	Alginate
BB	Brilliant Blue
BMP-7	Bone Morphogenetic Protein 7
CTE	Cartilage Tissue Engineering
CXB	Celecoxib
CLSM	Confocal Laser Scanning Microscope
DDW	Double Distilled Water
DMEM	Dulbecco's modified Eagle's medium
DAC	Dodecyltrimethyl-ammonium chloride
ECM	Extracellular Matrix
EE%	Encapsulation Efficiency
FITC	Fluorescein Isothiocyanate
GF	Growth Factor
HA	Hyaloronic Acid
hASCs	human adipose tissue-derived stem cells
HLB	Hydrophile-lipophile balance
IGF-I	Insuline like growth factor
MW	Molecular Weight
MSC	Mesenchymal Stem Cells
OA	Osteoarthritis
PCL	Polycaprolacton
PBS	Phosphate Buffered Saline
PLGA	Poly Lactic-*co*-Glycolic Acid

PVA   Polyvinyl Alcohol

SA   Sodium Alginate

SDS   Sodium dodecyl sulphate

TPP   Sodium tripolyphosphete

TGF-β1   Transforming growth factor beta 1

TGF-b3   Transforming growth factor beta 3

W/O   Water-in-oil

W/O/W   Water-in-oil-in-water

CHAPTER 1

INTRODUCTION

Articular cartilage (AC) is a highly specialized connective tissue that has a complex and stratified structure, and covers the distal ends of bones, such as knee and hip joints. It is avascular and anural in nature [1, 2]. In normal cartilage, the percentage of interstitial fluid ranges from 65-80% of the total weight with the remaining being collagen and proteoglycan [1]. Unlike other tissues, the interstitial fluid of AC is slightly acidic (pH 7.2-6.9). Water is the most abundant component (80% of the wet weight) in AC, and in addition to providing lubrication it also helps to transport and distribute nutrients through the cartilage [3].

AC is composed of only one type of cell, called chondrocyte. Chondrocytes are embedded in the extracellular matrix (ECM) consisting of collagens, proteoglycans, glycosaminoglycans, lipids, phospholipids and other non-collagenous proteins [1]. These chondrocytes respond to a variety of stimuli, such as growth factors, mechanical loads, piezoelectric force and hydrostatic press, thereby, synthesizing the ECM of cartilage, such as collagen and proteoglycans, in its immediate vicinity [1, 4]. Depending on the properties of collagens and proteoglycans, AC can respond to the tensile, shear and compressive forces during joint loading [2].

One of the problems associated with AC is that when this tissue wears off because of physical injury, improper joint loading or other degenerative joint diseases, its repair becomes challenging. This is most likely because of the poor regenerative capacity of cartilage, not only due to its avascular and aneural nature but also due to the complex stratified architecture comprising of 4 different zones [2, 1]. The composition and organization of the matrix, cell morphology, cell density, collagen orientation and thickness vary from zone to zone [2, 5]. Additionally, each zone has a unique mechanical property and gene expression pattern giving distinct properties of cartilage [6].

Osteoarthritis (OA), one of the most prevalent type of cartilage degenerative joint diseases, occurs due to breakdown and eventual loss of cartilage [4, 7, 8, 9]. At present, more than 250,000 knee and hip replacements are performed in the United States each year, and it has been expected that with an increase of aging population and obesity, the number of osteoarthritis cases will

increase drastically in upcoming years [10]. In a very recent study, it has been reported that by the age of 85, nearly 1 out of 4 people will have osteoarthritis in the hip and 1 out of 2 will have osteoarthritis in the knee, costing an estimated US $80 billion per year in healthcare related expenses [11]. As a result, cartilage defect repair is a thorny clinical issue. The existing therapies for cartilage defect repair include chondral shaving, subchondral drilling, microfracturing, mosaicplasty, prosthetic joint replacement, subchondral abrasion, autologous chondrocyte implantation [8, 12]. However, none of them can repair cartilage defect permanently because of their various limitations, such as unsuitable donor tissue availability, donor site morbidity, diseases transmission, implant loss due to limited durability of the prosthetics, and requirement to undergo many surgeries [12].

At present, among all therapeutic approaches, cartilage tissue engineering (CTE) has shown a great potential for cartilage defect repair. CTE aims to regenerate the neocartilaginous tissue by combining three basic components of tissue engineering, such as a suitable biocompatible scaffold, cells and an environment including a mechanical stimulus and a combination of appropriate bioactive factors (Figure 1.1).
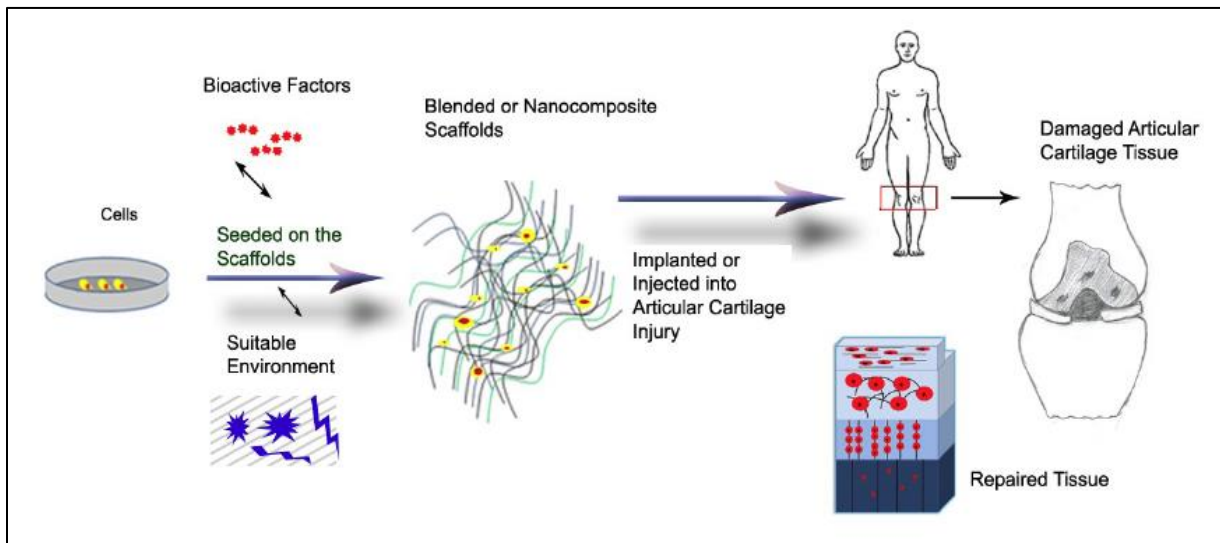
Figure 1.1: Schematic representation of cartilage regeneration by combining three basic components of CTE a suitable biocompatible scaffold, cells and an environment including mechanical stimuli and a combination of appropriate bioactive factors [2].

It is expected that through dynamic interaction of these three components, AC can be created to mimic the native one [2]. After decades of research on CTE, hybrid scaffolds, made of

hydrogels or polyester-based solid polymers, are gaining popularity because of their capacity to mimic the mechanical (e.g., polyester-based solid polymers) and biological properties (e.g., using hydrogels impregnated with cells) of native cartilage [13, 14]. In terms of scaffold fabrication, recently 3D bioprinting has shown great promises because of its ability to fabricate the complex scaffold architectures by manipulating mechanical and biological parameters of each zone independently [5]. A cell seeded PCL/alginate-based hybrid scaffold with improved biological properties has already been successfully fabricated in our lab [15]. However, one of the major challenges yet to overcome is to regulate the zone-specific gene expression in the engineered cartilage construct. This is most likely because of the change in chondrocyte phenotype that occurs either during a monolayer culture due to cellular dedifferentiation [16] or due to chondrocyte maturation when cultured for a prolonged period of time [15]. This dedifferentiation and the maturation of cells is responsible for expressing genes typical of deep and calcified zone of AC. Therefore, efforts have been made recently to prevent the phenotype change of cells, impregnated in engineered cartilage. One of the approaches could be using bioactive proteins that should prevent the phenotypic change of cells, thereby, regulating cellular differentiation, matrix synthesis and the zone-specific gene expression of chondrocytes [16, 17, 18, 19].

1.1 Roles of Bioactive Proteins in Cartilage Defect Repair

In addition to support the growing cells in a three-dimensional (3D) scaffold or hydrogel matrix, the success of CTE depends on obtaining the appropriate cells for implantation and directing the development of these cells to a chondrogenic pathway [20]. Currently, bioactive proteins such as growth factors (GFs) have become increasingly popular because of their ability to repair the cartilage defect. Their profound impact on the cartilage defect repair is mainly because of their regulatory effect on directing different cell types (e.g., chondrocytes, bone marrow derived stem cells, adipose, synovium, muscle and periosteum derived stem cells) down to the proper pathway [18, 21, 20]. GFs can bind to the cell surface receptors and can regulate the cellular proliferation, differentiation, migration, adhesion, and gene expression resulting in an improved ECM synthesis of AC [22, 23, 21]. There are a large number of GFs, such as Insulin like growth factor (IFG-1), Transforming growth factor beta 1 (TGF-β1), Bone morphogenic proteins (BMP-2,4 and 7), available that can regulate the cellular proliferation, differentiation, gene expression and ECM production of cartilage [10, 23, 19, 16, 24, 25, 26, 27, 20]. Among various GFs, BMPs

have been reported to have beneficial effects on the cartilage defect repair because of their potential to regulate gene expression and stimulate matrix synthesis, chondrogenic differentiation, maturation and maintenance [16, 28, 10]. For example, they can trigger MSCs to differentiate into chondrocytes and maintain their cellular phenotype [20].

BMPs are member of TGF-β superfamily and play an important role in cartilage defect repair. Among various BMPs, BMP-7 can stimulate both chondrogenesis and osteogenesis [29]. Unlike other BMPs, BMP-7 has the ability to up-regulate chondrocyte metabolism and protein synthesis without osteophytes formation and uncontrolled cell proliferation [29]. BMP-7 can maintain chondrocyte phenotype by regulating the chondrocyte specific genes. Several *in vitro* studies suggest that BMP-7 promotes the expression of genes typical of middle zone of cartilage such as *Col2a1*, *Col9a2* and *Sox9*, whereas BMP-7 suppresses the expression genes typical of deep and calcified zones [30, 31, 32, 33, 34].

In addition to having anabolic roles of exogenously expressed BMP-7 to maintain chondrocyte phenotype and chondrocyte specific gene expression, BMP-7 has also been reported to be endogenously present in the adult AC of different species [29]. The concentration of BMP-7 in normal cartilage is around 50ng/g dry tissue which is within the physiological range (50-200 ng/mL) for anabolic activities of BMP-7 [29]. This endogenously expressed BMP-7 is responsible for the normal cartilage homeostasis [29].

In order to manipulate different types of cells to chondrogenic pathway, BMP-7 needs to be introduced to the cell milieu by various manners, such as viral vectors, non-viral vectors, nucleofection and direct delivery [20]. A molecular understanding of BMPs' action on the regulation of chondrogenesis cannot be achieved without a proper understanding of the signaling pathways of BMPs. BMP signaling is mediated by two distinct pathways: a canonical Smad pathway and a mitogen-activated protein kinase (MAPK) pathway [35, 36] (Figure 1.2). In case of the canonical Smad pathway, BMP signaling is initiated by the binding of BMP to its receptors, such as BMPR-I and II [37]. Upon BMP binding, the type II receptor phosphorylate the serine/threonine residues in type I receptors resulting in an increased phosphorylation of the down-stream signaling molecules, including Smad1, 5 and 8 (R-Smads) [37, 36]. Afterwards, the R-Smads bind with Smad-4 or commonpartner Smads (Co-Smads) to form heteromeric complexes [37, 36]. These Smad complexes translocate into the nucleus, where they bind to the DNA directly, or interact with the DNA binding proteins, thereby, regulating the transcription of the target genes

[37, 36]. On the other hand, during a mitogen-activated protein kinase (MAPK) pathway, BMP regulates cellular process using the extracellular signal regulated kinases (ERKs), C-Jun-NH2-terminal kinases (JNKs) as well as the p38 MAPKs [38, 39, 35]. A precise modulation of the BMP signaling pathway is critical to cartilage defect repair and maintenance of cartilage homeostasis.
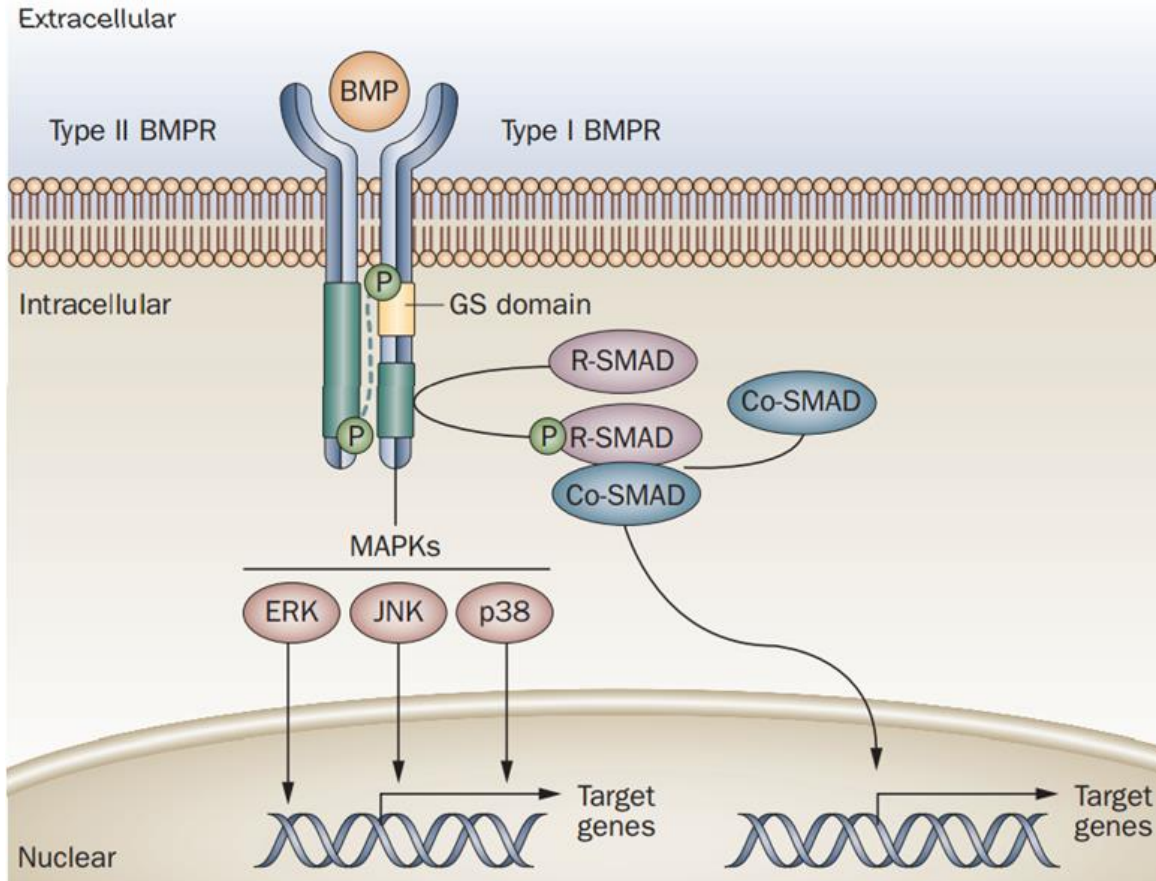
Figure 1.2 Schematic representation of BMP signaling pathway [35].

Undoubtedly, bioactive proteins e.g., GFs have a positive impact to repair the cartilage defect. However, this might not be true if their concentration is either too low or too high. The release of bioactive proteins at target site at a very high concentration can result in osteophytes formation and cartilage erosion [40]. The key to success of using bioactive proteins to mimic the native cartilage is that they must be delivered into the tissue site of interest at an optimal concentration and for a proper duration that can vary based on the bioactive protein used [27, 40]. For instance, in case of BMP-7, the duration of exposure and concentration needed to repair the cartilage defect are unknown. However, based on several *in vitro* and *in vivo* studies, release of

50-100 ng/mL BMP-7 over two weeks should be enough to repair the cartilage defect, preventing the progression of cartilage degeneration [29].

To repair cartilage defects, traditional approaches involve a systematic delivery of drug or bioactive proteins via a non-parental route of administration or direct injection into the tissue site of interest. However, the avascular nature of cartilage creates a significant barrier to biodistribution of the systematically delivered therapies (drug or GFs) to the target site, making the cartilage defect repair challenging [41, 7]. By direct injection, it is possible to locally deliver bioactive proteins into a tissue site of interest. However, the problem associated with this approach is that, after direct injection these bioactive proteins can get cleared from the tissue site via a pressure gradient that causes flow of synovial fluid, present in the cartilage [41]. Thus, due to short biological half-lives of proteins, repeated injection is required that is painful and patient incompatible [42]. Therefore, the development of control delivery devices is important to solve these issues of bioactive factors and increase their potential use in CTE.

1.2 Importance of Controlled Delivery of Bioactive Proteins

In TE, the controlled release of bioactive proteins plays an important role, both *in vitro* and *in vivo*. TE approach involves using macro- or microporous scaffolds or nanoporous hydrogel matrices [43]. Cells can be seeded into such scaffolds or matrices and cultured *in vitro* by simply adding an appropriate dose of bioactive proteins in a culture medium for the desired period of time without controlled release [43]. However, the problem associated with such approach is that bioactive proteins need to be added every time during medium change [43], which is expensive. Therefore, even if the controlled release of proteins is not necessary during *in vitro* cell culture, it is beneficial. The controlled release of bioactive proteins only locally within scaffolds or hydrogel matrices not only helps to manipulate the cells to behave in a desired way, but also reduces the required amount of bioactive proteins [43], such as GFs that are very expensive. Sometimes, TE approach involves using a cell free scaffold or hydrogel matrices containing a bioactive protein placed *in vivo*. It is believed that by providing bioactive proteins in a controlled manner it might be possible to manipulate cells to migrate into the scaffold or matrix and to behave in a desired way [43].

The controlled release of bioactive proteins means application of proteins in their optimal concentration. This concentration range is called a therapeutic window [43]. For bioactive proteins,

to exert their maximum bioactivity, they need to be present in their optimal concentration for a long period of time, thereby, increasing their effectiveness [43, 44]. In order to ensure a controlled delivery of proteins, they need to be incorporated into some kind of delivery device to minimize protein denaturation and loss of bioactivity [44, 9]. Unlike traditional approaches, a controlled delivery using various delivery devices can prolong half-lives of proteins, thereby, minimizing the required amount of expensive proteins [45, 40]. The difference in the release kinetics using either a traditional approach (i.e., direct injection) or a control delivery device has been shown in Figure 1.3.
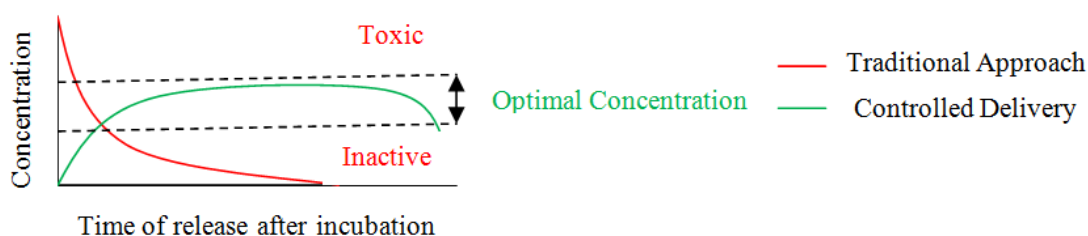
Figure 1.3: Difference in release kinetics of encapsulating materials using either a traditional approach or a controlled delivery device [43].

1.3 Design of Control Delivery Devices

Currently, several approaches have been developed for the controlled delivery of bioactive proteins. To ensure a controlled delivery, proteins can simply be admixed with the hydrogel matrix with which they have some biological affinity. They can also be loaded in the scaffolds or hydrogel matrices or they can be loaded in micro/nanospheres, prepared using either hydrophilic or hydrophobic polymers, which can then be loaded into various scaffolds or hydrogel matrices. The release of these bioactive factors can be controlled by diffusion, degradation, or erosion mechanism [43, 46, 22, 10, 27]. In order to design a delivery device, several factors need to be considered, such as the MW of protein molecules, biological half-life, dose requirement, and site and rate of administration [45]. Various configurations of control delivery devices are given below.

For a controlled delivery, proteins can be incorporated into a three-dimensional (3D) biodegradable scaffold along with the cells. The scaffold can be prepared using a hydrophobic or hydrophilic biodegradable polymer. In case of the scaffold prepared using a hydrophobic polymer, proteins can be applied directly before or after scaffold fabrication. However, using a hydrophobic

polymer it is difficult to incorporate the water-soluble proteins evenly before scaffold fabrication. In order to incorporate the water-soluble proteins homogeneously in a hydrophobic polymer, at first, proteins need to be dissolved in an aqueous solution and then mix with the polymer dissolved in an organic solvent [47]. However, the use of organic solvents during the hydrophobic polymer-based scaffold fabrication and the lack of compatibility between protein and polymer might lead to protein denaturation [45, 48]. Additionally, the incorporation of protein prior to a scaffold fabrication is hampered by other processing conditions (e.g., temperature, light, pressure, solvent, shear force, surface tension) used to prepare scaffolds [40]. In order to mitigate the protein denaturation due to the use these harsh processing conditions, they can be incorporated after scaffold fabrication. This can be done by dipping the prefabricated scaffold in a protein containing solution. However, this approach does not allow a great control over the release characteristics of proteins from scaffolds [40]. Furthermore, due to poor hydrophilicity and the lack of functional groups of hydrophobic polymers, the solution dipping method might result in a low loading efficiency and poor bioavailability of proteins [48]. For this type of hydrophobic scaffold, the degradation of polymer is a prerequisite for the delivery of protein into a target site. However, sometimes the degradation of this type of polymer may create an acidic microenvironment resulting in protein denaturation [45].

Unlike hydrophobic polymer-based scaffolds, a mild processing condition together with almost no use of organic solvents allows hydrophilic polymer-based scaffolds to encapsulate both proteins and cells at the same time, necessary for cartilage matrix formation [49, 40]. Using a hydrophilic polymer, it is possible to add both cells and bioactive proteins in the polymer solution prior to a scaffold fabrication. However, it is difficult to design a hydrophilic polymer-based scaffold such that it maintains the tissue growth and sustained protein release properties simultaneously. The reason is the polymer concentration of a scaffold needs to be high in order to reduce the pore size to ensure a prolonged protein release. On the other hand, cell prefers to remain in a comparatively aqueous polymer solution, so increased solution viscosity at a higher polymer concentration can hamper the cell viability [50]. This implies that the direct incorporation of proteins into a cell seeded hydrophilic scaffold limits its ability to control the release kinetics, due to different requirements for the cell viability and sustained protein release.

Hydrogels play a significant role as a delivery device for bioactive proteins. They are polymeric networks consisting of cross-linked hydrophilic polymers [51], such as synthetic

polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), alginate, collagen, chitosan and gelatin. They can swell and retain a significant fraction of water within their structure but will not dissolve in water [51, 52]. The ability of hydrogels to absorb water relies on the presence of hydrophilic groups, such as -OH, -CONH-, -CONH2- and -SO3H- [52]. This high-water retention capacity of hydrogels together with their soft and viscoelastic properties, as well as a low interfacial tension with biological fluids or water are the reasons they mimic living tissues [52].

In CTE, bioactive proteins and cell containing hydrogel matrix have been used both *in vitro* and *in vivo* to repair a cartilage defect [49]. Using hydrogels, the controlled delivery of encapsulating molecules can be achieved by tuning their physical properties, such as the degradation rate and pore size [7]. The incorporation of proteins into a hydrogel is done by physical encapsulation, physical or chemical cross-linking of hydrogel network or electrostatic interaction [7, 53]. The pore size of hydrogel depends on the distance between cross-link networks, and the distance between cross-link networks depends on the MW of polymeric chains that connect the cross-links [43]. Protein release from a hydrogel matrix can occur due to diffusion, swelling, hydrolytic or enzymatic degradation of hydrogel network [53]. Using hydrogel matrix, in order to maintain a controlled release for a long time, the distance among cross-link networks should be the same order of magnitude as the diameter of protein [43]. This means the pore size has to be very small [43], usually in a nanometer range. On the other hand, the optimal pore size for improved chondrocyte proliferation and cartilaginous ECM secretion is usually in micrometer range [50]. Therefore, it is hard to control the protein delivery when they are incorporated directly into hydrogels.

From the above discussions, it is clear that it is hard to design a protein loaded scaffold or hydrogel matrix having all the desired properties concurrently, necessary for the cell viability and controlled protein delivery. These issues can be resolved by designing a protein delivery device separately and then embedding it into a scaffold or hydrogel matrix [43, 54]. Designing protein delivery device separately increases the possibility and flexibility of acquiring a controlled release without compromising cell viability. Recently, scaffold or hydrogel matrix containing protein loaded micro/nanospheres has gained much popularity. This is most likely due to their ability to deliver bioactive proteins in a surrounding matrix, containing cells. in a precise and near physiological fashion [27]. This, in turn, facilitates the cellular ingrowth and neo-tissue formation by regulating cell proliferation and differentiation [27].

Several studies have confirmed that the scaffold or hydrogel matrix containing bioactive proteins loaded micro/nanospheres has the potential to provide the desirable release kinetics, resulting in an improved cartilage regeneration. For example, in case of hyaluronic acid (HA) hydrogel, compared to direct incorporation of TGF-β3 into the cell containing hydrogel, co-encapsulation of TGF-β3 loaded alginate microspheres improved the mechanical properties and cartilage matrix synthesis, both *in vitro* and *in vivo* [49]. In Fibrin matrix, the controlled release of TGF-β3 from heparin nanospheres resulted in the regeneration of hyaline cartilage tissue (both *in vitro* and *in vivo*), in another study when co-encapsulated along with cells [55]. Similarly, the seeding of poly (lactide-co-caprolactone) scaffold with a fibrin gel containing TGF-β1 loaded nanospheres and human adipose tissue-derived stem cells (hASCs) induced and sustained hASCs differentiation [56].

All of these studies indicate micro/nanospheres' significant roles as a protein delivery device in CTE to repair cartilage defect. Thus, it is important to understand the properties of micro/nanospheres as a protein delivery device, their release mechanism and how to control the release kinetics.

1.3.1 Micro/Nanospheres as a Protein Delivery Device

Micro/nanospheres have shown great promise as a protein delivery device in CTE, both *in vivo* and *in vitro* [57, 58, 59, 24, 49, 56, 55]. They are small spherical particles with a diameter ranging from micron to submicron scale. The development of such kind of delivery device helps reducing the required amount of proteins, which are expensive. Because of being small in size, their mechanical strength is comparatively high and the surface area to volume ratio increases resulting in high protein loading, bioavailability and surface reactivity [27, 60]. Also, using this kind of delivery devices protect the bioactive proteins against tissue regrowth and allow them to release in response to an environmental stimuli (e.g., pH or temperature) [54, 27]. Furthermore, by using micro/nanospheres, it is possible to maintain the release of protein at the target site for a long period of time, thereby, increasing their effectiveness [61].

The proteins encapsulated into micro/nanospheres are released based on the following mechanisms: (i) initial release of encapsulating molecules through the particle surface via protein desorption (ii) protein diffusion through the micro/nanosphere structure (iii) bulk degradation and the erosion of polymeric network [45, 62]. The diffusion of proteins through the

micro/nanospheres can be controlled by varying various process parameters, such as the polymer composition, concentration and MW, cross-linking density, and degradation rate.

The properties of micro/nanospheres as a delivery device can vary based on their fabrication techniques. Several techniques have been employed for the fabrication of micro/nanospheres, such as the ionotropic gelation [63, 64], emulsification [65, 64, 66], spray drying [67, 64, 66], phase separation [64], microfluidic preparation [64] and electrodispersion [64]. Among various fabrication techniques, the emulsification and spray drying methods are most popular [64].

Spray drying is one of the popular techniques that have been widely used to prepare micro/nanospheres, using both hydrophilic [68, 69, 70, 71] and hydrophobic [72] polymers. Using this method, a freeze-dried protein, in its solid state, is dispersed in an aqueous or organic polymer solution by homogenization. In case of an aqueous polymer solution, a cross-linking solution is also added [69]. This polymer-protein dispersion with or without a cross-linker is then fed into the nozzle with a peristaltic pump followed by atomization in a stream of heated air [69]. While the atomization helps to disrupt the liquid into small droplets, the heated air evaporates the solvents instantaneously yielding solid micro/nanospheres [69, 70]. The size of micro/nanospheres can vary based on the nozzle diameter [69]. One advantage of using the spray-drying method is its less dependency on the solubility characteristics of the encapsulating molecules [73]. Furthermore, this process is highly reproducible, and has a good control over the particle size. Using this method, it is possible to prepare micro/nanospheres in a narrow size range [69]. However, the harsh processing conditions, such as the heated air used in this technique might denature the sensitive proteins due to protein aggregation [67].

The emulsification is another popular method used in several studies to encapsulate proteins. This method is commonly used to prepare the hydrophilic protein loaded micro/nanospheres. Emulsification method can be of two types, such as water-in-oil (w/o) or water-in-oil-in-water (w/o/w) emulsification method. Based on the properties of polymers used to prepared micro/nanospheres, both water-in-oil (w/o) and water-in-oil-in-water (w/o/w) emulsion methods have been used in the past. The micro/nanospheres composed of a hydrophilic polymer are commonly prepared using the w/o emulsification method [74, 75, 76, 77, 78, 79, 80]. On the other hand, the micro/nanospheres composed of a hydrophobic polymer are commonly prepared using the w/o/w emulsification method [73, 81, 82].

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During micro/nanospheres fabrication using the w/o emulsification method, a polymer solution containing a protein or other chemical acts as an aqueous phase which is then dispersed in a continuous oil phase containing an oil soluble surfactant resulting in a w/o emulsion. In the organic phase the dispersion and break down of the aqueous phase into small particles can be done using a magnetic stirrer [74, 83] or high-energy stirring, such as homogenization [84, 77]. The continuous organic phase is composed of an organic oil, such as paraffin oil [77, 80, 79, 76], iso-octane [78, 85], ethyl acetate [74], n-hexadecane [76] or sunflower oil [76] with an oil soluble surfactant, such as Span 80 [76, 79, 80], Span 85 [85, 78] or Tween 80 [76] dissolved in the oil. During preparation of micro/nanospheres the surfactants play a significant role. They help to stabilize the emulsion droplets formed during the emulsification by forming a thin film around the emulsion droplets and by reducing the surface tension between the aqueous and oil phase [86]. Sometime, the w/o emulsification method is coupled with either an internal [87] or external gelation method [83, 84]. In such cases, the emulsion droplets (either microemulsion or nanoemulsion) formed can be cross-linked (either internally or externally) with various cross-linkers, such as $CaCl_2$, $CaCO_3$ or glutaraldehyde to form micro/nanospheres [74, 64, 76]. By contrast, sometimes the w/o emulsification is combined with a solvent diffusion method to prepare the micro/nanospheres [74]. When the emulsification method is combined with the solvent diffusion method micro/nanospheres are formed by diffusion out of water from the emulsion droplets to the continuous oil phase [74]. In order to remove oils, unreacted cross-linkers or other reagents, the prepared micro/nanospheres are then washed with deionized water, and various other organic solvents, such as isopropyl alcohol or n-hexane [88, 84, 77].

Unlike the w/o emulsification method, the w/o/w emulsification method is used to prepare the micro/nanospheres composed of a hydrophobic polymer [73]. In this process, proteins or other active molecules are first dissolved in an aqueous phase. To form a w/o emulsion, this aqueous protein solution is then emulsified with a non-miscible organic solvent, such as dichloromethane [89, 81, 90] or chloroform [90] containing a hydrophobic polymer [90, 91] dissolved in it which acts as an oil phase. The emulsification step can be carried out using a magnetic stirrer [91] or high energy stirring, such as homogenization or sonication [64, 81]. This primary emulsion is then mixed with a surfactant-containing aqueous solution followed by a high energy stirring to form a double emulsion of w/o/w. Various surfactants, such as Polyvinyl alcohol (PVA) [90, 91, 92], Tween 20 [93], sodium dodecyl sulfate (SDS) [93] and dodecyltrimethylammonium chloride

(DAC) [93] have been used in the past. Using this method, the removal of an organic solvent can be performed by either solvent evaporation or solvent extraction leaving the protein loaded micro/nanospheres in the aqueous continuous phase. These micro/nanospheres can be collected by filtering or centrifuging. However, using the w/o/w emulsification, mixing of the protein solution in the organic phase containing polymer is responsible for protein denaturation [89].

The size, morphology and swelling of micro/nanospheres play a direct role in controlling the rate and time of delivery of bioactive molecules [83]. During the fabrication of micro/nanospheres using an emulsification method, it is possible to control the size, morphology and degree of swelling by varying various process parameters, such as the stirring rate, concentration and MW of polymer, degree of cross-linking, concentration, type or hydrophile-lipophile balance (HLB) of surfactant. Therefore, based on a certain application the process parameters should be carefully selected.

The control of the size of micro/nanospheres is necessary in order to tune the release rate. For example, a larger surface area-to-volume ratio associated with the smaller particles exhibits a faster release compared to the larger particles [94]. During the fabrication of micro/nanospheres using the emulsification method, it is possible to manipulate the particle size and distribution pattern by varying the stirring speed (using magnetic stirrer or homogenizer) [54, 92, 95, 47]. Increasing the stirring speed increases the shear forces and turbulence, which in turn break down the emulsion into small droplets, thereby, decreasing the particle size [54]. In one study, the effect of the stirring speed on the microspheres size using both magnetic stirrer and high energy homogenizer has been studied. It was found that with the increase of the stirring speed of magnetic stirrer from 250 to 1250 rpm and the homogenizer speed from 6000 to 14000 rpm it was possible to reduce the size of PLGA microspheres from 80.85 to 62.04 µm and 15.47 to 4.56 µm, respectively [47]. In both cases, the high-power input at a higher stirring speed was explained as a reason to reduce the particle size [47].

The particle size can also be manipulated by using different type of surfactants, having different HLB value [96, 76], and at different concentrations [54, 92, 95, 86, 76]. By varying the surfactant type, HLB value and the concentration of surfactant it was possible to prepare microspheres in the range from 10-600 µm, in one study [76]. It has been reported that with the increase of surfactant concentration and the decrease of HLB value by using different types of surfactants it was possible to decrease the size of microspheres [76]. The effect of the type of

surfactants on the microspheres' size was tested in another study, and it was found that compared to the surfactant consisting of more fatty acid chains, the surfactant consisting of longer polyoxyethylene chains produced the smaller sized microspheres [96].

The MW and concentration of polymer play an important role because of their ability to control the size [54, 92, 47, 97] and matrix porosity of micro/nanospheres prepared using the emulsification method. The control of the particle size and matrix porosity is important as both of them can control the release rate [97]. With the increase of the polymer concentration and MW, the viscosity of the polymer solution increases, so a higher shear force is needed to break the polymer solution into small droplets [54, 47] resulting in larger sized particles. In one study, it was reported that increasing the MW of PLGA resulted in an increase of the microspheres' size due to an increased solution viscosity [47]. By increasing the concentration of polymethacrylate from 16.7 to 33.3 mg/ml the size of microspheres increased significantly from 97.6 to 125.8 µm, in another study [97]. Increasing the polymer concentration also decreased the microspheres' porosity resulting in a less release compared to the low polymer concentration counterparts, in another study [97]. These studies imply that the microspheres' size and porosity can be controlled by varying the polymer concentration, thereby affecting the release kinetics.

During the fabrication of micro/nanospheres using the emulsification method, the cross-linking condition, such as the concentration and type of cross-linker and cross-linking time was also found to affect the release rate. The effect of different cross-linkers, cross-linking concentrations and cross-linking times was evaluated in one study [98]. When different types of cross-linkers were used, due to a lower degree of cross-linking using Formaldehyde, it showed significantly faster release compared to the Glutaraldehyde cross-linked microspheres. Also, when the effect of different cross-linking concentrations and cross-linking times was evaluated in that same study, with the increase of the cross-linking concentration and cross-linking time the release of encapsulating molecules decreased [98]. In another study, the increased cross-linking density with the increased cross-linker concentration also decreased the release of encapsulating molecules [77]. Both of these studies imply that during fabrication of micro/nanospheres, the type and concentration of cross-linker and cross-linking time can affect the release kinetics. Reduced swelling and pore size at a higher degree of cross-linking might also reduce the release rate [99, 100]. On the other hand, an insufficient cross-linking time might be responsible for an incomplete cross-linking [101], resulting in an increased release of encapsulating molecules. Therefore, the

cross-linking time should be carefully selected to prevent an incomplete cross-linking of micro/nanospheres.

After fabrication of micro/nanospheres, they are either used in their wet state [65] or dried using different drying conditions, such as air dry at different temperatures [65], oven dry at different temperatures to different times [77, 102] or freeze dry [103]. After fabrication of polymer-based micro/nanospheres, the post treatment also affects their morphology and control the release rate [86]. While dehydrating gelatin microspheres using an acetone/water solution and then acetone, in one study, resulted in microspheres with non-porous, very smooth, and uniform morphology, their treatment with a pure acetone only resulted in wrinkled microspheres [86]. Also, in that same study, the freeze drying of microspheres resulted in microspheres with a porous morphology having the pore size in the range of 10-50 µm [86]. Compared to air drying, the freeze drying of chitosan microspheres, prepared using emulsification/internal gelation, increased the release rate of Hb in another study [65]. Therefore, to control the micro/nanospheres morphology and release rate the post treatment should be carefully selected for certain applications.

The performance of micro/nanospheres as a protein delivery device can vary based on the type of polymers used. In order to develop micro/nanospheres as a protein delivery device, both natural polymer, such as alginate, collagen, chitosan gelatin and albumin, starch, dextran, inulin, cellulose, hyaluronic and synthetic biodegradable polymers, such as PLGA, PLA, PLG, have been used in the past [104, 45]. During fabrication of micro/nanospheres the natural hydrophilic polymers are preferable over the synthetic polymer. Non-toxicity, inert nature towards proteins, chemical modification ability, biocompatibility, and bioavailability of natural polymers make them ideal candidates to prepare a protein delivery device [44]. Moreover, the use of this kind of polymers allows to fabricate micro/nanospheres without using organic solvents [44], responsible for protein denaturation. However, a better understanding of the protein release kinetics and more effective ways to reduce the initial burst release is important in order to get a desired protein release kinetics using natural polymer-based micro/nanospheres.

1.4 Alginate as a Delivery Device of Protein and Other Chemicals

Alginates have been declared by FDA (Food and Drug Administration) as a GRAS (generally regarded as safe) ingredient [105] and have been widely used as a delivery device of protein and other chemicals. Diffusion of several proteins from alginate beads has been reported

in literature, including basic fibroblast growth factor (bFGF) [106, 107], acidic FGF [107], endothelial cell growth factor (ECGF) [108], BSA [109], Insulin [110], and fibrinogen [109]. The reason for widespread application of alginate-based delivery devices relies on their relatively simple protein encapsulation process and excellent biocompatibility towards proteins [64]. Additionally, a mild encapsulation process free of organic solvents allows protein incorporation into an alginate without denaturation [111]. In order to design an alginate-based delivery device, it is important to understand the physical and chemical properties of alginate. Among various forms of alginates, sodium alginate (SA), which is a sodium salt of alginic acid [101], is mostly used.

Alginate is a naturally occurring anionic copolymer which is hydrophilic in nature and composed of 1–4 glycosidically linked mannuronic acid (M-unit) and guluronic acid (G-unit) units [112, 113, 64] (Figure 1.4 A). They can be obtained from various natural sources, such as brown seaweed and certain species of bacteria [114, 113, 101]. Depending on their sources, their MW and the relative percentage of G and M units can vary [112, 64]. The MW of commercially available alginates varies from 32,000 to 400,000 g/mol [113, 64].

Incorporation of proteins into alginate-based micro/nanospheres can be done under a relatively mild gelation process that can be performed in ambient temperature and does not require any toxic chemicals, such as organic solvents, thereby, preventing protein denaturation. During gelation, the G-units of one polymer chain form junctions with the G-units of the adjacent polymer by binding with the cross-linker cations [113]. This, in turn, results in a cross-linked gel structure with protein encapsulated within the matrix (See Figure 1.4B). Gelation of alginates can be induced by using various divalent cation of cross-linkers; e.g., Ba^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+} or Sr^{+2} can be used for gelation [64]. Among various cross-linkers, Ca^{2+} is mainly used because of its non-toxicity, clinical safety, easy availability, and economical attraction [64, 115].

The function of an alginate as a protein delivery device depends on its MW, and the relative percentage of G and M units. It has been reported that the protein release rate, mechanical strength, elasticity, swell ability, matrix porosity and degradability of alginate matrices can vary based on their relative (G/M) ratio [111, 104, 114]. Compared to the low MW alginates having a lower percentage of G-units, the high MW alginates having a relatively higher percentage of G-units are more effective in retarding protein diffusion [114]. This is most likely because only G-units of an alginate participate during cross-linking with Ca^{2+} and with the increase of G-units the degree of

cross-linking increases. This, in turn, increases mechanical strength resulting in an alginate gel that is less prone to swelling and erosion [113, 111]. However, alginate gels prepared with more than 70% G-unit content become brittle and they lose their swelling ability after drying. Furthermore, in presence of high G-units the porosity of alginate gels increases as they adopt a more open pore structure showing minimal shrinkage ability [104, 64, 115]. On the contrary, alginate gels prepared using a high percentage of M units become swellable, less porous, softer, and more elastic. However, as a result of high water absorption, alginate gels containing a higher percentage of M-units dissolve quickly compared to ones containing a higher percentage of G-units [64, 105, 111]. Therefore, a proper balance among the relative percentages of G and M units in the alginate matrix is necessary so that they minimize gel brittleness and show comparatively high mechanical strength, stability and less porosity in the gel matrix.
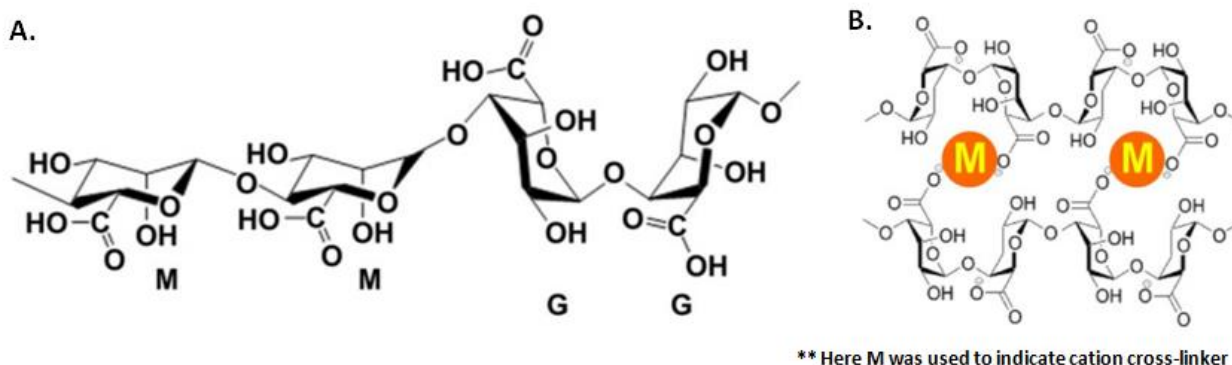
Figure 1.4. (**A**) Chemical structure of sodium alginate (**B**) Mechanism of ionic cross-linking between alginate and cation cross-linkers [116].

Several techniques are available to fabricate alginate-based micro/nanospheres. They can be prepared using ionotropic gelation, emulsification, electrodispersion or spray drying methods [49, 64]. Among them, the emulsification and spray drying are very popular. However, as discussed earlier, the heated air and drying process used in the spray drying method might denature some of the proteins [67]. By contrast, the alginate-based delivery devices prepared using the emulsification method can protect the bioactive proteins from degradation improving their biological half-lives. Using the emulsification method, bioactive proteins can be mixed evenly in the alginate solution and the solution can be emulsified under dispersive force (e.g., sonification or homogenization) [116, 84].

In most cases, the emulsification method is used in conjunction with external or internal gelation. Micro/nanoemulsion prepared using the emulsification process can be cross-linked by either external gelation [107, 83] or internal gelation [103, 117, 85]. During external gelation, micro/nanospheres are produced by adding $CaCl_2$ externally into the emulsion droplets of protein loaded alginate formed after emulsification [83]. On the other hand, during internal gelation, instead of adding $CaCl_2$ externally after emulsification, acid soluble calcium salts (e.g., calcium carbonate) are uniformly mixed with alginate prior to emulsification. Using this method, a controlled release of soluble calcium ions is usually obtained by a change in the pH by adding an oil soluble acid in order to free Ca^{2+} from insoluble calcium salts [117, 60, 118].

Properties of alginate spheres, such as strength, stiffness, pore size, and permeability of encapsulated molecules can vary based on their gelation process [118, 60]. Micro/nanospheres prepared using external gelation are inhomogeneous in structure. This is because during external gelation, a higher percentage of both Ca^{2+} and alginate are present in the surface and a lower percentage in the core resulting denser layer at surface and looser layer in core [118, 60]. Unlike external gelation, a concentration gradient is hardly present when internal gelation is used, resulting in a homogeneous structure of microspheres [118]. However, internal gelation has several limitations. Firstly, the pore size of microspheres prepared using internal gelation has been reported to be larger than the microspheres prepared using external gelation, resulting in the high permeability of release media into the microspheres [118]. Secondly, liberation of CO_2 from $CaCO_3$ during internal gelation might weaken the matrix [85]. Therefore, during fabrication of alginate micro/nanospheres a gelation method should be carefully selected.

In mammals, the degradation mechanism for an alginate is not enzymatic. This is most likely because, mammals do not have alginase enzyme necessary to degrade its polymer chain [113]. The degradation mechanism of an ionically cross-linked alginate is dissolution or hydrolysis. Dissolution occurs during the release of divalent cations of cross-linked alginates due to an exchange reaction with monovalent cation, such as Na^+ ion [113].

Even though alginate micro/nanospheres have been widely used as a protein delivery device, one of the major limitations of alginates as a delivery device is their inherent high porosity and instability at high pH medium. The morphology and release rate of encapsulating molecules using alginate spheres largely depend on the pH of the medium [114]. The release rate at a low pH decreases significantly due to shrinkage of alginate spheres [114]. On the contrary, at a higher pH,

a high degree of swelling and rapid dissolution of alginate spheres occurs [114, 119]. This is most likely due to an enhanced relaxation of the polymer chain of the alginate and an enhanced exchange of the divalent ions of alginate with the monovalent ions in surrounding media, resulting in a burst release of encapsulating molecules [114, 119]. In one study, Fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) was encapsulated in Ca-alginate microspheres and incubated in a phosphate buffer at pH 7.2 [120]. Low stability of the alginate above pH 5 has been reported to be the reason why all of the loaded FITC-BSA was released within 6 hours [120]. Considering the pH value (pH 7.2-6.9) of the interstitial fluid of cartilage [3], it is most likely that the use of alginate-based micro/nanospheres may increase release of the encapsulated proteins due to the poor stability of alginates, resulting in rapid swelling and dissolution.

In spite of having some limitations, alginates are still considered an excellent candidate as a protein delivery device because of their temperature independent mild gelation process, low price, relatively inert aqueous environment towards proteins, and ability to control protein release rates by varying various process parameters [113, 104, 44, 121, 111]. Many approaches have been undertaken to reduce the degradation rate of alginates. For example, it has been reported that increasing the alginate concentration can reduce the rate of degradation [122]. Increasing the degree of cross-linking, in another study, has been reported to decrease the degree of swelling by improving the mechanical properties of alginate [123], which can also reduce the degradation rate. The concentration of alginates and the cross-linking time used in the previous study, where 100% FITC-BSA was released from alginate microspheres were 2% and 10-minute respectively. Also, the resultant microspheres were dried completely by freeze drying [120]. The alginate concentration and cross-linking time that were used in that study might not be enough. Additionally, the pore size of the freeze-dried microspheres was probably larger resulting in a faster release rate [86, 65]. Another probable reason for the faster release might be because the microspheres were dried completely during freeze drying, so they developed surface cracking [102]. Increasing the alginate concentration and cross-linking time might reduce the release rate by decreasing the degradation rate and increasing the mechanical strength of alginate micro/nanospheres. Moreover, instead of complete dehydration, partial drying might reduce the pore size [113] of micro/nanospheres resulting in a less release. In order to effectively control the burst release of alginate, it is important to understand the factors responsible for a burst release and ways to prevent it.

Burst release can be referred to as the release of a very high percentage of encapsulating molecules within a short time [124]. Burst release occurs due to surface protein desorption and diffusion from the outer surface of micro/nanospheres [125, 62]. Due to its toxic side effect, in most cases this high initial burst release is undesirable [124]. Moreover, due to the burst release, a very high percentage of protein release occurs in a very short time, thereby, decreasing the effectiveness of the delivery device [124, 114]. The ideal protein release profile should show a zero-order release kinetics, i.e., a constant protein release with time as shown in Figure 1.5 [66]. However, practically this kind of protein release profile is difficult to achieve. In most cases, upon placement of micro/nanospheres in a release medium, an initial burst release of a very high percentage of proteins occurs before they achieve a constant release rate [124]. The difference between protein release profile maintaining a zero-order kinetics or one having a high initial burst release have been shown in Figure 1.5.
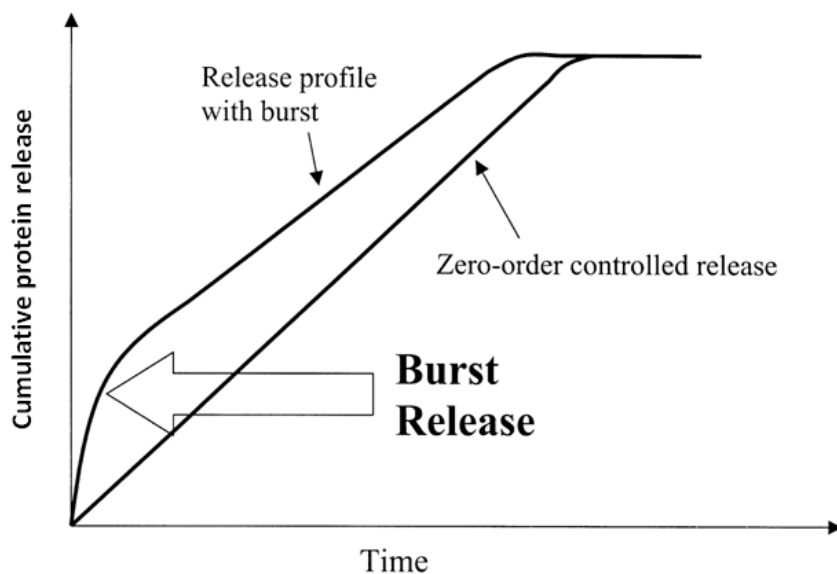
Figure 1.5: Schematic representation showing the difference in release profiles between zero-order controlled release and due to initial burst release [124].

Because of the inherent high porosity and high degradability of alginates, in most cases, coating or reinforcing alginates with several natural polymers, such as chitosan [63, 117], Dextran sulfate [63] or methylcellulose hydroxyethyl cellulose [126], has been used to prolong the protein

release and reduce the initial burst release. Due to surface coating or reinforcing of alginate-based micro/nanospheres, it is possible to reduce their degradability and porosity and increase their mechanical strength [104, 127, 44]. The effect of polycation coating on alginate beads using either poly (L-lysine HBr) or poly (vinyl amine) was evaluated in one study and reported a decrease in the burst release with an increase in polycation concentration [128]. However, coating or reinforcing of alginate-based micro/nanospheres add additional processing steps and cost. Moreover, improper coating of alginate-based micro/nanospheres should result in protein loss or an incomplete protein release profile [117, 83]. Therefore, alternative approaches to reduce protein diffusion need to be explored.

1.5 Approaches to Minimize Initial Burst Release and Protein Diffusion Through Microsphere Matrix

Various approaches have been considered to reduce the initial burst release and the diffusion of protein or other chemicals through the micro/nanospheres matrix. The initial burst release and protein diffusion from micro/nanospheres largely depend on the properties of proteins to be encapsulated. Additionally, the diffusion rate and the initial burst release can be controlled by regulating electrostatic interaction among the protein-polymers [104, 129], by varying polymer concentration [130, 131], MW [131], cross-linking density [132], particle size, swelling rate, protein-to-polymer ratio and drying condition [65]. Therefore, a thorough understanding of the relationship among these process parameters and the release mechanisms is important to design micro/nanospheres showing less burst release.

1.5.1 Effect of properties of protein

Properties of the proteins to be encapsulated, such as their MW [124], pI value [104, 117, 44, 129] and the percentage of protein loading [133, 134, 135], play a vital role in the protein release rate. Burst release has been reported to be decreased for the high MW proteins due to their greater hindrance to the pore diffusion [124, 136]. The effect of TGF-b2 and Bone morphogenetic protein-7 (BMP-7) on protein release from nanospheres was evaluated in one study. Due to comparatively a high MW of TGF-b2 compared to BMP-7, TGF-b2 loaded nanospheres showed slower release rate compared to the BMP-7 loaded counterparts [26]. However, an increase in the protein diffusion with the increase of protein MW has also been reported in another study when Ca-alginate macrobeads (d=3.30±0.20mm) were used. The diffusion of high MW γ-globulin

(MW=154kDa) and fibrinogen (MW=341kDa) was reported faster compared to low MW albumin (MW=69kDa). It was speculated that this might be due to the change in the Ca-alginate gel structure, when higher MW proteins were used for encapsulation, making the diffusion of γ-globulin and fibrinogen faster compared to albumin [109]. The solubility of chemicals has also been reported to have an effect on their diffusion through the microsphere matrix. For example, due to high water solubility, the release period of FITC-dextran was lasted only for 1-2 days in the release medium. By contrast, using the same condition the release period for the sparingly soluble Brilliant blue (BB) was more than 2 months [137]. From these studies it can be hypothesized that an increased protein solubility in an aqueous release medium might also increase protein release.

The net charge of proteins and their pI values can also influence their diffusion through the micro/nanosphere matrix [104]. For example, a protein with a high pI value and overall net positive charge can interact with an anionic polymer thereby inhibiting its diffusion through the polymer matrix. By contrast, a protein with a low pI value can release more rapidly though the anionic polymer matrix [104]. However, a sustained protein release by electrostatic interaction between proteins and polymers might cause protein denaturation because of the affinity of the polymer to bind with the protein [138]. Therefore, properties of proteins need to be considered carefully while designing a protein delivery device.

1.5.2 Effect of process parameters

Process parameters play a significant role in burst release. Burst release is a surface phenomenon and the entire release profile is proportional to the surface area exposed to the release medium. The least the surface area-to-volume ratio is exposed to the release medium the least is the burst release [62, 139]. It has been reported that compared to non-porous micro/nanospheres, porous micro/nanospheres have larger surface area-to-volume and consequently high permeability to release medium resulting in an increased initial burst release [140]. The presence of the pre-existing pores and channels in PLGA-based microspheres were found to be responsible for a high initial burst release of about 60% drugs [141]. Therefore, decreasing the porosity of micro/nanospheres might reduce their permeability to release medium resulting in a decreased protein release.

Like microspheres porosity, the degree of swelling also has a profound impact on the protein release [62, 139]. The mechanism of protein release involves swelling of the porous structure of microspheres followed by dissolution and diffusion of protein to reach the release

medium [142]. However, more swelling means less compact gel structure and more permeability of the release medium through the gel network resulting in more release [142]. Therefore, decreasing the degree of swelling of microspheres is another prerequisite to reduce the protein release rate.

An increase in the loading percentage of protein or a decrease in the polymer/protein ratio of micro/nanospheres is also responsible for a high initial burst release. The effects of various percentages of BSA, such as 2.64%, 0.66% or 0.45% loading on the release profile of PLGA-based microspheres were evaluated in one study. It was reported that the 2.64% BSA loaded microspheres showed a considerably higher initial burst and more rapid release compared to the 0.66% or 0.45% BSA loaded microspheres. This higher initial burst and rapid protein diffusion using a high percentage of BSA loading are most likely due to an increase in BSA concentration gradient between the microspheres and the outer aqueous phase of the release medium [133]. Therefore, the percentage of protein used to design protein loaded microspheres also needs to be carefully considered to reduce initial burst release.

Earlier studies confirmed that the polymer concentration [108, 109, 140, 63, 62, 133] and MW [121, 62] play a significant role in controlling the diffusion rate of encapsulating proteins through the micro/nanosphere matrix. This is because increasing the polymer concentration and MW will increase the solution viscosity, thereby, reducing the tendency of the protein or other encapsulated molecules to be carried toward the micro/nanospheres surface, hence, less burst release [62, 121, 143]. By varying the polymer concentration and MW, it is possible to control the internal gel structure and porosity of microspheres [112], cross-linking density [62, 112, 121], size [121], swelling ability [62], and degradation rate [144]. In case of PLGA, a hydrophobic polymer, increasing the MW from 5000 to 15000 Daltons decreased the initial burst release of FITC-BSA from 70% to less than 20%. The author attributed the decreased water uptake and degradation rate at a higher MW to be the reason of the reduced burst release and total release [144]. The decrease in the matrix porosity due to an increase in the PCL concentration from 16.7mg/ml to 33.3mg/ml were found to decrease the protein release [133]. In case of the hybrid chitosan/tripolyphosphate microspheres by increasing the chitosan concentration from 0.05 to 0.30 % and its MW, it was possible to reduce the initial burst release and total BSA release [62].

The effect of concentration and MW of alginates on the protein release has been reported in a few studies. For example, increasing the MW of an alginate reduced the initial burst release

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of BSA from the alginate microsphere prepared using an emulsification method [121]. In case of the Ca-alginate macrosbeads, prepared using the external gelation method, increasing the alginate concentration from 2 to 4% showed a considerable decrease in the release of BSA [109]. *However, to our knowledge, the effect of the alginate concentration on the initial burst release of BSA and other proteins from micro/nanospheres prepared using a combination of the w/o emulsification and external gelation method has not been investigated yet*. Since, the properties of micro/nanospheres and their initial burst release can vary based on the fabrication method used [145, 118, 146], therefore, it is worthwhile to test how varying alginate concentration can affect the initial burst release and total protein release. Also, while a considerable effect of the alginate concentration on the protein release has been reported in a previous study [109], another study found no effect of the alginate concentration on the protein release [108]. This implies that the effect of alginate concentration on the protein release can vary based on the type of protein used. Moreover, most often the encapsulating molecules that have been used to determine the effect of MW and alginate concentration on the release behavior of alginate micro/nanospheres are chemicals other than proteins. For example, the effect of alginate concentration on the release of Brilliant Blue (BB) dye was tested in one study. BB release decreased with an increase in alginate concentration and MW [131]. The effect of various alginate concentrations on the release of Furosemide was evaluated in another study and found that the drug release prolonged with an increase in alginate concentration [130]. However, no effect of the alginate concentration on the release of Blue Dextran was reported in another study [102]. Since the properties of these chemicals are quite different from proteins and the effect of alginate concentration on the protein release from micro/nanospheres prepared using different fabrication methods have not been studied enough, therefore, more investigation is required. This will allow to evaluate if the variation in alginate concentration shows similar effect on the release kinetics for proteins as it does for other chemicals. Furthermore, this will help to better understand if the varying alginate concentration shows similar effect on the initial burst release and total protein release when different fabrication methods are used. Therefore, the evaluation of the effect of alginate concentration on the protein release kinetics using different fabrication methods might help to design micro/nanosphere-based delivery devices having a less initial burst release.

Cross-linking conditions have also been reported to have a significant effect on the initial burst release and total release of proteins or other chemicals. Because of the more constricted gel

network of the cross-linked micro/nanospheres they show more capacity to delay the protein diffusion compared to the ones without cross-linking [114, 74]. The use of different types of cross-linkers at various concentrations [147, 62] or cross-linking times [101, 102, 127, 147, 148, 137] can reduce the initial burst release and total release of the encapsulating molecules. This is most likely because of their ability to control the cross-linking density and matrix porosity. It has been reported that due to an increase in the cross-linking density and a decrease in the chain mobility of polymers using a higher cross-linking concentration or cross-linking time will form stable, rigid and less swell able micro/nanospheres [149, 150]. These rigid and less swell able micro/nanospheres at a higher degree of cross-linking will reduce protein diffusion rate due to less available free space for protein diffusion [149, 150]. To test the effect of cross-linking time on protein release, BSA loaded chitosan microspheres were prepared in one study using an incubation method [147]. The incubation method involves suspending blank microspheres in a protein solution. In that study, the increase of cross-linking time increased the degree of cross-linking and reduced swell ability of microspheres. However, unexpectedly, at a higher cross-linking time instead of decreasing release rate, an increase in the initial BSA release was observed. This is because, at a higher cross-linking time, compact microsphere surface and less microsphere swell ability prevented the diffusion of BSA from the protein solution into the microspheres. Therefore, protein adsorbed near the microspheres surface instead [147]. Even though the result of this study was negative it helps to understand the effect of cross-linking time on the properties of micro/nanospheres. In another study, the release of lactic acid from gelatin microspheres, cross-linked with glutaraldehyde, decreased due to a reduction in microspheres swelling at a higher cross-linker concentration or cross-linking time [149]. By increasing cross-linker concentration or cross-linking time it was possible to reduce initial burst release by 20% and 30% respectively [149].

The effect of different cross-linkers, cross-linking concentrations and cross-linking times on the release behavior of various encapsulating chemicals from alginate microspheres also have been studied in previous studies. For example, the effect of cross-linker concentration and cross-linking time on the release of Celecoxib (CXB) from chitosan/alginate microspheres was evaluated in one study [127]. Increasing the cross-linker concentration or cross-linking time was found to reduce the diffusion rate of CXB due to the increase in cross-linking density and rigidity of the hybrid microsphere [127]. However, no effect of the cross-linking time on the release of blue

dextran from alginate beads was also reported [102]. The effect of various cross-linkers, such as BaCl$_2$, CaCl$_2$ or Al$_2$(SO4)$_3$ on the release of Furosemide from alginate microspheres was reported in another study [130]. It was found that a higher degree of cross-linking of microspheres prepared using either BaCl$_2$ or Al$_2$(SO4)$_3$ cross-linker showed less release compared to the CaCl$_2$ cross-linked counterparts [130]. The effect of 10, 20 and 30-minute cross-linking times on the degree of swelling of alginate beads and the release rate of liquid pesticides were tested in one study [148]. By increasing the cross-linking time from 10 to 30 minutes, it was possible to decrease the degree of swelling and the release rate significantly. Denser structure of the alginate beads with the increase of cross-linking time has been explained as a reason to the decreased diffusion of the encapsulated molecules [148]. *However, to our knowledge, the effect of cross-linking time on the initial burst release of the protein loaded alginate nanospheres has not been reported yet.* Therefore, the evaluation of the effect of cross-linking time on protein release is important.

Drying conditions also have an effect on the release rate of protein, or other chemicals [130, 65]. By drying of micro/nanospheres, it is possible to control the matrix porosity and distribution of the encapsulated molecules. During drying, water is removed from the gel network resulting in an increase in polymer concentration which eventually reduces the average pore size of the gel network causing a less release [65, 104, 124, 114]. Even though the drying of micro/nanospheres can reduce the release of encapsulating molecules, opposite results have also been reported. For example, freeze drying of the chitosan coated alginate microspheres showed a faster release of Hemoglobin (Hb), similar to wet microspheres, in one study [65]. However, when the microspheres were air dried at 4ºC to 6ºC they showed least Hb release compared to wet or freeze-dried microspheres. Since the freeze-drying temperature is higher compared to the air drying one, the author speculated that the less Hb release using the air drying method is attributed to its slower drying rate [65]. Moreover, the freezing process during the drying of micro/nanospheres using the freeze-drying method might break the particles, causing the leakage of their content that is undesirable [151]. Therefore, during designing protein delivery devices drying conditions should be carefully selected.

Even though the drying of micro/nanospheres have the potential to reduce the protein release rate, it might not be the case if they are completely dehydrated. Theory indicates that while complete dehydration might destroy the structural integrity of microspheres due to surface cracking causing faster release, partial drying might reduce the matrix porosity resulting less

release [114, 104, 130, 102]. For example, the effect of degree of dehydration on the release of furosemide from alginate microspheres was evaluated in one study [130]. It was observed that due to complete dehydration microspheres dried at 80°C for 2 hours showed faster Furosemide release compared to the air-dried microspheres [130]. In another study, to evaluate the influence of drying time on the release of Blue Dextran from alginate beads, three different drying conditions, such as drying at 80°C for either 6 or 12 hours or undried microspheres were evaluated [102]. It was found that the drying time has significant effect on the release of encapsulating molecules. Due to complete dehydration, drying of alginate beads at 80ºC for 12 hours resulted in faster release of Blue Dextran compared to 0 or 6 hour drying time group, with 0-hr drying time showing the least release. The surface cracking of microspheres during the drying was explained as a reason responsible for faster release for both drying time groups [102]. Therefore, the drying condition should be carefully selected to prevent rapid release. In case of protein loaded micro/nanospheres, they cannot be dried under such high temperature, since it might cause protein denaturation. *Even though it has been reported in literature that partial drying can reduce microsphere porosity and is better than complete dehydration, in which drying condition and how long the protein loaded alginate micro/nanospheres should be dried to prevent burst release is still unknown.* Therefore, the evaluation of the appropriate drying condition and drying time to prevent protein denaturation and reduce the initial burst release and total protein release is important.

In addition to morphology, the size of micro/nanospheres also has a profound impact on the release kinetics [86, 152]. The increase in particle size has been reported to reduce the initial burst release and release rate of encapsulating molecules [101, 64, 104, 113, 121, 130, 153]. This is most likely due to the reduced surface area-to-volume ratio and increased diffusion path distance with the increase in size [154, 155, 140]. Also, due to an increase in micro/nanospheres size, the degradation time of micro/nanospheres increases resulting in a prolonged release [121]. The size of micro/nanospheres depends on various process parameters, such as the stirring speed [65, 81], polymer concentration and MW [121], concentration and type of surfactants during emulsification [81, 121], the degree of cross-linking using different concentration of cross-linker [78] and different cross-linking time [100]. Drying conditions [151, 130] and the drying time [102] have also been reported to have an effect on the size of micro/nanospheres. During determination of the effect of various process parameters on the initial burst release and protein release kinetics it might be interesting to see if the change in micro/nanospheres' size or morphology plays a dominant role

to control the release rate. Normalization of the initial burst release and protein release kinetics based on the same relative size of micro/nanospheres will allow to determine the effect of various process parameters when their size is equal. This approach will indirectly help to confirm if the change in size of micro/nanospheres by varying various process parameters is affecting the initial burst release and protein release kinetics. *However, to our knowledge, normalization of the initial burst release and protein release kinetics based on the same relative size of micro/nanospheres has not been investigated yet to determine if the change in size of micro/nanospheres by varying alginate concentration, cross-linking time or drying time is affecting the release.*

As mentioned earlier, release rate depends on the surface area-to-volume ratio of micro/nanospheres exposed to release medium [152]. More surface area-to-volume ratio exposed to release medium results in more release [152]. The surface area-to-volume ratio of micro/nanospheres is also dependent on the number of micro/nanospheres. With the increase of the number of micro/nanospheres the surface area-to-volume ratio will increase resulting more release. This implies that the number of nanospheres might affect the release rate. The relative number of micro/nanospheres prepared using various processing conditions might also vary depending on the process parameters used. During determination of the effect of various process parameters on the initial burst release and protein release kinetics it might be interesting to see if the change in the relative number of micro/nanospheres affects the release rate. Normalization based on the same relative number of micro/nanospheres will allow to determine the effect of various process parameters on the initial burst release and protein release kinetics when the relative number of micro/nanospheres is equal. This approach will indirectly help to confirm if the change in relative number of nanospheres is affecting the release. *However, to our knowledge, normalization of the initial burst release and protein release kinetics based on the same relative number of micro/nanospheres has not been investigated yet to determine if the change in relative number of micro/nanospheres by varying alginate concentration, cross-linking time or drying time is affecting the initial burst release and protein release kinetics.*

From the above discussion, we can conclude that by varying various process parameters, such as the polymer concentrations, MW, type of cross-linker, cross-linking concentration, cross-linking time, drying condition and drying time, it is possible to control the initial burst release and protein release kinetics of micro/nanospheres. This is because these process parameters allow controlling the size, number of micro/nanospheres, porosity, cross-linking density and relative

percentage of protein in micro/nanospheres which in turn can affect the initial burst release and protein release kinetics. However, most often the encapsulating molecules that have been used to study the effect of these process parameters are not protein but small MW drugs, dye and other chemicals [131, 130, 112, 127, 153, 102, 101, 148]. Since, the MW, charge, PI value of proteins are quite different than those of other chemicals, therefore, it is important to investigate if the effect of these process parameters show similar trend on the initial burst release and protein release kinetics as it does for other chemicals. Moreover, the effect of the alginate concentration, cross-linking time or drying time on the initial burst release and total protein release from alginate micro/nanospheres prepared using a combination of w/o emulsification and external gelation method have not been investigated yet. Variation of these process parameters might affect the size and number of micro/nanospheres which might also affect the release. However, normalization of the initial burst release based on the same relative size or number of nanospheres has not been investigated yet to determine if the change in size or relative number of micro/nanospheres by varying the alginate concentration, cross-linking time or drying time is affecting the release rate. Evaluating these effects is important to understand their significance on the protein release kinetics and to develop alginate micro/nanospheres having a less initial burst release. Therefore, a thorough investigation of the effect of these process parameters on the initial burst release and protein release kinetics from alginate nanospheres prepared using various process parameters is necessary.

The initial release and total protein release of micro/nanospheres also depend on the properties of the bioactive proteins to be encapsulated. BSA has been used widely used in past as a model protein of BMP-7 [156] and the MW of BSA is close to BMP-7, an important bioactive protein for the cartilage defect repair. Hence, it was expected that during the development of alginate nanospheres as a delivery device, use of BSA would help to better understand the effect of these process parameters on the initial release and release kinetics of alginate nanospheres. This would provide useful information to test the potential of alginate nanospheres to use as a delivery device for the small MW BMP-7 and other proteins.

1.6 Research Objectives

The aim of this thesis was to develop alginate nanospheres as a protein delivery device with a reduced initial burst release by varying various process parameters for potential applications in CTE.

In this study, the protein loaded alginate nanospheres were prepared using a combination of water-in-oil emulsification and external gelation. The concentration of alginates used to prepare alginate-based micro/nanospheres ranges from 2% to 5% [127, 130, 63, 101, 109]. Since the effect of alginate concentration on the release rate of protein from alginate nanospheres prepared using the emulsification method has not been investigated yet, therefore, this thesis investigated the effect of alginate concentration on the initial burst release, protein release kinetics and EE%. The use of 2% alginate in a previous study was responsible for very fast release of 100% FITC labeled BSA (within 6 hours) [120]. Therefore, in this present study, during the determination of concentration effect, 3% alginate was selected as the lowest value and 5% alginate as the highest value. Alginate concentration higher than 5 % was not selected because at alginate concentration higher than 5%, the solution becomes very viscous and it was very difficult to dissolve the alginate uniformly using a magnetic stirrer.

In case of alginate-based microspheres, cross-linking time that was tested in previous studies to determine the release of encapsulating chemicals, ranged from 5-60 minutes [127, 102, 101]. However, the effect of cross-linking time on the protein release from alginate nanospheres has not been investigated yet. Therefore, this thesis investigated the effect of 1, 10 or 30-minute cross-linking time on the initial burst release and protein release kinetics. Here, 1-minute cross-linking time was selected as a lowest value to show how incomplete cross-linking might affect the initial burst release and 30-minute cross-linking time was selected as the highest value. Cross-linking time higher than 30-minute was not tested since previous studies already confirmed that 30-minute is the maximum time required to uptake Ca2+ by alginate gel matrix [157, 158].

This thesis also investigated the effect of drying condition and drying time on the initial burst release and protein release kinetics of alginate nanospheres. To determine the effect of drying time, nanospheres were dried at 37°C at different times, such as 0-hour, 0.5-hour, 1.5-hour, 4.5-hour and 24-hour. Since, the effect of drying time on the nanospheres dried at 37 ℃ was not investigated before and it was not clear how long the nanospheres should be dried to reduce release, therefore, drying time was selected based on log interval as it allows to visualize the effect of certain parameters on certain outcome by minimizing the number of groups necessary. Here the 24-hour drying time was selected as a completely dehydrated group (as no mass change of alginate nanospheres was observed after 24-hour) to show how complete drying might affect the initial burst release.

By varying the alginate concentration, cross-linking time or drying time the size and the relative number of nanospheres can vary, which can also affect the initial burst release and protein release kinetics. Therefore, this thesis also investigated the effect of the size and relative number of nanospheres on the initial burst release and protein release kinetics by normalizing them based on the relative size and relative number of nanospheres.

1.7 Research Hypothesis

Increasing the alginate concentration, cross-linking time or drying time reduces the initial burst release independently of associated changes to the size and number of nanospheres.

1.8 Thesis Organization

The rest of the thesis organized as follows. Chapter 2 discusses the experimental design, materials and methods used for the development and characterization of protein loaded alginate nanospheres. Chapter 3 provides the results of the effects of various process parameters on the relative number of nanospheres and their size and distribution pattern, protein release kinetics, initial burst release and EE% along with a detailed and comparative explanation of results obtained. This chapter also discusses the limitations of present study and finally, Chapter 4 summarizes the thesis providing a conclusion and discussing some future directions.

MATERIALS AND METHODS

This chapter discusses the materials and methods used to fabricate and characterize the protein loaded alginate nanospheres. Section 2.1 describes the materials and fabrication method used to fabricate the BSA loaded alginate nanospheres; and Section 2.2 describes the methods used to characterize the performance of BSA loaded alginate nanospheres as a protein delivery device.

2.1 Materials and Methods

2.1.1 Materials

Low viscosity Sodium Alginate (B25266) from Fisher scientific, Paraffin oil, Span 80 from, BSA (A2153), Dulbecco's modified Eagle's medium (D5648) and Penicillin-Streptomycin from Sigma Aldrich, 24 and 96 well plates from Thermo Fisher Scientific, isopropyl alcohol, double distilled water (DDW), calcium chloride ($CaCl_2$), Phosphate buffered saline (PBS) and Bradford assay reagent.

2.1.2 Preparation of protein loaded alginate nanospheres

In order to prepare protein loaded alginate nanospheres, a combination of w/o emulsification and external gelation method was used as reported in [84]. Briefly, SA was dissolved in DDW with the help of a magnetic stirrer to get an alginate concentration of either 3% or 5%. BSA, a model protein was then added into the alginate solution and then mixed completely with the help of a magnetic stirrer to get a protein concentration of 300 µg/mL of alginate. This BSA containing alginate solution acted as an aqueous phase during emulsification. Then in a 24 well plate, 50 µL of a nonionic Span 80 surfactant was added in 1 mL of Paraffin oil to get a Span 80 concentration of 5%. The mixture was homogenized at 5000 rpm for 20s using a probe type tissue homogenizer (Fisherbrand™ 850 homogenizer with powerful 850-watt motor, probe dimension: 7 x 110 mm). The mixture of paraffin oil and 5% Span 80 acted as a continuous oil phase during emulsification. Next, 300 µL of aqueous alginate/BSA solution was added into the non-aqueous paraffin oil/Span 80 mixture and was homogenized again for another 40s to break

down the solution into small emulsion droplets, thereby, forming a water-in-oil emulsion. During emulsification, the Span 80 was added as a surfactant to stabilize the emulsion mixture by decreasing the interfacial tension among the aqueous droplets and the surrounding oil phase, thereby, forming stable alginate droplets. It is important to note that the concentration of surfactant plays a significant role to prepare stable emulsion droplets. The concentration of a surfactant should be equal or above its CMC to prepare stable emulsion droplets. The CMC of Span 80 at waters-paraffin oil has been reported to be 0.5% (w/w), and a previous study from our lab confirmed that the 5% span 80 is enough to prepare spherical alginate microspheres without aggregation [159]. Therefore, the 5% Span 80 was assumed to be enough to prepare highly stable aqueous droplets without coalescence. Afterwards, to cross-link the alginate droplets, 150µL of 500mM $CaCl_2$ solution was added externally and the mixture was further homogenized for 20s. The mixture was then left for cross-linking for a specific period of time to form the cross-linked alginate nanospheres. During cross-linking, the Ca^{2+} replaces the Na^+ of alginate, and forms an ionically cross-linked three-dimensional matrix of alginate gel spheres entrapping the protein [121]. After cross-linking for a specific period of time, 300 µL of pure isopropyl alcohol was added to harden the alginate nanospheres. The resultant nanospheres were then collected by centrifugation at 1983 x g for 10 minutes. To remove some of the trapped oil droplets, unreacted reagents and uncomplexed polymers, the nanospheres were then washed two times with 1mL isopropyl alcohol and one time with 1 mL DDW. After fabrication, the nanospheres were either used directly without drying or dried at 37°C for a specific period of time, and then used for nanospheres' characterization. Preliminary study showed that alginate interferes during the Bradford assay (see Appendix A). Therefore, alginate nanospheres without any BSA were used as a negative control. Alginate nanospheres without any BSA were prepared the same way as mentioned above except no BSA was mixed with the alginate. The schematic diagram of the fabrication method used to prepare alginate nanospheres is given in Figure 2.1.

It is important to note that during determination of the effect of various process parameters on the performance of alginate nanospheres, at least 2 batches of nanospheres (where each batch was performed in triplicate and contains 3 independent samples) were prepared. The only exception was for nanospheres prepared using the 3% or 5% alginate, 10-minute cross-linking time and 0-hour drying time, where total 3 batches (n= 3 x 3 = 9 independent samples) of nanospheres were prepared to determine the protein release kinetics, initial burst release and EE%. For these

groups, in order to get an equal sample size, the data of 6 independent samples out of 9 was randomly selected using the SPSS software. Reporting the number of batches and the number of independent samples at the same time is quite confusing. Therefore, from now on, the number of independent samples used, which in this case is always 6, is reported.
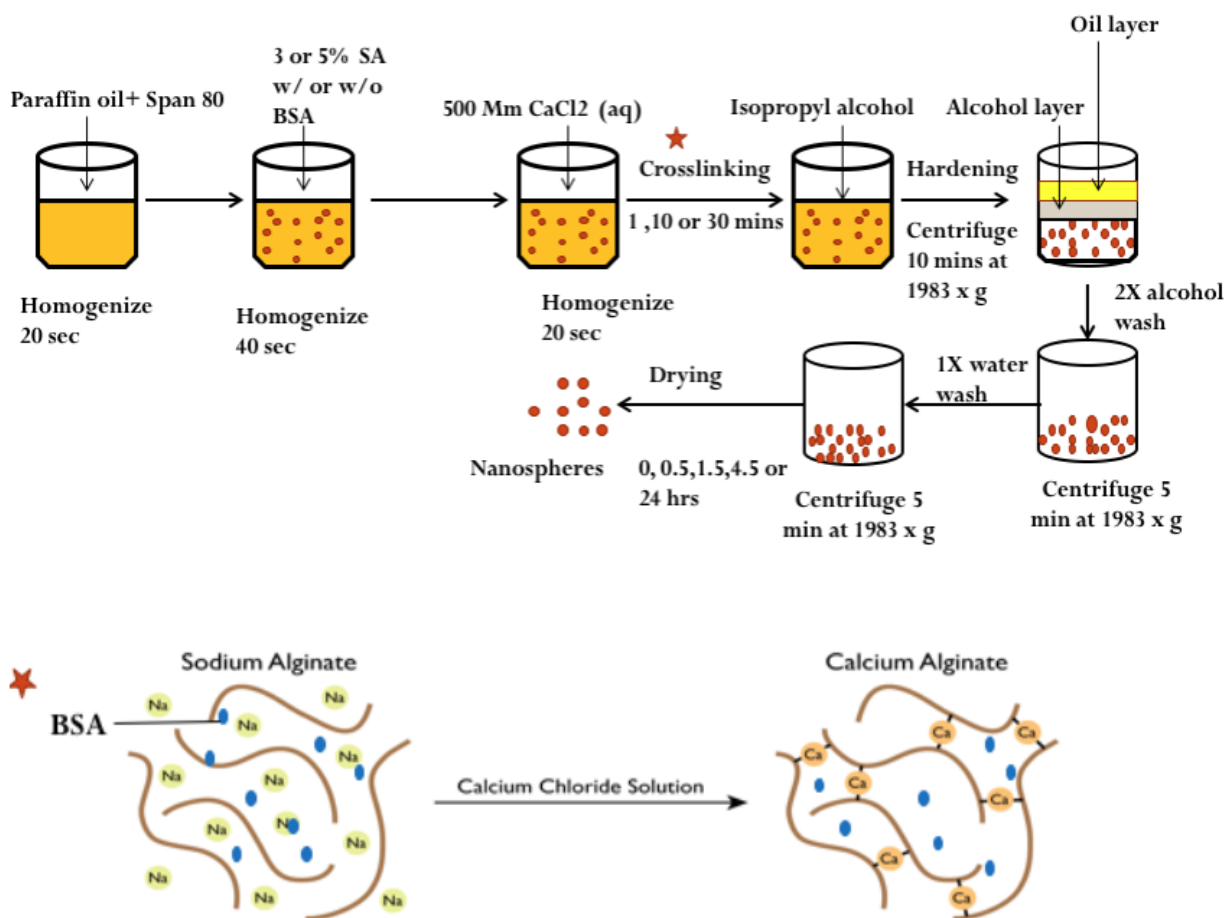
Figure 2.1: Experimental design of microsphere fabrication. A. Schematic diagram of nanosphere fabrication. B. Representation of Cross-linking process of Sodium alginate with $CaCl_2$ [160].

2.1.3 Experimental Design

A summary of the process parameters used to fabricate BSA loaded alginate nanospheres is given in Table 2.1. When the effect of one factor was evaluated only its own parameters were altered, keeping the other parameters constant. The first step of the experiment was to determine the effect of alginate concentration on the initial burst release. After determining the effect of

alginate concentration, the concentration of alginate that showed a minimal initial burst release was used in the second step to determine the effect of cross-linking time on the initial burst release. Finally, the cross-linking time that showed a minimal initial burst release was used along with the alginate concentration selected in the first step to determine the effect of drying time on the initial burst release.

Table 2.1 Experimental design used to determine the effect of various process parameters on the performance of BSA loaded alginate nanospheres

	Factors Tested	Alginate Concentration (%)	Cross-linking time (Min)	Drying time (hr)
1st Step ⟶	Concentration	3	10	0
		5	10	0
2nd Step ⟶	Cross-linking Time	3 or 5	1	0
		3 or 5	10	0
		3 or 5	30	0
3rd Step ⟶	Drying Time	3 or 5	1 or 10 or 30	0
		3 or 5	1 or 10 or 30	0.5
		3 or 5	1 or 10 or 30	1.5
		3 or 5	1 or 10 or 30	4.5
		3 or 5	1 or 10 or 30	24

2.2 Characterization of Alginate Nanospheres

2.2.1 Morphology, size and number of nanospheres

Morphology, size and the relative number of alginate nanospheres prepared using various processing conditions were determined using an optical microscope (Zeiss) equipped with a camera. Briefly, the nanospheres prepared using various processing conditions (e.g., alginate concentration, cross-linking time and drying time) were suspended in 1 mL DMEM (pH 7.2). From

the suspended nanospheres, 3 µL was taken in a microscope slide and micrographs were taken using the optical microscope. The volume of the suspended nanospheres was kept constant at 3µL, and 63X magnification was used to take all of the images. For each sample, a total of 3 micrographs were taken. The size of nanospheres and their distribution pattern and the relative number of nanospheres prepared using various processing conditions were then analyzed from these micrographs using the ImageJ software. The ImageJ software provides the area and relative number of nanospheres of a micrograph. The area of nanospheres found using the ImageJ software was then used to calculate the diameter of nanospheres using Excel. For each experimental condition, to determine the average diameter of nanospheres and their distribution pattern and the relative number of nanospheres, micrographs of 6 independent samples (6 X 3=18 micrographs) were analyzed. The distribution pattern of nanospheres prepared using different experimental conditions was analyzed using the StatPlus software. For different experimental conditions to produce a histogram the lower bin limit was set to 100 and the upper bin limit to 2000 with a bin interval of 100.

The relative size and relative number of nanospheres prepared using various processing conditions were evaluated to normalize the protein release kinetics and the initial burst release based on the relative size and relative number of nanospheres. After analyzing the average diameter of nanospheres, in order to determine the relative size of nanospheres for a specific processing condition (alginate concentration, cross-linking time or drying time), the process condition that provided the lowest average diameter of nanospheres was considered as 1 and the diameter of nanospheres prepared using other conditions was compared relative to 1. Similarly, after analyzing the relative number of nanospheres for a specific process parameter, the process condition that provided the least relative number of nanospheres was considered as 1 and the relative number of nanospheres prepared using other conditions was compared relative to 1.

2.2.2 Protein release Study from BSA loaded nanospheres *in vitro*

In this study, the supernatant protocol [57] (i.e. removing whole supernatant from the same batch of nanospheres at different timepoints) was used to determine the protein release kinetics. It is important to note that at the beginning the individual tube protocol, i.e., use of different batches of nanospheres prepared using the same processing condition to collect supernatant at different timepoints, was used to determine the protein release kinetics. However, the individual tube protocol could not show an expected trend in the protein release kinetics as this protocol had lots

of variability issues because of using different batches of nanospheres at different timepoints (See Appendix B). On the other hand, after a lot of troubleshooting the supernatant protocol showed an expected trend in the protein release kinetics and it was reproducible (See Appendix C). Therefore, in the present study the supernatant protocol was used. Using the supernatant protocol, nanospheres were suspended in 1mL of DMEM containing 1% Penicillin-Streptomycin (PS) and incubated in a shaking incubator operating at 37°C and 100 rpm. PS was used in DMEM to prevent the contamination by bacteria or fungi. Four different timepoints, such as 5-hour, 24-hour, 48-hour and 96-hour were selected in order to determine the protein release kinetics. At predetermined time periods, whole 1mL supernatant was collected for analysis by centrifugation at 1983 x g for 10 minutes. The collected supernatant was centrifuged again 2X at 16873 x g for 15 minutes each to get a clear supernatant solution. After supernatant collection, the pellet of nanospheres was re-suspended in 1mL fresh DMEM and put back into the shaking incubator and the process was repeated until the end of the experiment. The protein contents in the collected supernatant were analyzed using the Bradford protein assay which has an absorption maximum at 595nm. The linear concentration range used is 1.56–100 µg/mL of protein, using BSA as the standard protein molecule. As mentioned earlier, alginate interferes during the Bradford assay, therefore, alginate nanospheres without any BSA were used as a negative control for all cases.

2.2.3 Determination of burst release

For nanospheres prepared using various processing conditions, more than 60% of BSA was released within first 5 hours. Burst release means the release of a very high percentage of encapsulated protein at initial timepoint, therefore, the percentage of total released BSA at 5-hour was presumed to be the initial burst release. In order to characterize the burst release, nanospheres were suspended in 1 mL DMEM and incubated in a shaking incubator operating at 37°C and 100 rpm. The supernatant was collected at 5-hour and protein content in the supernatant was analyzed using Bradford protein assay (as described in section 2.2.2). The effect of various processing conditions on burst release was determined by calculating what percentage of the total released protein was released at the 5-hour timepoint. The burst release was determined from an average of 6 independent samples and was calculated using the following equation:

$$Burst\ Release\ (\%) = \frac{Mass\ of\ protein\ released\ at\ 5\ hour\ timepoint}{Mass\ of\ total\ released\ protein\ after\ 96\ hour}\ X\ 100 ....(2.1)$$

2.2.4 Normalization of data to determine initial burst release and protein release kinetics

In order to determine the protein release kinetics and initial burst release, the amount of protein released from nanospheres prepared using different processing conditions were normalized in 3 different ways:

2.2.4.1 Normalization based on nanospheres' weight

In order to normalize data, at first, the amount of protein released from the total amount of recovered nanospheres was evaluated. There was variability in the amount of nanospheres created in each tube. Therefore, to normalize the protein release kinetics based on the nanospheres' weight, the protein released at different timepoints from the total amount of recovered nanospheres was divided by the nanospheres' weight to get protein release per mg of nanospheres. Drying time was one of the variables to be tested, therefore, nanospheres were not dried completely before the protein release assay. Once the protein release assay was completed, nanospheres used for the assay were dried completely to obtain their weight. After determination of the protein release kinetics, the burst release per mg of nanospheres was calculated using equation 2.1.

2.2.4.2 Normalization based on the relative size of nanospheres

To determine the effect of nanospheres' size (by varying the alginate concentration, cross-linking time or drying time) on the protein release kinetics and initial burst release, protein release data normalized based on the nanospheres' weight was normalized again based on the relative size of nanospheres. To normalize the protein release kinetics based on the relative size of nanospheres, protein released per mg of nanospheres at different timepoints were divided by the relative size of nanospheres. Similarly, to normalize the initial burst release based on the relative size of nanospheres, the initial burst release per mg of nanospheres was divided by the relative size of nanospheres.

2.2.4.3 Normalization based on the relative number of nanospheres

The protein release data normalized based on the nanospheres' weight was also normalized based on the relative number of nanospheres to determine the effect of relative number of nanospheres on the protein release kinetics and initial burst release. To normalize the protein release kinetics based on the relative number of nanospheres, the protein released per mg of nanospheres at different timepoints were divided by the relative number of nanospheres. Similarly,

to normalize the initial burst release based on the relative number of nanospheres, the initial burst release per mg of nanospheres was divided by the relative number of nanospheres.

2.2.5 Determination of encapsulation efficiency

In order to determine the encapsulation efficiency (EE%), the total amount of BSA released in the released medium, i.e. DMEM after 96 hours was presumed to be the total amount of encapsulated protein. This is because no BSA release was found after 96 hours (see Appendix D). BSA was added in the alginate solution at a concentration of 300 µg/mL of alginate. 300 µL of the protein containing alginate solution was used to prepare each sample, therefore, theoretically maximum amount of BSA that could be present in each formulation is 90 µg. To evaluate the EE%, the total amount of recovered nanospheres prepared using a specific experimental condition, was suspended in DMEM containing PS and then incubated in the shaking incubator (operating at 100 rpm speed and 37ºC) for 96 hours to promote the release of all of the loaded protein. Instead of determining the protein release after 96 hours, the protein release was determined at four different timepoints, i.e. 5-hour, 24-hour, 48-hour and 96-hour to fulfill a proper sink condition. At predetermined time intervals, whole 1 mL supernatant was collected for analysis by centrifugation at 1983 x g for 10 minutes. The collected supernatant was centrifuged again 2X at 16873 x g for 15 minutes each to get a clear supernatant solution. The protein content in the supernatant was analyzed using the Bradford protein assay as discussed in section 2.2.2. The alginate nanospheres without any BSA were used as a negative control. The EE% was determined from the average of 6 independent samples and was calculated using the following equation:

$$Encapsulation\ Efficiency\ (\%) = \frac{Mass\ of\ protein\ released\ from\ total\ amount\ of\ recovered\ nanosheres\ after\ 96\ hours}{Mass\ of\ protein\ added\ in\ each\ formulation}\ X\ 100 ....(2.2)$$

2.2.6 Statistical Analysis

In this study, the statistical analysis was performed using SPSS V.22 (SPSS, Chicago, IL, USA). The distribution of data obtained using various processing conditions were tested for normality using the Shapiro-Wilk test. All data is presented as mean ± SEM (standard error of the mean). A difference was considered statistically significant for $p \leq 0.05$.

During determination of the effect of each process parameter on the initial burst release, EE% and diameter of nanospheres, the independent-sample t-test was performed when the effects of two independent groups were compared (e.g., alginate concentrations). On the other hand, the one-way analysis of variance (ANOVA) was used when the effects of more than 2 groups were compared (e.g., cross-linking and drying time effect). In the case of one-way ANOVA, when significance was found the Tukey's multiple comparison test was utilized to assess where the difference occurred between the groups.

During determination of the effect of alginate concentration on the relative number of nanospheres, the data for 3% alginate did not fall into normal distribution. Thus, the Mann-Whitney U test (equivalent to the independent-sample t-test) was used. On the other hand, during determination of the effects of cross-linking time and drying time on the relative number of nanospheres, all data fell into normal distribution. Therefore, in the case of cross-linking time and drying time the one-way ANOVA followed by Tukey's post-hoc test was performed when significance was shown.

During determination of the effect of various processing conditions on the protein release, the protein release data of some of the timepoints was not normally distributed. Therefore, the Mann-Whitney U test (equivalent to the independent-sample t-test) was used when two groups were compared (e.g., alginate concentration effect). By contrast, the Kruskal Wallis test (equivalent to one-way ANOVA) was used when more than two groups were compared (e.g., cross-linking and drying time effect). In the case of Kruskal Wallis test, when significance was shown the Dunn's multiple comparison test with Bonferroni correction was utilized to assess where the difference occurred between groups.

To determine whether there was a significant difference in the amount of protein released within the same experimental group and at different timepoints, the one-way repeated measures ANOVA was used when the protein release data for all the timepoints fell into normal distribution. On the other hand, the Friedman test (equivalent to one-way repeated measures ANOVA) was used when the protein release data for all the timepoints did not fall into normal distribution. In the case of one-way repeated measures ANOVA, when significance was shown multiple analysis using Bonferroni correction was utilized to determine whether there was a significant difference in the amount of protein released between 5 and 24-hour, 24 and 48-hour and 48 and 96-hour timepoints. On the other hand, in the case of Friedman test when significance was shown, the

Wilcoxon signed-rank test was performed for multiple comparisons with Bonferroni correction applied to show significant difference in the amount of protein released at different timepoints.

CHAPTER 3

EXPERIMENTAL RESULTS AND DISCUSSION

This chapter reports the results obtained from this study and provides a detailed discussion of these results. Section 3.1 discusses the effects of alginate concentration, cross-linking time and drying time on the performance of alginate nanospheres as a protein delivery device; Section 3.2 provides a detailed discussion explaining the results obtained in Section 3.1; and Section 3.3 explains the limitations of the present study and factors that might have affected the results.

3.1 Experimental Results

3.1.1 Effect of alginate concentration

This subsection describes the results related to the effect of alginate concentration on the nanospheres morphology, size and distribution pattern, relative number of nanospheres, protein release kinetics, burst release and EE%. Table 3.1 summarizes the effect of alginate concentration on the initial burst release, size and relative number of nanospheres.

3.1.1.1 Morphology, size and relative number of nanospheres

Nanospheres prepared using various alginate concentrations were smooth and spherical in shape, as shown in Figure 3.1. Also, it was found that the nanospheres prepared using a higher alginate concentration had a higher apparent contrast compared to their lower alginate concentration counterparts. In addition, both the 3% and 5% alginate resulted in nanospheres having a normal size distribution pattern, as shown in Figure 3.2, with most of the nanospheres being in the range between 700 to 900 nm. Analysis of the particle size indicated that variation of the alginate concentration had a significant effect on the mean diameter of nanospheres. As expected [130], with an increase of alginate concentration from 3 to 5% the mean diameter of nanospheres increased from 674.30 ± 12.37 to 855.30 ± 24.22 nm (Figure 3.3). The relative number of nanospheres that was found after analyzing the micrographs was 2898.33 ± 285.23 and 1973.39 ± 166.37 for 3 and 5% alginate respectively. Increasing the alginate concentration showed a significant decrease in the relative number of nanospheres. It was found that the relative number

of nanospheres prepared using different alginate concentrations was inversely proportional to their mean diameters.

A.   B.
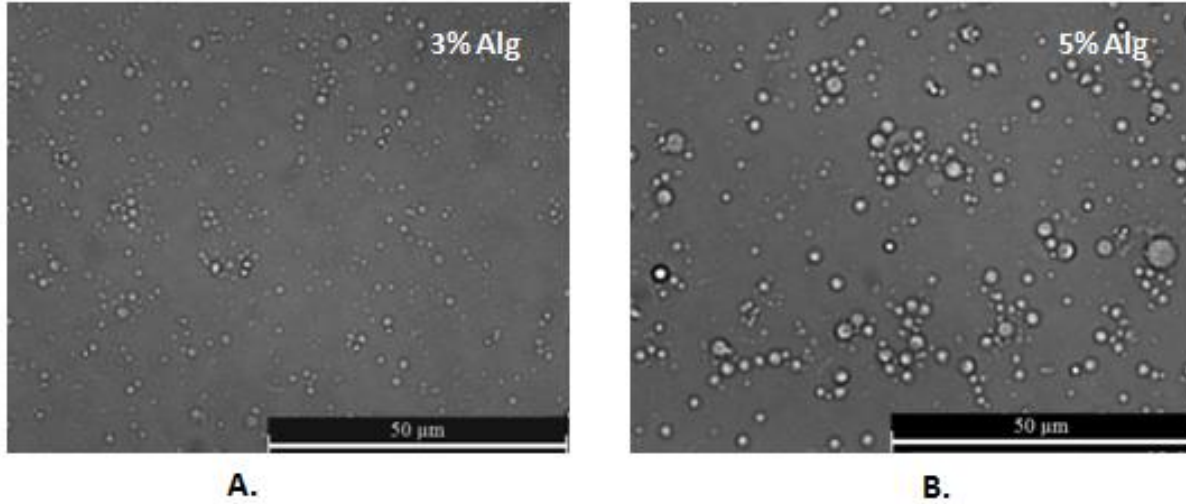
Figure 3.1: The effect of alginate concentration on nanospheres' morphology prepared with (A) 3%, and (B) 5% Alg. Optical microscope with a 63X magnification was used to take all the micrographs. The alginate nanospheres were prepared under the following conditions: 10 min Cross-linking time and 0-hour drying time.
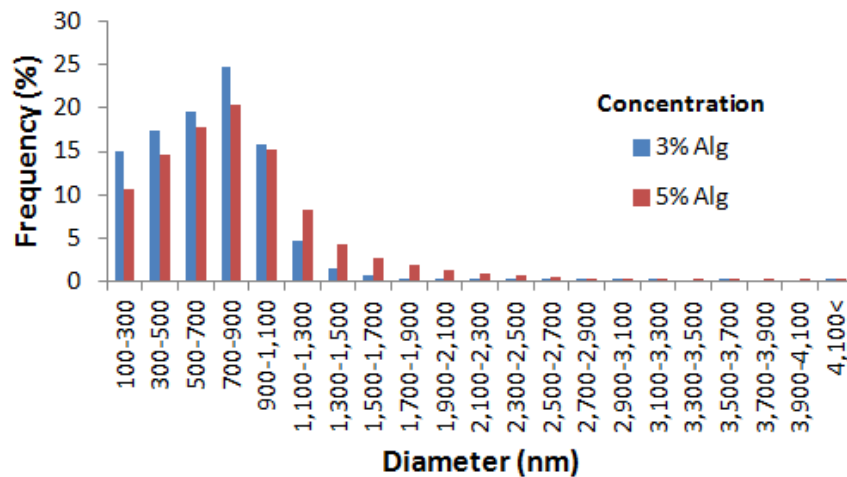
Figure 3.2: The effect of alginate concentration on the size distribution of alginate nanospheres.

Data represents mean, error bars are SEM (number of independent samples, n = 6). Asterisks (*) indicate significant difference for different alginate concentration.
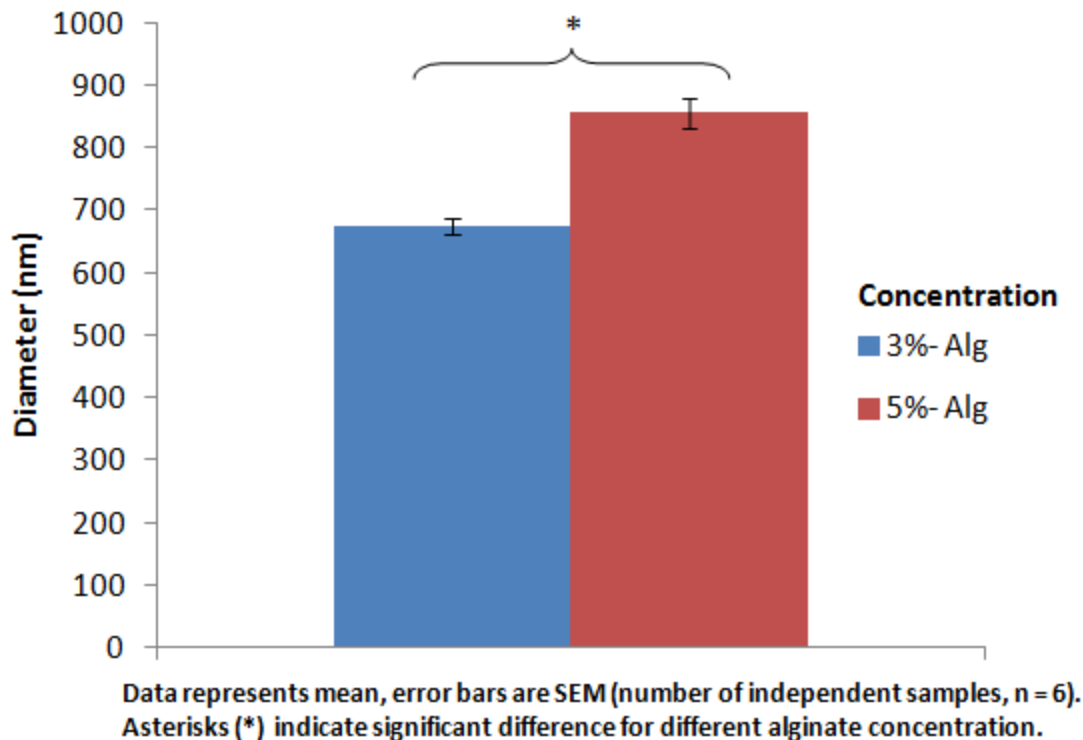
Figure 3.3: The effect of alginate concentration on the diameter of alginate nanospheres.

3.1.1.2 Protein release kinetics, burst release and EE%

The effect of alginate concentration on the *in vitro* protein release kinetics has been reported in Figure 3.4 (A-C), where the cross-linking time and drying time were kept constant at 10 minutes and 0 hour respectively. The concentration of alginate appeared to had an effect on the *in vitro* protein release kinetics when the data was normalized based on the nanospheres' weight, as shown in Figure 3.4A. With the increase of the alginate concentration from 3 to 5%, the protein release at the initial timepoint (5-hour) and the total protein release was found to be decreased. However, increased alginate concentration showed no significant difference in the protein release at any of the timepoints. Theoretically, the maximum amount of BSA that could be present in each formulation is 90 µg (see section 2.2.5). Based on the measured nanospheres weight, the maximum amount of BSA (if 100% of BSA got encapsulated) that could be present per mg of nanospheres are 5.59 and 4.29 µg for 3 and 5% alginate concentrations respectively. However, the total protein release per mg of nanospheres after 96 hours was 2.56 and 2.10 µg for 3 and 5% alginate concentrations respectively.

When the initial burst release was normalized based on the nanospheres' weight, increasing the alginate concentration from 3 to 5% showed a significant decrease in the initial burst release, as shown in Figure 3.5A. With an increase of alginate concentration from 3 to 5%, it was possible to decrease the initial burst release from 85.55 ± 3.62 to $74.50 \pm 1.53\%$.

To evaluate if the size of nanospheres affected the release kinetics, the protein release at different timepoints was also normalized based on the relative size of nanospheres, as shown in Figure 3.4B. For the same relative size of nanospheres, the pattern of protein release kinetics was similar with the 3% alginate showing more initial and total protein release compared to the 5% alginate. At the initial time point (5-hour), for the same relative size of nanospheres, the 5% alginate showed a significant decrease in the protein release compared to the 3% alginate. Like before (Figure 3.4A), the difference in protein release at later timepoints remained unchanged. For the same relative size of nanospheres prepared using 3 and 5% alginate concentrations, the total protein release after 96 hours was 2.56 and 1.65 µg respectively.

When the initial burst release was normalized based on the relative size of nanospheres, like before (see Figure 3.5A), it was found that for the same relative size of nanospheres the 5% alginate showed a significant decrease in the initial burst release compared to the 3% alginate (Figure 3.5B). The initial burst release for 3 and 5% alginate was $85.55 \pm 3.62\%$ and $58.66 \pm 1.20\%$ respectively.

In order to evaluate if the relative number of nanospheres affected the release, the protein release at different timepoints was also normalized based on the relative number of nanospheres, as shown in Figure 3.4C. The pattern of the protein release kinetics changed when the effect of the same relative number of nanospheres was evaluated. For the same relative number of nanospheres, the 5% alginate showed an increase in the protein release compared to the 3% alginate. However, for the same relative number of nanospheres, prepared using either 3 or 5% alginate, the difference in the protein release was not significant for any of the timepoints. For the same relative number of nanospheres, the total protein release using the 3 and 5% alginate concentrations was 1.74 µg and 2.10 µg respectively.

When the initial burst release for the same relative number of nanospheres was calculated it was found that unlike before (see Figure 3.5A) for the same relative number of nanospheres increasing the alginate concentration to 5% significantly increased the initial burst release compared to the ones prepared using the 3% alginate (Figure 3.5C). The initial burst release for

the same relative number of nanospheres prepared using 3 and 5% alginate concentrations was 58.20 ± 2.46 and 74.50 ±1.53 % respectively.

Data represents mean, error bars are SEM (number of independent samples, n=6).
Asterisk (*) indicates significant difference for different alginate concentration.
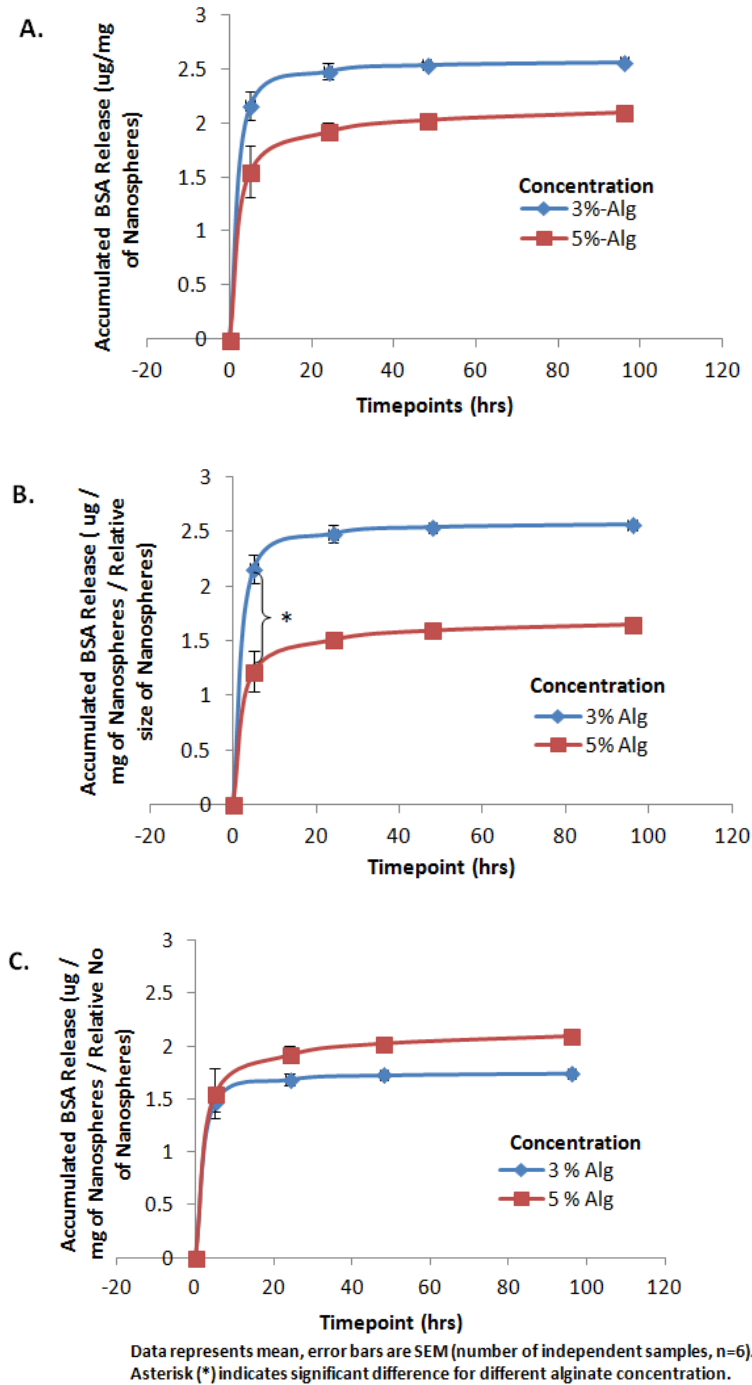
Figure 3.4: The effect of alginate concentration on the protein release kinetics normalized based on the (A) nanosphere weight; (B) relative size of nanospheres; (C) relative number of nanospheres.

Data represents mean, error bars are SEM (number of independent samples, n=6).
Asterisks (*) indicate significant difference for different alginate concentration.
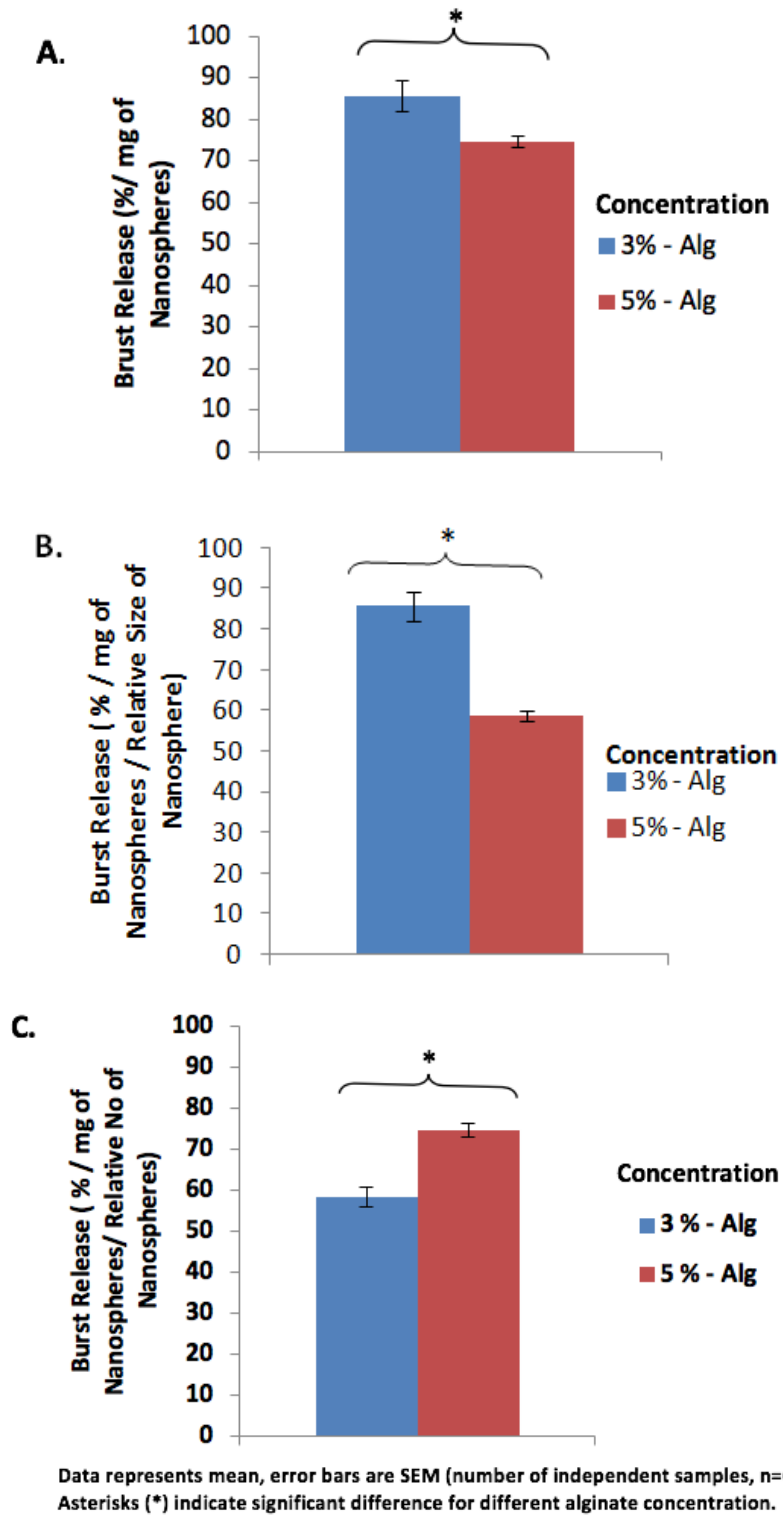
Figure 3.5: The effect of alginate concentration on the initial burst release normalized based on the (A) nanospheres weight; (B) relative size of nanospheres; (C) relative number of nanospheres.

The difference in the protein release at different timepoints within the same experimental group prepared using a specific processing condition (3 or 5% alginate), was also evaluated. In the case of 3% alginate, the Friedman test was performed to test if there was any significant difference in the protein release at different timepoints. Unexpectedly, even if the Friedman test showed significance, the Wilcoxon test followed by Bonferroni correction showed no significant difference in the protein release at any of the timepoints. On the other hand, in the case of 5% alginate, the one-way repeated measures ANOVA followed by multiple comparison using Bonferroni correction showed a significant difference in the protein release only between 5 and 24-hour timepoints. During normalization, the protein release at different timepoints was divided by the same value; therefore, the result remained unchanged irrespective of the normalization method.

Data represents mean, error bars are SEM (number of independent samples, n = 6).
No significant difference was found for different alginate concentration (p > 0.05)
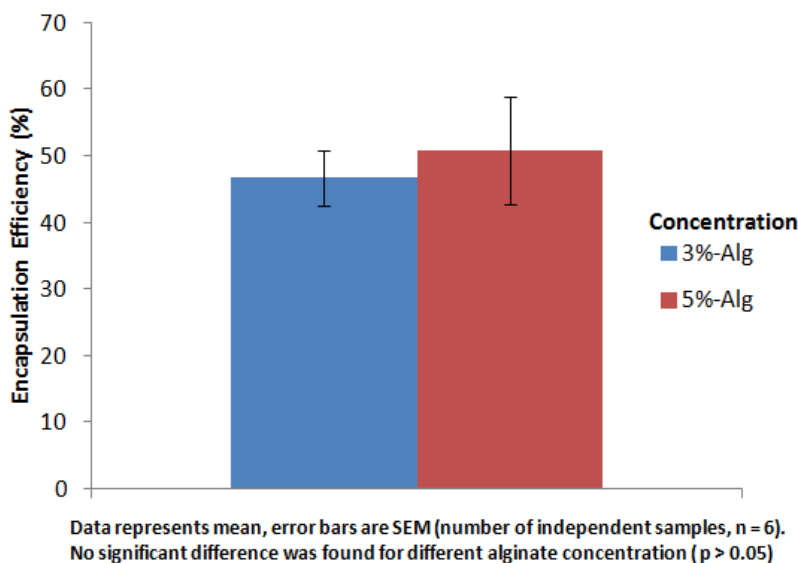
Figure 3.6: The effect of alginate concentration on the EE% of alginate nanospheres.

The effect of alginate concentration on the EE% was also tested in this study, and it was found that with the increase of alginate concentration from 3 to 5% the EE% increased from 46.64 $\pm$ 4.16 to 50.70 $\pm$ 8.09% (Figure 3.6). This might be because the increased solution viscosity using a higher alginate concentration prevented the protein leaching during nanospheres fabrication [143]. However, the increased alginate concentration showed no significant difference in the EE % of alginate nanospheres.

Table 3.1: Summary of the concentration effect of alginate on the performance of alginate nanospheres. The data is presented as mean ± SEM.

Alginate Concent-ration (%)	Burst Release (%)			Relative # of nanospheres	Diameter (nm)
	Normalization Method				
	Based on nanospheres weight	Based on nanospheres size	Based on relative number of nanospheres		
3	85.55 ± 3.62	85.55 ± 3.62	58.20 ±2.46	2898.33 ± 285.23	674.30 ± 12.37
5	74.50 ±1.53	58.66 ± 1.20	74.50 ±1.53	1973.39 ± 166.37	855.30 ± 24.22

3.1.2 Effect of cross-linking time

This subsection describes the results related to the effect of cross-linking time on the nanospheres' morphology, size and distribution pattern, relative number of nanospheres, protein release kinetics, burst release and EE%. Table 3.2 summarizes the effect of cross-linking time on the initial burst release, size and relative number of nanospheres.

3.1.2.1 Morphology, size and relative number of nanospheres

During determination of the effect of cross-linking time on the nanospheres morphology, size, distribution pattern and the relative number of nanospheres, freshly prepared alginate nanospheres, using the 5% (w/v) alginate, were used without drying (0-hour drying time). Nanospheres prepared using various cross-linking times were discrete and spherical in shape with nanospheres aggregation at some of the places. With the increase of cross-linking time nanospheres became opaque showing a whitish color. The intensity of the white color increased with the increase of cross-linking time as shown in Figure 3.7 (A-C). This might be because with the increase of cross-linking time Ca^{2+} uptake increases showing a white color. However, increasing the cross-linking time did not affect the distribution pattern of nanospheres and nanospheres prepared using different cross-linking times showed normal distributions implying homogeneity (Figure 3.8). For different cross-linking times most of the nanospheres were in the range between 700 and 900 nm. Results indicated that with the increase of cross-linking time from 1 to 10 minutes, the mean diameter of alginate nanospheres decreased very slightly and then increased again when the cross-linking time was increased to 30 minutes (Figure 3.9). However, the effect of cross-linking time on the mean diameter of nanospheres was not statistically

significant. The mean diameter of nanospheres prepared using various cross-linking times were in the range between 855.29 ± 24.22 and 919.10 ± 7.40 nm. The relative number of nanospheres that was found after analyzing the micrographs was 1671.11 ± 115.62, 1973.39 ± 166.37 and 1533.50 ± 63.78 for 1, 10 and 30- minute cross-linking times respectively. Increasing the cross-linking time did not show a significant difference in the relative number of nanospheres. Like before, the relative number of nanospheres prepared using different cross-linking times was inversely proportional to their mean diameter.

A.   B.   C.
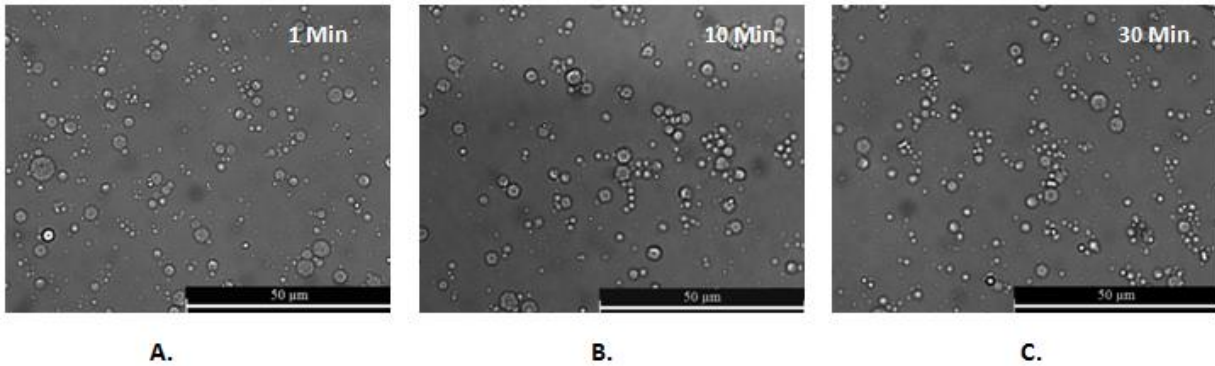
Figure 3.7: The effect of cross-linking time on the nanospheres' morphology prepared with (A) 1-Min cross-linking time, (B) 10-Min cross-linking time and (C) 30-Min cross-linking time. Optical microscope with a 63X magnification was used to take all micrographs. Alginate nanospheres were prepared under the following conditions: 5% Alg and 0-hour drying time.
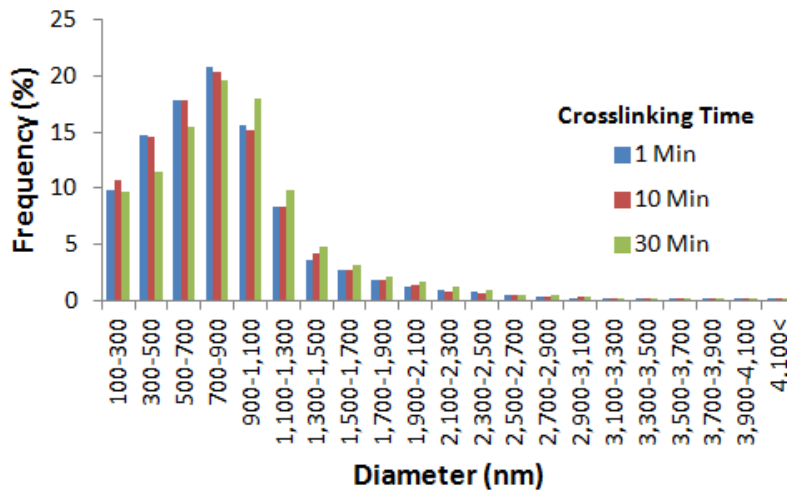
Figure 3.8: The effect of the cross-linking time on the size distribution of alginate nanospheres.

Data represents mean, error bars are SEM (number of independent samples, n=6).
No significant difference was found for different cross-linking time (p > 0.05)
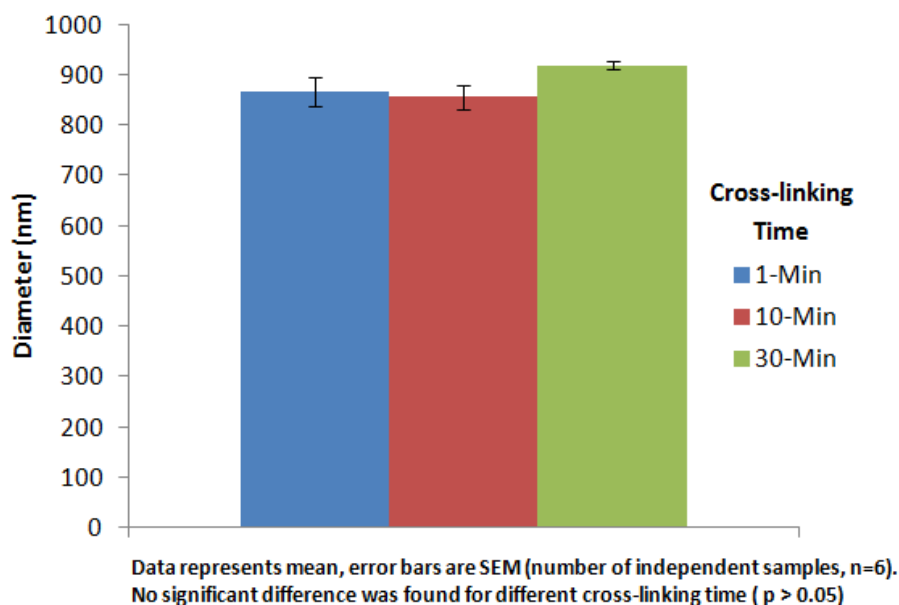
Figure 3.9: The effect of the cross-linking time on the diameter of alginate nanospheres.

3.1.2.2 Protein release kinetics, burst release and EE%

To determine the effect of cross-linking time on the protein release kinetics, the time of cross-linking varied from 1 to 30 minutes, while keeping the levels of other parameters constant, i.e., the alginate concentration and drying time constant at 5% (w/v) and 0 hour respectively. The effect of cross-linking time on the protein release kinetics has been reported in Figure 3.10 (A-C). The cross-linking time appeared to have a very little effect on the release kinetics of alginate nanospheres when the data was normalized based on the nanospheres' weight (see Figure 3.10A). At the initial timepoint, increasing the cross-linking time from 1 to 10 minutes decreased the protein release slightly and then increased again when the cross-linking time was increased to 30 minutes. The total protein release for different cross-linking times also showed similar pattern with 30 minutes cross-linking time showing the highest total protein release compared to the other two. As discussed previously, theoretically the maximum amount of BSA that could be present in each formulation is 90 µg (section 2.2.5). Based on the measured nanospheres weight (after protein release study), the maximum amount of BSA (if 100% of BSA got encapsulated) that could be present per mg of measured nanospheres are 5.24, 4.29 and 5.4 µg for 1, 10 and 30-minute cross-

linking times respectively. However, the total protein release after 96 hours for 1, 10 and 30-minute cross-linking times was 2.19, 2.10 and 2.40 µg respectively (Figure 3.10 A).

When the initial burst release was normalized based on the nanospheres' weight, it was found that increasing the cross-linking time decreased the initial burst release very slightly. Compared to the 1-minute cross-linking time, both the 10 and 30-minute cross-linking times showed less burst release with the 10-minute cross-linking time showing the least. The initial burst release for 1, 10 and 30-minute cross-linking times was 84.60 ± 5.21, 74.50 ± 1.53 and $81.33 \pm 4.81\%$ respectively (Figure 3.11 A). However, the effect of cross-linking time on the initial burst release was not significant.

In order to evaluate if the size of nanospheres affected the release kinetics, the protein release at different timepoints was also normalized based on the relative size of nanospheres, as shown in Figure 3.10B. For the same relative size of nanospheres, the pattern of protein release kinetics using different cross-linking times remained same as before (see Figure 3.10A). Like before, the total protein release was highest for the 30-minute cross-linking time and lowest for the 10-minutes cross-linking time. Also, the difference in the protein release was not significant at any of the timepoints. The total protein release for the same relative size of nanospheres prepared using 1, 10 and 30-minute cross-linking times was 2.17, 2.10 and 2.24 µg respectively.

When the initial burst release for different cross-linking times was normalized based on the nanospheres' size it was found that the size of nanospheres prepared using different cross-linking times had no significant effect on the initial burst release (Figure 3.11 B). The initial burst release for the same relative size of nanospheres prepared using 1, 10 and 30-minute cross-linking times was 73.76 ± 1.51, 74.50 ± 1.53 and $76.00 \pm 4.49\%$ respectively.

In order to evaluate if the relative number of nanospheres affected the release kinetics, the protein release was also normalized based on the relative number of nanospheres, as shown in Figure 3.10C. For the same relative number of nanospheres prepared using different cross-linking times, the pattern of protein release kinetics remained unchanged. Also, for the same relative number of nanospheres the difference in the protein release using different cross-linking times was not significant at any of the timepoints. For the same relative number of nanospheres, the total protein release using 1, 10 and 30-minute cross-linking times was 2.01, 1.62 and 2.40 µg respectively.
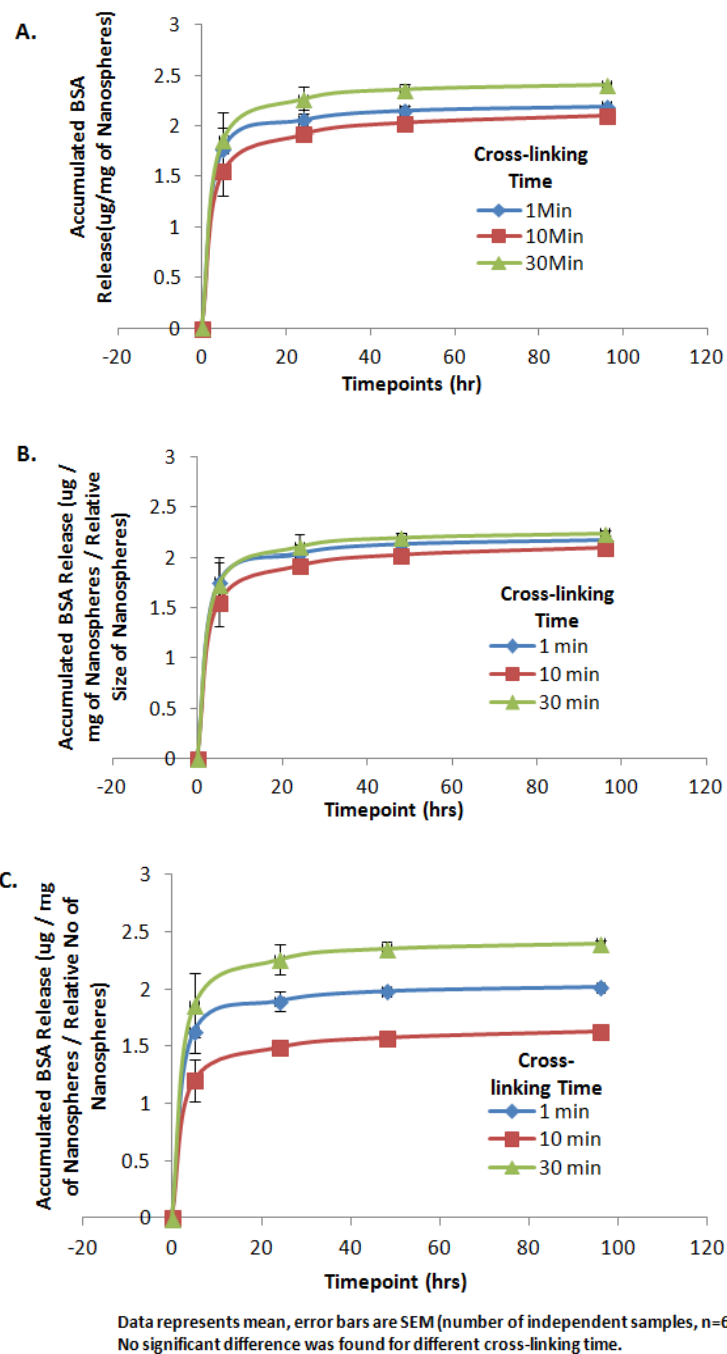
Figure 3.10: The effect of cross-linking time on the protein release kinetics normalized based on the (A) nanosphere weight; (B) relative size of nanospheres; (C) relative number of nanospheres.

When the initial burst release was normalized based on the relative number of nanospheres, for the same relative number of nanospheres, the 10-minute cross-linking time showed a significant decrease in the initial burst release compared to the 1 and 30-minute cross-linking times

(Figure 3.11C). The initial burst release for the same relative number of nanospheres prepared using 1, 10 and 30 minutes cross-linking times was 77.61 ± 4.78, 57.75 ± 1.18 and $81.33 \pm 4.81\%$ respectively.

Data represents mean, error bars are SEM (number of independent samples, n=6).
Asterisks (*) indicate significant difference for different cross-linking time.
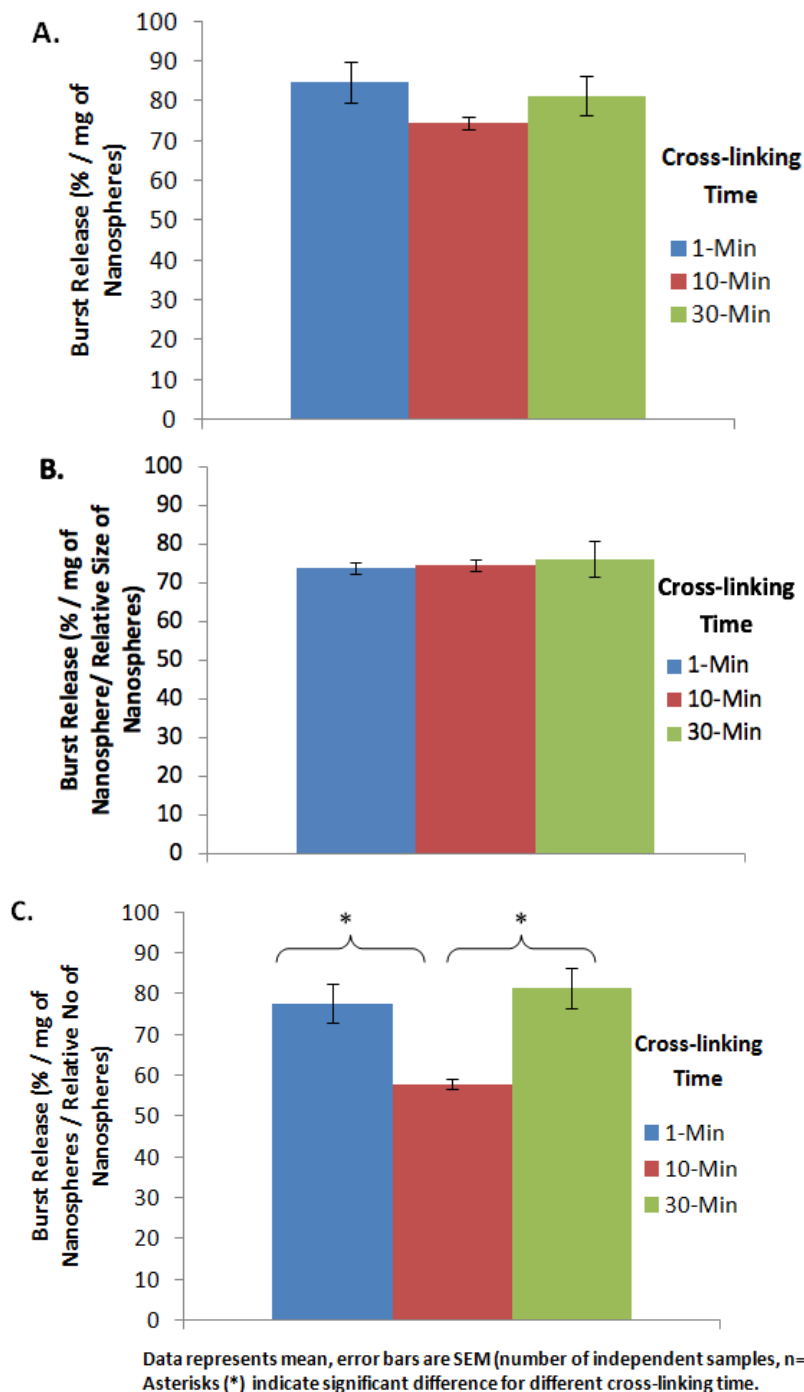
Figure 3.11: The effect of cross-linking time on the initial burst release when normalized based on the (A) nanospheres weight; (B) relative size of nanospheres; (C) relative number of nanospheres.

The difference in the protein release at different timepoints and within the same experimental group prepared using specific processing conditions (1, 10 and 30-minute cross-linking time), was also tested. In the case of 1 and 30-minute cross-linking times, the Friedman test was performed to test if there was any significant difference in the protein release at different timepoints. Even though the Friedman test showed significance, the Wilcoxon test followed by Bonferroni correction showed no significant difference in the protein release at any of the timepoints. By contrast, in the case of 10-minute cross-linking time, the one-way repeated measures ANOVA followed by multiple comparison using Bonferroni correction showed a significant difference in the protein release between 5 and 24-hour timepoints. After 24-hour, no significant difference in the protein release was found at any other timepoints. During normalization, the protein release at different time points was divided by the same value; therefore, the result remained unchanged irrespective of the normalization method.

Data represents mean, error bars are SEM (number of independent samples, n=6). No significant difference was found for different cross-linking time (p > 0.05).
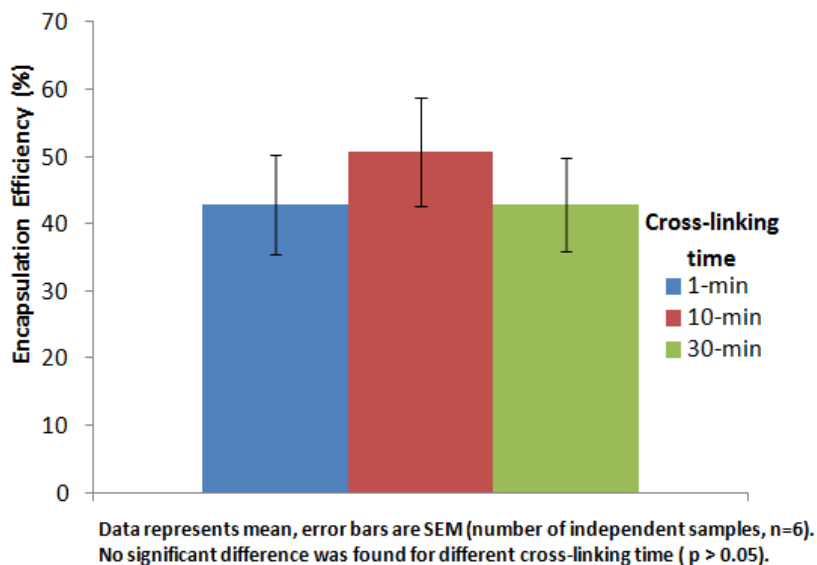
Figure 3.12: The effect of cross-linking time on the EE% of alginate nanospheres.

When the effect of cross-linking time on the EE% was tested, increasing the cross-linking time showed no significant difference in the EE%. With the increase of cross-linking time from 1 to 10 minutes, the EE% increased and then decreased again for 30-minute cross-linking time. The

EE% for 1, 10 and 30-minute cross-linking times was 42.87 ± 7.50, 50.70 ± 8.09 and 42.84 ± 6.96 respectively.

Table 3.2: Summary of the effect of cross-linking time on the performance of alginate nanospheres. The data is presented as mean ± SEM.

Cross-linking Time (Min)	Burst Release (%)			Relative # of nanospheres	Diameter (nm)
	Normalization Method				
	Based on nanospheres weight	Based on nanospheres size	Based on relative number of nanospheres		
1	84.60 ± 5.21	73.76 ± 1.51	77.61 ± 4.78	1671.11 ± 115.62	865.89 ± 29.00
10	74.50 ± 1.53	74.50 ± 1.53	57.75 ± 1.18	1973.39 ± 166.37	855.29 ± 24.22
30	81.30 ± 4.80	76.00 ± 4.49	81.33 ± 4.81	1533.50 ± 63.78	919.10 ± 7.40

3.1.3 Effect of drying time

This subsection describes the results related to the effect of drying time on the nanospheres morphology, size, distribution pattern, relative number of nanospheres, protein release kinetics, burst release and EE%. Table 3.3 summarizes the effect of drying time on the initial burst release, size and relative number of nanospheres.

3.1.3.1 Morphology, size and relative number of nanospheres

In order to determine the effect of drying time on the nanospheres size and morphology, nanospheres were used either immediately after fabrication (0 hour drying) or after drying at 37ºC for several hours (0.5,1.5,4.5 and 24 hours). The effect of drying time on the nanospheres morphology and size distribution pattern have been shown in Figure 3.13 and 3.14 respectively, where the alginate concentration and cross-linking time were kept constant at 5% (w/v) and 10 minutes respectively. The nanospheres prepared using the 0-hour drying time were discrete and spherical in shape, with nanospheres aggregation at some of the places. However, with the increase of drying time the tendency of nanospheres aggregation increased, as can be seen in Figure 3.13 (A-E). All of the drying time groups showed a normal distribution pattern, implying the

homogeneity of nanospheres (Figure 3.14). For the 0, 4.5 and 24-hour drying times, most of the nanospheres were in the range between 700 to 900 nm. On the other hand, for the 0.5 and 1.5-hour drying times most of the nanospheres were in the range between 900 and 1100 nm. With increasing the drying time from 0 to 1.5 hours the size of nanospheres was found to be increased and then decreased again with the further increase. The effect of drying time on the mean diameter of nanospheres has been shown in Figure 3.15. Compared to the 0-hour drying time, both the 0.5 and 1.5-hour drying times showed a significant increase ($p \leq 0.05$) in the mean diameter. However, the effects of other drying times on the mean diameter of nanospheres were not significant. The relative number of nanospheres found after analyzing the micrographs was 1973.39 ± 166.37, 1029.23 ± 95.74, 896.91 ± 74.45, 1329.86 ± 94.19 and 1468.08 ± 98.52 for 0, 0.5, 1.5, 4.5 and 24-hour drying times respectively. Compared to the undried nanospheres, increasing the drying time showed a significant decrease in the relative number of nanospheres for all of the other drying time groups. Like in all other cases, for all drying time groups the relative number of nanospheres was inversely proportional to their mean diameter.
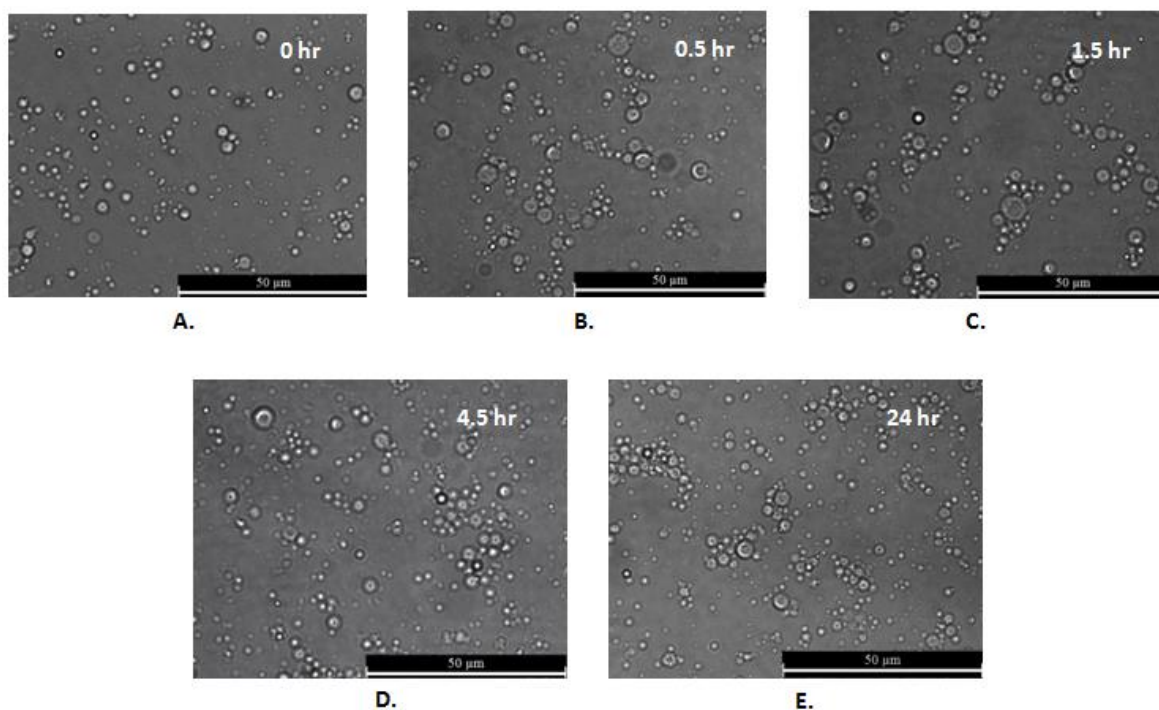
Figure 3.13: The effect of drying time on the nanospheres' morphology prepared with (A) 0 hr drying time, (B) 0.5 hr drying time and (C) 1.5 hr drying time, (D) 4.5 hr drying time and (E) 24 hr drying time. Optical microscope with a 63X magnification was used to take all of the micrographs.
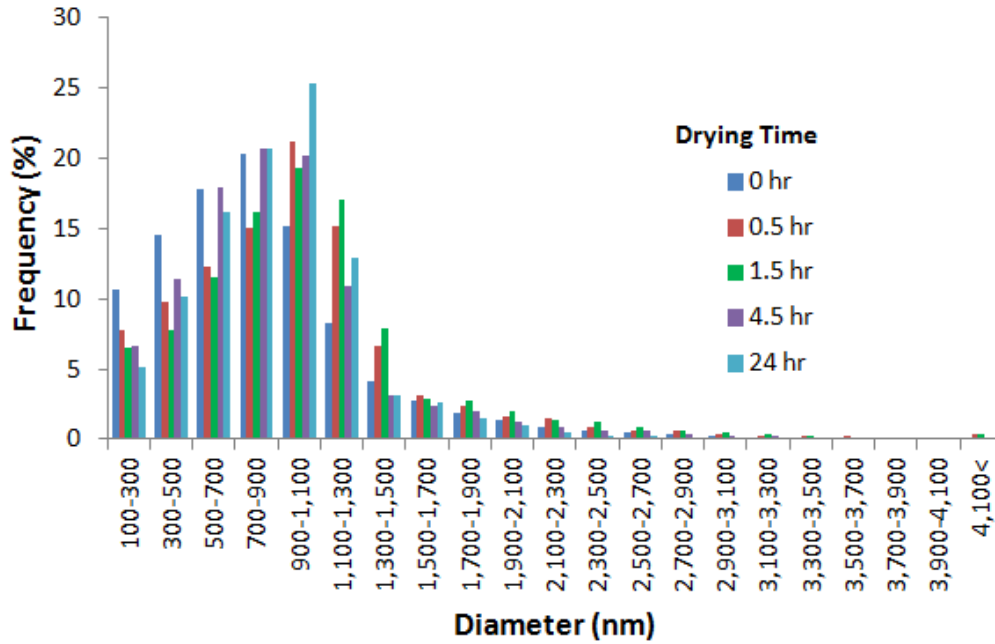
Figure 3.14: The effect of drying time on the size distribution of alginate nanospheres.

Data represents mean, error bars are SEM (number of independent samples, n=6). Asterisks (*) indicate significant difference for different drying time.
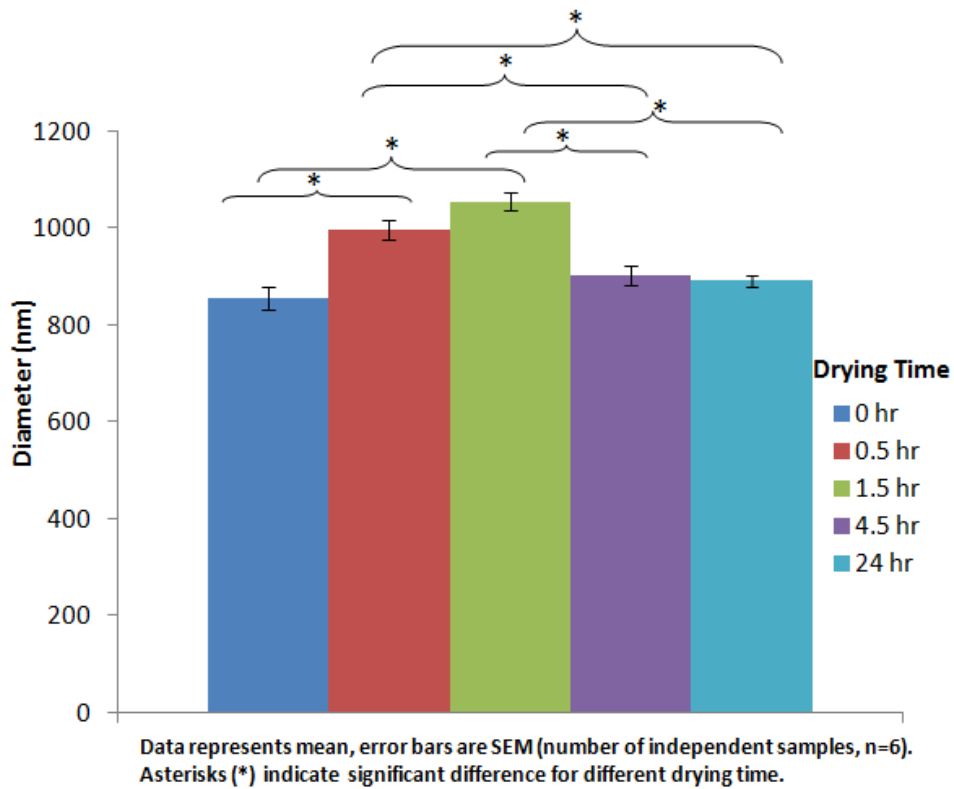
Figure 3.15. The effect of drying time on the diameter of alginate nanospheres.

3.1.3.2 Protein release kinetics, burst release and EE%

In order to determine the effect of drying time on the protein release kinetics, nanospheres were either used immediately after fabrication (without drying) or dried for different times (0.5, 1.5, 4.5 and 24 hours). The effect of drying time on the protein release kinetics, using various normalization methods, have been shown in Figure 3.16 (A-C). During the evaluation of drying time effect, levels of other parameters e.g., the alginate concentration and cross-linking time were kept constant at 5% (w/v) and 10 minutes respectively. When the protein release data was normalized based on the nanospheres' weight (Figure 3.16A), drying time showed a significant difference in protein release at the initial timepoint (5-hour). At the initial timepoint, the 24-hour drying time showed a significant decrease in the protein release compared to the 0.5 and 4.5-hour drying times. Other drying time groups showed no significant difference in the protein release. For different drying times, the difference in protein release after 5 hours remained unchanged. As discussed previously, theoretically the maximum amount of BSA that could be present in each formulation is 90 µg (see section 2.2.5). Based on the measured nanospheres' weight (dried and measured at the end of protein release study), the maximum amount of BSA that could be present per mg of nanospheres are 4.29, 5.24, 5.00, 5.05 and 5.63 µg for 0, 0.5, 1.5, 4.5 and 24-hour drying times respectively. However, the total protein release after 96 hours was 2.10, 2.74, 2.17 and 2.40 and 0.51 µg for 0, 0.5, 1.5, 4.5 and 24-hour drying times respectively. Among the various drying time groups, drying of nanospheres till 24-hour resulted in an incomplete protein release.

In terms of the initial burst release, it was found that increasing the drying time showed no definite pattern when normalized based on the nanospheres' weight (Figure 3.17A). With the increase of drying time, only the 1.5 and 24-hour drying time groups showed slightly less burst release compared to the undried nanospheres (0-hour drying time group). On the other hand, the 0.5 and 4.5-hour drying time groups showed more initial burst release compared to the undried nanospheres. The initial burst release for the nanospheres prepared using 0, 0.5, 1.5,4.5 and 24-hour drying times was 74.50 ±1.53, 80.5±4.95, 73.1±4.17, 75.3±5.24 and 67.6±10.57% respectively. Increasing the drying time showed no significant difference in the initial burst release compared to the 0-hour drying time.

In order to evaluate if the size of nanospheres prepared using various drying times affected the protein release kinetics, the protein release at different timepoints was also normalized based on the relative size of nanospheres, as shown in Figure 3.16B. For the same relative size of

nanospheres, increasing the drying time showed a significant difference in protein release, at the initial timepoint. For the same relative size of nanospheres, increasing the drying time to 24-hour showed a significant decrease in the protein release compared to the 0, 0.5 and 4.5-hour drying time groups. After the initial timepoint no significant difference in the protein release between any other drying times was found. The total protein release for the same relative size of nanospheres prepared using 0, 0.5, 1.5, 4.5 and 24-hour drying times was 2.10, 2.36, 1.77, 2.29 and 0.49 µg respectively.

When the initial burst release was normalized based on the relative size of nanospheres it was found that the size of nanospheres had no significant effect on the initial burst release (Figure 3.17 B). For the same relative size of nanospheres, all of the drying time groups showed less initial burst release compared to the undried nanospheres. The initial burst release for the same relative size of nanospheres prepared using 0, 0.5, 1.5, 4.5 and 24-hour drying times was 74.50 ± 1.53, 69.42 ± 4.26, 59.42 ± 3.39, 71.76 ± 4.99 and 64.96 ± 10.16 % respectively.

For different drying time groups, in order to evaluate the effect of the relative number of nanospheres on the protein release kinetics, the protein release at different timepoints was also normalized based on the relative number of nanospheres, as shown in Figure 3.16C. In case of different drying time groups, for the same relative number of nanospheres the protein release kinetics differed significantly. At the initial timepoint, for the same relative number of nanospheres increasing the drying time to 24-hour showed a significant decrease in protein release compared to the 0.5 and 1.5-hour drying time groups. Additionally, the 0-hour drying time group showed a significant decrease in protein release compared to the 0.5-hour drying time group, at the initial timepoint. The total protein release for the same relative number of nanospheres prepared using 0, 0.5, 1.5, 4.5 and 24-hour drying times was 0.92, 2.58, 2.17, 1.68 and 0.33 µg respectively.

For different drying time groups, when the initial burst release was normalized based on the relative number of nanospheres it was found that the relative number of nanospheres had a significant effect on the initial burst release (Figure 3.17C). For the same relative number of nanospheres, the 0.5 and 1.5-hour drying time groups showed a significant increase in the initial burst release compared to the undried nanospheres (0-hour drying time). After 1.5 hours, the initial burst release started decreasing with the 4.5 and 24-hour drying time groups showing a significant decrease compared to the 1.5-hour drying time group. For the same relative number of nanospheres, all of the drying time groups showed more initial burst release compared to the 0-

hour drying time. The initial burst release for 0, 0.5, 1.5, 4.5 and 24-hour drying times was 33.86 $\pm$ 0.69, 47.37 $\pm$ 2.91, 73.08 $\pm$ 4.17, 50.91 $\pm$ 3.54 and 40.22 $\pm$ 6.29 % respectively.

The difference in the protein release at different timepoints and within the same experimental group prepared using specific processing conditions (0, 0.5,1.5,4.5 and 24-hours drying time) was also tested in this study. It was found that drying time affected the protein release kinetics within groups at different timepoints. In the case of 0, 0.5 and 1.5-hour drying times, the one-way repeated measures ANOVA followed by multiple comparison using Bonferroni correction showed a significant difference in the protein release between 5 and 24-hour timepoints. By contrast, in the case of the 4.5 and 24-hour drying times, the Friedman test was performed to test if there was any significant difference in the protein release within groups. However, despite the Friedman test showed significance, the Wilcoxon test followed by Bonferroni correction showed no significant difference in the protein release at any of the timepoints. During normalization, the protein release at different timepoints was divided by the same value; therefore, the result remained unchanged irrespective of the normalization method.

The drying time appeared to have a significant effect on the percentage of total protein released at 96 hours (Figure 3.18). The total protein release percentage was calculated based on how much BSA was released from all of the recovered nanospheres compared to the total amount of BSA added in each formulation. Drying of nanospheres for 24 hours showed a significant decrease in the total protein release compared to all other drying times. The percentage of the total BSA release for 0, 0.5, 1.5, 4.5 and 24-hour drying times was 50.70 $\pm$ 8.09, 53.51 $\pm$ 9.99, 44.00 $\pm$ 6.55, 48.50 $\pm$ 8.04 and 9.19 $\pm$ 2.67 % respectively.
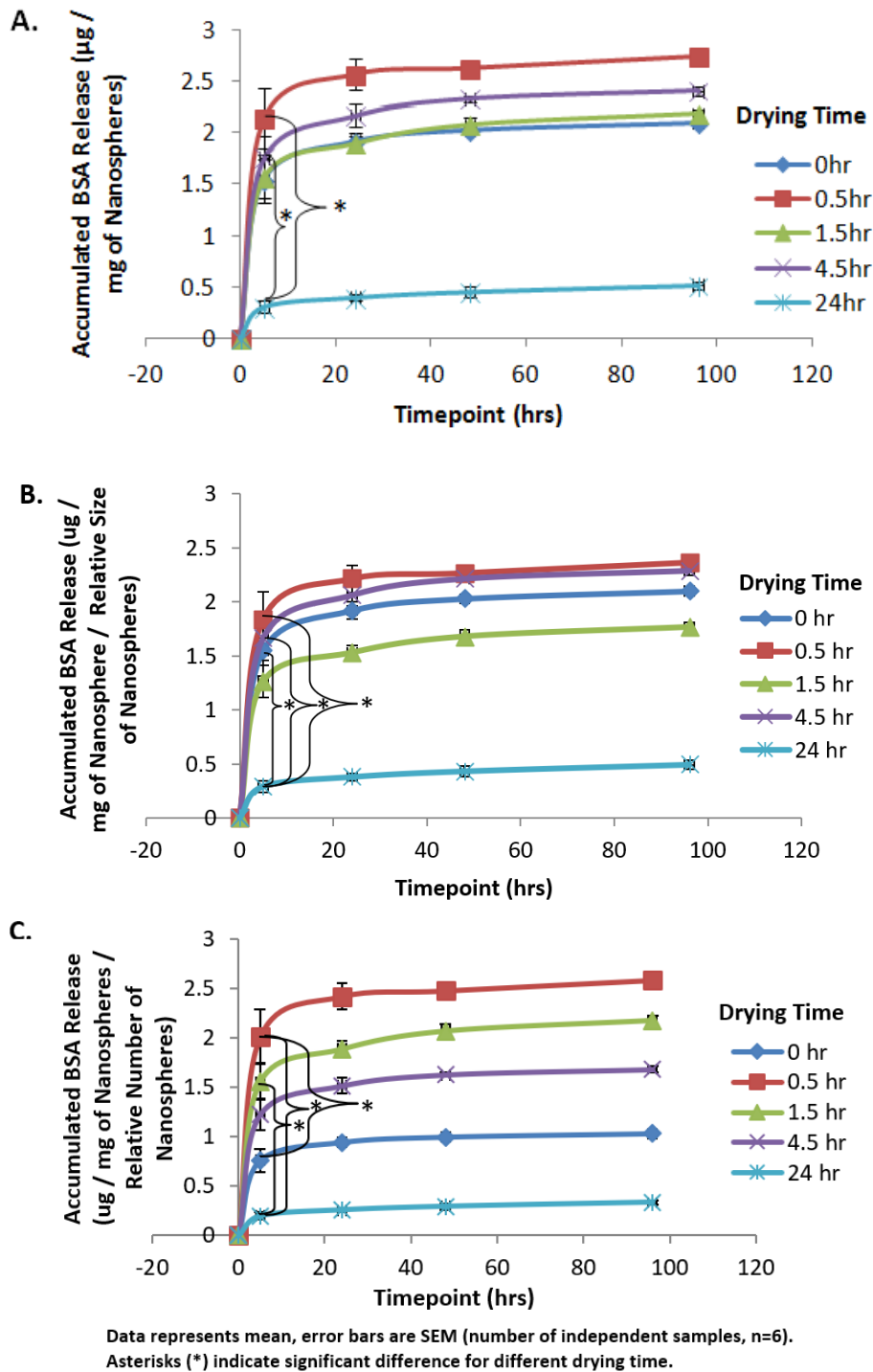
Figure 3.16: The effect of drying time on protein release kinetics normalized based on the (A) nanosphere weight; (B) relative size of nanospheres; (C) relative number of nanospheres.

Data represents mean, error bars are SEM (number of independent samples, n=6).
Asterisks (*) indicate significant difference for different drying time.
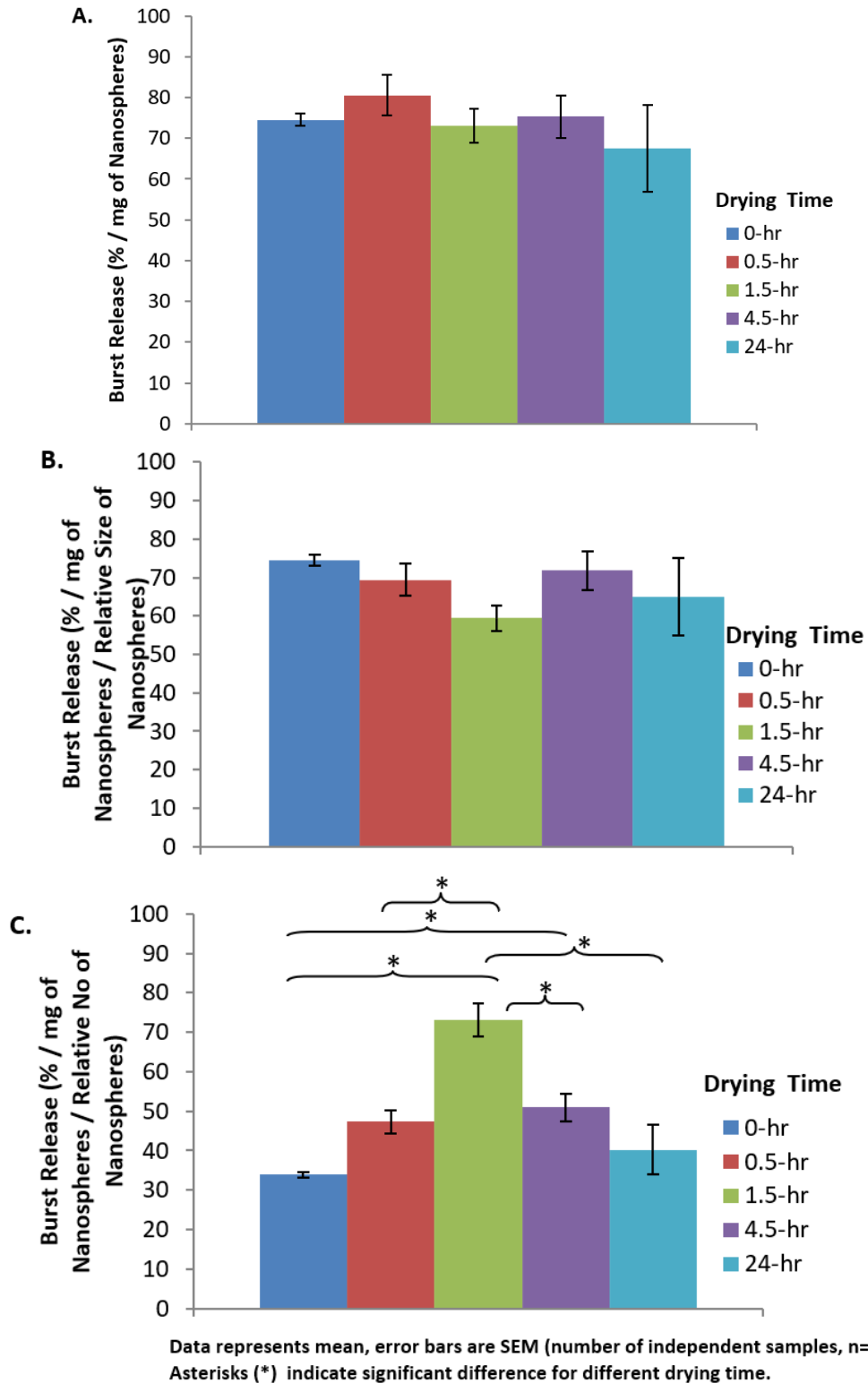
Figure 3.17: The effect of drying time on the initial burst release when normalized based on the (A) nanospheres weight; (B) relative size of nanospheres; (C) relative number of nanospheres.

Data represents mean, error bars are SEM (number of independent samples, n=6).
Asterisks (*) indicate significant difference for different drying time.
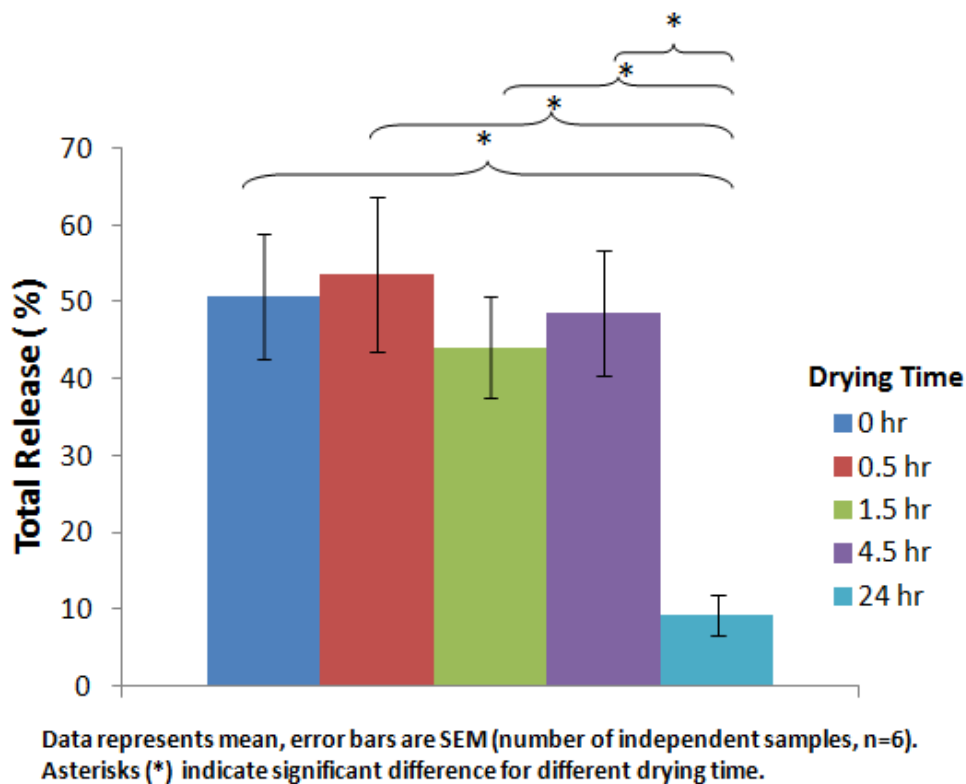
Figure 3.18: The effect of drying time on the total protein release of alginate nanospheres after 96-hour.

Table 3.3: The effect of drying time on the performance of alginate nanospheres. The data is presented as mean ± SEM.

| Drying Time (hr) | Burst release (%) | | | Relative # of nanospheres | Diameter (nm) |
| | Normalization Method | | | | |
	Based on nanospheres weight	Based on nanospheres size	Based on relative number of nanospheres		
0	74.50 ± 1.53	74.50 ± 1.53	33.86± 0.69	1973.39 ± 166.37	855.30 ± 24.22
0.5	80.5±4.95	69.42 ± 4.26	47.37 ± 2.91	1029.23 ± 95.74	996.19 ± 18.77
1.5	73.1 ± 4.17	59.42 ± 3.39	73.08 ± 4.17	896.91 ± 74.45	1054.03 ± 18.29
4.5	75.3 ± 5.24	71.76 ± 4.99	50.91 ± 3.54	1329.86 ± 94.19	902.21 ± 18.77
24	67.6±10.57	64.96 ±10.16	40.22 ± 6.29	1468.08 ± 98.52	890.39 ± 12.06

3.2 Discussion

Alginate micro/nanospheres have been widely used as a protein delivery device because of their excellent biocompatibility, inert nature towards bioactive proteins and most importantly, a mild and easy protein encapsulation process without protein denaturation [64, 111]. However, one of the major limitations of using alginate-based micro/nanospheres as a delivery device is the high initial burst release of encapsulated proteins and other chemicals from alginate micro/nanospheres within a very short time [120]. Various factors such as the high matrix porosity, instability and high degree of swelling at a higher pH release media are responsible for the high initial burst release of alginate micro/nanospheres. Various approaches have been reported in literature that were used to reduce the initial burst release and total protein release of alginate micro/nanospheres. One of the most common approaches that has been used to reduce the matrix porosity and initial burst effect of alginate micro/nanospheres is the coating or reinforcing alginate with other polymers [104, 44, 63, 117, 127, 126]. The problem associated with the coating and reinforcing is that they add extra cost and processing steps. Also, in one study, an improper coating of alginate microspheres was reported to be responsible for the protein loss [117]. Other than the coating and reinforcing using other polymers, the initial burst release and total release can also be controlled by varying various other process parameters, such as the polymer concentration [62], cross-linking time [149] and drying condition [65] and drying time [102]. Therefore, the aim of this research was to reduce the initial burst release of alginate nanospheres by varying the alginate concentration, cross-linking time and drying time, for their intended application in CTE. In order to fulfill this aim, the effects of alginate concentration, cross-linking time and drying time on the protein release kinetics, initial burst release and EE% were evaluated. Since BSA has been used previously as a model protein of BMP-7 [156] and its MW is similar to BMP-7, necessary for the cartilage defect repair [29], therefore, BSA was used as a model protein in this study.

3.2.1 Effect of alginate concentration

The concentration and MW of polymer have considerable effects on the initial burst release and total release because of their ability to control the porosity [112], swelling ability [62], size [121], and degradation rate [144]. Most importantly, with the increase of concentration and MW of alginate the degree of cross-linking has been reported to be increased [62, 112, 121]. This increased cross-linking density at a higher alginate concentration and MW might be the reason the

porosity, degradability and swell ability of alginate micro/nanospheres decreases resulting in decreased initial burst release and total release. Since most often the encapsulating molecules that have been used to study the concentration effect of alginate micro/nanospheres was not protein, therefore, it seemed worthwhile to test the effect of alginate concentration on the initial burst release and total release from the protein loaded alginate nanospheres.

Even though a higher MW or higher viscosity grade is preferred over a lower MW or lower viscosity grade alginate, in this study, the low viscosity alginate was selected over the medium viscosity alginate. This is most likely because the medium viscosity alginate showed very little or almost no release of protein (see Appendix E). Very high viscosity and comparatively a high degree of cross-linking using the medium viscosity alginate might be the reason protein diffusion through alginate microspheres was hindered. During determining the concentration effect, only the 3% and 5% alginate were tested, as an alginate at a concentration higher than 5% becomes extremely viscous making it difficult to dissolve using a magnetic stirrer.

It was found that nanospheres prepared using the 5% alginate showed a higher apparent contrast compared to the ones prepared using the 3% alginate. This might be due to the denser internal structure of alginate nanospheres at a higher alginate concentration. Also, increased alginate concentration showed a significant increase in the mean diameter of nanospheres (Figure 3.3A). This agrees with several other studies that found similar results explaining the phenomenon might be due to an increase in the solution viscosity at a higher alginate concentration resulting in larger aqueous droplets in emulsion [101, 130]. High mean diameter of nanospheres means they will be able to accommodate a smaller number of nanospheres per unit area. This explains why in all cases; the relative number of nanospheres was inversely proportional to their mean diameter. The relative number of nanospheres decreased significantly with the increase of alginate concentration.

In this study, the increase in alginate concentration decreased the initial and total protein release, when normalized based on the nanospheres' weight (Figure 3.4A). This is because with the increase of alginate concentration the degree of cross-linking and the mean diameter of nanospheres increases delaying the protein diffusion [130, 161]. However, the effect of alginate concentration on the protein release was not significant at any of the timepoints.

In our study, increasing the alginate concentration from 3% to 5% decreased the initial burst release significantly when normalized based in the nanospheres' weight (Figure 3.5A), which

aligns with our hypothesis. By increasing the alginate concentration from 3% to 5 % it was possible to reduce the initial burst release by 9%.

Based on literature, burst release is a surface phenomenon and the entire protein release is proportional to the surface area-to-volume ratio exposed to a release medium. The more surface area-to-volume ratio of micro/nanospheres exposed to a release medium the more release [62]. It has been reported that the porous microspheres have a higher surface area-to-volume ratio compared to the non-porous microspheres [140]. In order to calculate the total surface area-to-volume ratio of nanospheres, it is important to determine the porosity, size and total number of nanospheres created in each experimental condition. However, the porosity of nanospheres was not calculated in our study. Also, the total number of nanospheres created in each tube is unknown. If we do not consider the nanospheres porosity and total number of nanospheres for different experimental conditions (e.g., alginate concentration, cross-linking time and drying time), then based on the relative number of nanospheres, the relative surface area can be calculated using the following equation:

Relative surface area, A= $4\pi r^2$ x relative number of nanospheres............(3.1)

Where, r = radius of nanospheres.

Therefore, using equation (3.1), the relative surface area of nanospheres prepared using the 3% and 5% alginates was 4140 and 4540 μm^2 respectively. Based on the relative surface area, the 5% alginate should release more protein compared to the 3% alginate. However, the 5% alginate showed a significant decrease in the initial burst release. This result implies that with the increase of alginate concentration from 3 to 5% the porosity of nanospheres, which also affects the surface area of nanospheres, reduced resulting in a less release. Increased cross-linking density with the increase of alginate concentration might be the reason the 5% alginate showed reduced porosity compared to the 3% alginate [130]. We believe in the case of 5% alginate, a less surface area-to-volume ratio was exposed to the release medium compared to the 3% alginate resulting in a significant decrease in the initial burst release. However, this is just an assumption, as nanosphere porosity was not evaluated in this study. Another probable reason of getting a less initial burst release from nanospheres prepared using the 5% alginate might be due to their higher mean diameter compared to the 3% alginate. This might also cause a delay in protein diffusion through the nanospheres matrix [161]. Furthermore, with the increase of alginate concentration, the relative percentage of polymer to encapsulate protein increases. This increase in the relative percentage of

alginate at a higher alginate concentration might be another reason of getting a less burst release using 5% alginate [102, 147, 134].

The protein release data of different alginate concentrations was also normalized based on the relative size of nanospheres to determine the effect of the size of nanospheres on the protein release kinetics. At the initial timepoint, for the same relative size of nanospheres increasing the alginate concentration significantly reduced the protein release (Figure 3.4B). However, when the protein release data was normalized based on the nanosphere' weight, no significant difference in the protein release was found at any of the timepoints (Figure 3.4A). This implies that the size of nanospheres had a significant effect on the protein release kinetics, specially at the initial timepoint. When the initial burst release was normalized based on the nanospheres' size, for the same relative size of nanosphere increasing the alginate concentration to 5% alginate showed a significant decrease in the initial burst release compared to the 3% alginate (Figure 3.5B). Since increasing the alginate concentration showed a significant decrease in the initial burst release before (see Figure 3.5A) and after normalization based on the relative size of nanospheres (see Figure 3.5B), therefore, the change in nanospheres' size by varying the alginate concentration had no significant effect on the initial burst release, which aligns with our proposed hypothesis.

When the protein release data was normalized based on the relative number of nanospheres, for the same relative number of nanospheres the pattern of the protein release kinetics found to be altered completely (Figure 3.4C). While compared to the 3% alginate, the 5% alginate showed a less protein release before (Figure 3.4A), for the same relative number of nanospheres the 5% alginate showed a higher protein release compared to the 3% alginate (Figure 3.4C). However, the effect was not significant. Also, when the protein release data was normalized based on the relative number of nanospheres increasing the alginate concentration to 5% for the same relative number of nanospheres significantly increased the initial burst release compared to the nanospheres prepared using the 3% alginate (Figure 3.5C). However, when the initial burst release was normalized based on the nanospheres' weight, increasing the alginate concentration significantly decreased the burst release (Figure 3.5A). This finding implies that the relative number of nanospheres by varying the alginate concentration has a significant effect on the initial burst release, which doesn't align with our research hypothesis. The diameter of alginate nanospheres prepared using the 5% alginate was significantly large, which allows loading more protein

compared to the 3% alginate. This explains why for the same relative number of nanospheres, the 5% alginate showed more protein release.

3.2.2 Effect of cross-linking time

The swelling of micro/nanospheres also controls the release rate when cross-linked hydrogel is used as a delivery device [139]. Due to the micro/nanospheres' swelling, the mesh size of the cross-linked network increases. This increased mesh size of the cross-linked network increases the penetration ability of the release medium through the micro/nanospheres matrix, resulting in a faster protein release. Several earlier studies have confirmed that increasing the cross-linking time increases the cross-linking density and decreases the micro/nanosphere swelling during incubation [148, 147]. This high degree of cross-linking and reduced swelling might delay the initial and total protein release. The effect of the cross-linking time on the protein release using alginate nanospheres has not been investigated yet, therefore, it seemed worthwhile to test the effect of this parameter on the initial burst release and protein release kinetics of alginate nanospheres.

In order to test the effect of cross-linking time, the time of cross-linking varied from 1 to 30 minutes. Due to practical reasons, the 1-minute cross-linking time was selected as a lowest value as processing steps after adding the cross-linker (such as, hardening of nanospheres using isopropyl alcohol and transferring the nanospheres from the 24-well plate to Eppendorf tubes) requires at least 1-minute. The intention of using the 1-minute cross-linking time was to show how an incomplete cross-linking might affect the protein release from alginate nanospheres. The cross-linking time higher than 30 minutes was not tested in this study because based on the literature, 30 minutes are the maximum time required to uptake Ca^{2+} by an alginate gel matrix [157, 158].

The cross-linking time did not have any significant effect on the mean diameter of nanospheres. While with increasing the cross-linking time from 1 to 10-minute the mean diameter of nanospheres decreased slightly and was almost similar, in the case of 30-minute cross-linking time, the mean diameter of nanospheres increased again. This comparatively high mean diameter using the 30-minute cross-linking time might be due to a higher degree of cross-linking of the G-units of SA at a higher cross-linking time [162]. The relative number of nanospheres prepared using various cross-linking times was also not statistically significant.

During determination of the effect of cross-linking time on the protein release kinetics, the protein release at different timepoints was normalized based on the nanospheres' weight (Figure

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3.10 A). Increasing the cross-linking time from 1 to 10 minutes decreased the initial and total protein release slightly. This might be due to an increase in the degree of cross-linking and decrease in the degree of swelling of nanospheres resulting in a less release [101, 148]. However, unexpectedly, the 30-minute cross-linking time showed slightly higher initial and total protein release. Even though, the 10 and 30-minute cross-linking times showed less initial and total protein release compared to the 1-minute cross-linking time, the difference in the protein release was not statistically significant at any of the timepoints. This comparatively high release for the nanospheres prepared using the 30-minute cross-linking time might be because, with the increase of cross-linking time the $CaCl_2$ contents, that is hygroscopic, in nanosphere increased. Because of the increased hygroscopicity of nanospheres they might be showing a greater attraction towards the release medium. This attraction of nanospheres to the release medium using 30-minute cross-linking time might increase the nanospheres' swelling resulting in more release. For the same reason, the 30-minute cross-linking time might show a slightly more initial burst release compared to the 10-minute cross-linking time (3.11A). However, this is just a speculation, as the effect of cross-linking time on the nanospheres' swelling was not investigated in this study. Therefore, the degree of swelling needs to be investigated in future to better explain the effect of cross-linking time on the initial burst release and protein release kinetics of alginate nanospheres.

By increasing the cross-linking time, it was possible to slightly reduce the initial burst release, when normalized based on the nanospheres' weight. Both the 10 and 30-minute cross-linking time groups showed less initial burst releases compared to the 1-minute cross-linking time group (Figure 3.11A). Also, the 10 minutes cross-linking time was found to be the maximum time required to reduce the initial burst release. However, the reduction in the initial burst release by increasing the cross-linking time was not significant, which is not in an agreement with our proposed hypothesis.

We know that the burst release is a surface phenomenon, therefore, to explain the result, the effect of cross-linking time on the relative surface area of nanospheres was also calculated using equation 3.1. The relative surface area of nanospheres prepared using the 1, 10 and 30-minute cross-linking times was 3940, 4540 and 4070 μm^2 respectively. Based on the relative surface area, increasing the cross-linking time should increase the burst release. The opposite happened in this study, therefore, this result implies that with the increase of cross-linking time, the porosity of nanospheres slightly decreased due to a comparatively higher degree of cross-

linking resulting in a less burst release. However, probably the decrease of nanospheres' porosity was not enough to exert a significant effect.

To determine if the change in nanospheres size by varying the cross-linking time affected the result, the protein release data of nanospheres prepared using various cross-linking times was also normalized based on the relative size of nanospheres (Figure 3.10B). Results obtained suggest that for the same relative size of nanospheres, increasing the cross-linking time showed no significant difference in the protein release at any timepoints. This implies that change in nanospheres size by varying the cross-linking time had no significant effect on the protein release kinetics. When the initial burst release for different cross-linking times was normalized based on the relative size of nanospheres (Figure 3.11B), increasing the cross-linking time for the same relative size of nanospheres had no significant effect on the initial burst release. Increasing the cross-linking time also showed no significant difference in the initial burst release when normalized based on the nanospheres' weight (see Figure 3.11A), which implies that the change in the nanospheres' size by varying the cross-linking time showed no significant effect on the burst release, which aligns with our research hypothesis.

When the effect of the relative number of nanospheres (by varying the cross-linking times) on the protein release kinetics was tested, it was found that the relative number of nanospheres did not affect the protein release kinetics significantly at any of the timepoints (Figure 3.10C). However, when the initial burst release was normalized based on the relative number of nanospheres, unlike before (Figure 3.11A), for the same relative number of nanospheres, the 10-minute cross-linking time showed a significant decrease in the initial burst release compared to the 1 and 30-minute cross-linking times (Figure 3.11C). This implies that the change in the relative number by varying the cross-linking time influenced the initial burst release significantly obscuring the effect of cross-linking time on the initial burst release, which doesn't align with our research hypothesis.

3.2.3 Effect of drying time

By modifying the chemical property of alginate nanospheres, such as increasing the alginate concentration and cross-linking time it was possible to reduce the initial burst release to some extent; however, the initial burst release was still quite high (74.50 ± 1.53 %). Based on the previous studies, the degree of drying can influence the matrix porosity. Earlier studies confirmed that while complete dehydration increases the burst release, due to the destruction of the structural

integrity of microspheres, partial drying might reduce the release of the encapsulated molecules due to the decreased matrix porosity [102]. However, the effect of the drying time on the protein release from alginate nanospheres has not been investigated yet. Therefore, an effort was made in this study to determine the optimal drying time necessary to reduce the initial burst release. In order to better visualize the drying time effect, drying times were selected based on the log interval as it allows minimization of the number of groups necessary to determine the effect of a certain parameter on a certain outcome. Previous studies reported that freeze dried microspheres behave similar to wet microspheres and increase the protein release [163, 65]. Therefore, instead of freeze drying, nanospheres were dried at 37°C. Another reason of choosing 37°C is that it matches with the physiological temperature, hence, the probability of protein denaturation should be minimal at this temperature.

In this study, compared to the undried nanospheres, the mean diameter of nanospheres prepared using various drying times was comparatively high with a significant increase for the 0.5 and 1.5-hour drying times (Figure 3.15). Based on literature, due to water loss during drying the mean diameter of dried nanospheres should be less compared to their undried counterparts [130]. Imaging using an optical microscope is time consuming due to low throughput and the requirement for multiple measurements to calculate the mean diameter [164]. If the time required to take microphotographs affecting the nanospheres' size was tested first in order to explain this unusual result. However, it was found that the time required to take microphotographs didn't significantly affect the size (see appendix F). The effect of drying time also showed an unusual effect on the relative number of nanospheres. All of the nanospheres were prepared prior to drying and using the same processing conditions (5% alginate and 10-minute cross-linking time). Therefore, there should not have been any significant difference in the relative number of nanospheres. However, in this study, other drying time groups showed significant decreases in the relative number of nanospheres compared to the undried nanospheres. Compared to the 0-hour drying time, the reason for getting significantly high mean diameters for nanospheres prepared using the 0.5 and 1.5-hour drying times, and significantly less relative number of nanospheres for all the drying time groups might be due to nanospheres' aggregation. Probably when the external surface of nanospheres dried due to water loss, the distance between nanospheres decreased. This, in turn, might form new hydrogen bonds between alginate nanospheres due to the presence of hydroxyl groups in alginate, resulting in a comparatively high mean diameter due to nanospheres' aggregation. Using the

optical microscope, it is possible to observe the particle aggregation [164]. However, for very small sized nanospheres it might not be possible to detect the nanospheres' aggregation as they might appear as a single nanosphere, thereby, increasing the mean diameter and decreasing the relative number of nanospheres. With a farther increase in drying time more water loss occurred from the internal surface of these initially aggregated nanospheres resulting in a farther decrease of the mean diameter. However, this is just an assumption, as the real reason is still unknown. In order to confirm this assumption, the DLS and zeta potential analyzer can be used in future to determine the size and tendency of aggregate formation of nanospheres using different drying times.

In terms of protein release, it was found that increasing the drying time from 0 to 4.5 hours showed no significant difference in the protein release, when normalized based on the nanospheres' weight (Figure 3.16A). Based on literature, drying of microspheres reduces the water content from the gel network resulting in an increase in the gel concentration which eventually reduces the average pore size of the gel network [65, 104]. Therefore, with the increase of drying time the protein release should decrease. However, no significant difference in the protein release was found for different drying times. The reason behind this might be drying of nanospheres from 0 hour to 4.5 hours was not enough to reduce the nanospheres' porosity to such an extent that it affected the protein release. However, the effect of drying time on nanospheres' porosity was not tested in this study, so should be tested in future. In this study, the 24-hour drying time was considered as a completely dehydrated group of nanospheres (as no change of nanospheres mass was observed after 24 hours). Based on literature, complete dehydration increases the release of encapsulating molecules due to the destruction of structural integrity [102]. This 24-hour drying time was selected in order to confirm this negative effect of complete dehydration and to show why partial drying is better. Unexpectedly, at the initial timepoint, the 24-hour drying time group showed an incomplete protein release resulting in a significant decrease in the protein release compared to the 0.5 and 4.5-hour drying time groups. This might be due to the nanospheres' aggregation during drying, so they did not disperse properly when placed in a release medium. Therefore, probably DMEM could not impregnate through all the nanospheres preventing BSA release [65]. Another probable reason might be drying nanospheres for such a long period shrunk the nanospheres to such an extent that it was irreversible when placed in a release medium. This might be the reason they could not release all of the encapsulated BSA. This explains why in the

case of 24-hour drying time the percentage of the total protein release decreased significantly compared to all other drying time groups (Figure 3.18). However, using a low-resolution optical microscope it was not possible to get a detailed information about the nanospheres morphology due to the small size of nanospheres. Other high-resolution imaging techniques, such as the Scanning Electron Microscope (SEM) or Transmission Electron Microscope (TEM) would probably give a better information on the nanospheres morphology and should be used in the future. When the initial burst release was normalized based on the nanospheres' weight increasing the drying time showed no significant effect on the initial burst release (Figure 3.17A), which is not in an agreement with our proposed hypothesis. With the increase of drying time some of the drying time groups showed more and some showed less burst release compared to the 0-hour drying time. One possible explanation for the increasing drying time showing no definite pattern on the initial burst release might be because during drying protein diffuses by convection with the water, leaving an uneven distribution of protein throughout nanospheres [124]. This uneven protein distribution using different drying times might be the reason increasing the drying time showed no definite pattern in the initial burst release. However, the effect of drying time on the protein distribution should be tested in future to better understand the drying time effect on the protein release. Nanospheres prepared using the 24-hour drying time showed an incomplete protein release, therefore, the decrease in the initial burst release using the 24-hour drying time was excluded from the result.

The surface area of nanospheres also affects the initial burst release, therefore, in order to explain the effect of nanospheres' drying, the relative surface area of nanospheres for different drying times was calculated using equation (3.1). The relative surface area of nanospheres prepared using 0, 0.5, 1.5, 4.5-hour drying times was 4540, 3210, 3130 and 3400 μm^2 respectively. Based on the relative surface area (when porosity and total number of nanospheres were not considered), compared to the 0-hour drying time all other drying time groups should show a less release. However, all of the drying time groups showed similar or more release compared to the 0-hour drying time group. As discussed previously, the effect of drying time on the mean diameter and relative number of nanospheres showed contradictory results. Therefore, the relative surface area of nanospheres for different drying time groups, which were calculated using size and relative number of nanospheres, needs to be confirmed.

When the protein release data was normalized based on nanospheres' weight (Figure 3.16 A), the 0-hour drying time showed no significant difference in the protein release compared to any other drying time groups at the initial timepoint. However, at the initial timepoint (5-hour), for the same relative size of nanospheres the 0-hour drying time group showed a significant increase in the protein release compared to the 24-hour drying time group, when normalized based on the relative size of nanospheres. This implies that the change in the size of nanospheres by varying the drying time had a significant effect on the protein release kinetics. When the initial burst release for different drying time groups was normalized based on the relative size of nanospheres, like before (Figure 3.17A), increasing the drying time for the same relative size of nanospheres also showed no significant difference on the initial burst release (Figure 3.17B). This result implies that the change in nanospheres size by varying the drying time had no significant effect on the initial burst release, which aligns with our proposed hypothesis. However, as discussed previously, there is a possibility that the effect of nanospheres' size on the protein release kinetics and initial burst release might not be providing the actual effect of the nanospheres' size and should be confirmed in future.

When the protein release kinetics was normalized based on the relative number of nanospheres (3.16C), at the initial timepoint, unlike before (Figure 3.16A), for the same relative number of nanospheres the 0 and 24-hour drying time groups showed a significant decrease in protein release compared to the 1.5 and 0.5-hour drying times respectively. This implies that the relative number of nanospheres had a significant effect on the protein release kinetics. When the initial burst release was normalized based on the relative number of nanospheres, varying the drying time for the same relative number of nanospheres significantly affected the initial burst release (Figure 3.17C). However, when the initial burst release was normalized based on the nanospheres' weight (Figure 3.17A) it showed no significant effect on the burst release. This implies that the change in the relative number of nanospheres by varying the drying time influenced the initial burst release significantly obscuring the effect of the drying time on the initial burst release, which doesn't align with our research hypothesis. However, as discussed previously, the protein release kinetics and initial burst release when normalized based on the relative number of nanospheres might not be providing the actual effect of the relative number of nanospheres and should be confirmed in future.

3.3 Limitations of the Present Study

While the results of this study confirm that nanospheres prepared using various processing conditions affect the protein release kinetics and initial burst release to some extent and increasing the size of nanospheres reduced the initial burst release only in the case of alginate concentration, there are several limitations that might have affected the results. Those limitations is discussed below:

The size of micro/nanospheres plays a prominent role in determining the release kinetics of encapsulating molecules [94]. Both the type and concentration of surfactant affects the particle size and thereby, release kinetics [165]. However, only one type of surfactant and one fixed concentration have been used in this study. Therefore, further studies are needed to test the effect of other surfactants at different concentrations on the nanospheres' size which can eventually affect the release kinetics. Also, in this study, increasing the drying time showed a significant increase in the mean diameter of nanospheres. Further studies are required to explain this unusual effect of drying time on the nanospheres' size.

Determination of the percentage recovery is important as it helps to know about the efficiency of the preparation method. However, in this study, the percentage recovery of alginate and BSA was not calculated. In order to determine the percentage recovery, it is important to know the dry weight of BSA containing alginate nanospheres and the total weight of alginate and BSA. However, the dry weight of BSA loaded alginate nanospheres was not calculated in this study. The dry weight of BSA containing alginate nanospheres could have been determined by drying the nanospheres immediately after fabrication. For each group at least 3 to 6 independent samples could have been used to calculate the percentage recovery of alginate nanospheres and BSA.

All the possible combinations of alginate concentration, cross-linking time and drying time were not tested in this study, as it becomes hard to predict the effect of certain process parameter if other process parameters also vary concurrently. As a result, nanospheres prepared using the 3% alginate were not subjected to different cross-linking and drying times. Also, nanospheres prepared using different drying times were not subjected to different cross-linking times. It might be interesting to see how other possible combinations of the alginate concentration, cross-linking and drying time, other than the ones tested in this study, affect the protein release kinetics and initial burst release.

In this study, an optical microscope was used to determine the nanospheres' morphology. Due to the poor resolution of optical microscope it was not possible to correctly characterize the nanospheres' morphology in terms of the pore size and surface structure. Despite the SEM being one of the most popular techniques used to visualize the surface morphology and pore size of micro/nanospheres [166], this technique was not possible to use in this study. This is because in order to take an image using SEM, nanospheres need to be dehydrated completely, which is not applicable in this study as the drying time was one of the parameters to be tested. Therefore, complete dehydration would prevent determining the morphological change for different drying times. The Flow Imaging Microscopy (FIM) can be used in future to determine the nanospheres morphology as it allows to take the image of small particles without drying [167].

An optical microscope was also used to determine the size and relative number of nanospheres. As discussed previously (see section 3.2.3), the poor resolution of an optical microscope might be the reason it was not possible to detect the nanospheres' aggregation formed using different drying time groups. The DLS technique and zeta potential analyzer can be used in future in order to correctly determine the size and aggregation tendency of nanospheres respectively.

Determination of the degradation behavior of nanospheres is important as it also affects the release rate [94]. However, the degradation behavior of nanospheres was not tested in this study. Further studies are needed to determine the degradation rate of nanospheres. The Gel Permeation Chromatography (GPC) is one of the analytical techniques that can be used to determine the degradation rate of micro/nanospheres by determining the MW of a polymer at different times [168].

In this study, during determination of the effect of various process parameters on the protein release kinetics and initial burst release, only four timepoints (5, 24, 48 and 96-hour) were selected, with the 5-hour as an initial time point to determine the initial burst release. However, more than 60% of BSA was released at 5-hour and in most cases no significant difference in the protein release was found after 24 hours. Since the aim of this study was to determine the effect of various process parameters on the initial burst release, therefore, earlier time points before 5 hours should have been selected in this study.

In this study, the Bradford protein assay was used to determine BSA release at different timepoints. One of the limitations of Bradford assay is that it can be easily affected by surfactants,

oil residue, un-reacted cross-linkers and some of the uncomplexed polymers present in the nanospheres surface during processing (see Appendix E). Therefore, nanospheres in this study were re-suspended during washing to get clean nanospheres. However, possible residual presence after cleaning of nanospheres was not analyzed that might also have interfered the results.

Based on the previous studies, the physiological range of BMP-7 needed to exert the desired effect ranges from 50-100 ng/mL [29]. However, due to a low detection limit of the Bradford protein assay using this method it was not possible to determine the protein release below 1 µg/mL of protein. As a result, it was not possible to keep the amount of protein in each formulation to nanogram range. Instead, the amount of protein in each formulation was increased to microgram range. This higher percentage of protein loading might also be responsible for the high initial burst release [135]. Due to a high protein loading, the amount of protein released per mg of nanospheres at the initial timepoint (at 5-hour) ranged from 1.55 to 2.16 µg/ mL (excluding initial protein release for 24-hour drying time) which is way above than the physiological range of BMP-7 necessary for the cartilage defect repair. The release of bioactive proteins at a high concentration can exert toxic side effects, such as osteophytes formation and cartilage erosion [40]. Therefore, a more sensitive detection method, such as the Fluorometric analysis should be used in future studies so that the amount of protein loading in each formulation can be decreased to nanogram level.

Traditionally, the EE% can be determined either directly by dissolving nanosphere using an appropriate solvent or indirectly by determining the loss of protein during manufacturing and washing. However, due to the use of Bradford protein assay as a protein detection method, it was not possible to determine the EE% using any of these methods. Therefore, in this study, for different process conditions the percentage of total BSA released after 96 hours from the total recovered nanospheres has been reported as EE%. However, practically the EE% and the percentage of total BSA release are quite different. It is not possible to confirm if all of the encapsulated protein was released after 96 hours.

Due to the use of Bradford protein assay as a protein detection method, it was not possible to determine how much BSA was actually lost in isopropyl alcohol and oil layer during fabrication and washing using isopropyl alcohol. This is most likely because, BSA is insoluble in the isopropyl alcohol and organic oil, necessary to prepare the standard curve. Therefore, the calculation of mass balance was not possible in this study.

In this study, BSA was used as a model protein since its MW (66.5 kDa) is close to the MW of BMP-7 (49.3 kDa). Also, BSA has been used as a model protein of BMP-7 in previous studies [156]. However, the pI values of BSA (pI 4.8) and BMP-7 (pI 7.74) are quite different. Since the pI value of BSA was less than the pH value of the release medium (pH value of release medium was around 7.2), the net charge of BSA encapsulated in anionic alginate nanospheres should be negative resulting in an electrostatic repulsion between the alginate and BSA. This electrostatic repulsion between alginate and BSA might be the reason a very small percentage of BSA was in the core matrix of nanospheres, and most of the BSA was accumulated near the nanosphere surface resulting in a higher initial burst release. On the other hand, due to the high pI value of BMP-7 and net positive charge it would physically cross-link with the alginate resulting in a less release. Probably for the same reason the release rate of BMP-7 was less compared to BSA in one study [156]. All of this information implies that compared to the MW, the pI value of protein might play a more dominant role in controlling the release rate, as reported in one study [129]. However, this is just an assumption as the net charge of BSA in the release medium and the distribution pattern of BSA within the alginate nanospheres was not evaluated in this study.

In the present study, another reason of getting a very high burst release might be because of the release medium (high glucose DMEM) used to determine the protein release. This is most likely due to the sensitivity of alginate nanospheres towards some monovalent cations, such as Na^+ ion present in DMEM [104]. The degradation of alginate nanospheres due to the removal of Ca^{2+} from alginate gel matrix with monovalent ions present in DMEM might also be the reason for a comparatively higher burst release in this study. This might explain the fact that even though the alginate concentration, cross-linking time and drying time could reduce the matrix porosity to some extent, the burst release is still higher due to the degradation of gel matrix when incubated in DMEM.

3.4. Contributions of the Present Research

Attempts have been made in this thesis to reduce the high initial burst release of alginate nanospheres by varying the alginate concentration, cross-linking time, drying time and size of nanospheres. The main contributions of this thesis are as follows:

- In an experimental set up this is the first time the effect of alginate concentration, cross-linking time and drying time on the initial burst release of protein from the alginate nanospheres was evaluated.

- By varying the alginate concentration, cross-linking time or the drying time it was possible to reduce the initial burst release of alginate nanospheres by 13%.

- By normalizing the initial burst release based on the size and relative number of nanospheres it was possible to confirm that the change in the relative number of nanospheres (by varying the alginate concentration, cross-linking time or drying time) significantly affected the initial burst release.

CHAPTER 4

SUMMARY, CONCLUSIONS AND FUTURE WORK

4.1 Summary and Conclusions

In this study, alginate nanospheres were developed as a protein delivery device by using a combination of emulsification and external gelation method. The aim of this study was to investigate the influence of alginate concentration, cross-linking time, drying time on the initial burst release and rigorously selecting these parameters to reduce the initial burst release. BSA was used as a model protein for the preparation of protein loaded alginate nanospheres. The hypothesis was that, "*Increasing the alginate concentration, cross-linking time and drying time reduces the initial burst release independently of associated change to the size and number of nanospheres.*"

By increasing the alginate concentration from 3 to 5%, cross-linking time from 1 to 30 minute and the drying time from 0 to 24-hour, it was possible to prepare nanospheres with mean diameters ranged from 674.3 ± 12.37 to 1054.03 ± 18.29 nm. While increasing the alginate concentration and drying time showed significant effect on the nanospheres' size, increasing the cross-linking time had no significant effect on the size of nanospheres. In all cases, the relative number of nanospheres was inversely proportional to their mean diameter.

The effect of various process parameters, such as alginate concentration, cross-linking time or drying time on the protein release kinetics was evaluated. Increasing the alginate concentration and cross-linking time showed no significant effect on the protein release at any of the timepoints. However, increasing the drying time showed a significant effect on the protein release at the initial timepoint, with the 24-hour drying time showing an incomplete protein release.

By increasing the alginate concentration, cross-linking time and drying time it was possible to reduce the initial burst release from 85.55 ± 3.62 to 73.10 ± 4.17 %. Among various processing conditions, only increasing the alginate concentration showed a significant decrease in the initial burst release. By increasing the cross-linking and drying time, it was possible to decrease the initial burst release slightly.

When the effect of the size of nanospheres (by varying the alginate concentration, cross-linking time and drying time) on the initial burst release was tested it showed no significant effect

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on the initial burst release. However, when the effect of the relative number of nanospheres, prepared using different alginate concentration, cross-linking time and drying time, was tested it was found that the change in the relative number of nanospheres significantly affected the initial burst release for all the parameters tested in this study.

When the EE% was calculated it was found that increasing the alginate concentration and cross-linking time showed no significant difference in the EE%. However, increasing the drying time showed a significant difference in the percentage of total protein release.

In conclusion, we can tell that regulating process parameters is a mean to control initial burst release. By varying alginate concentration, cross-linking and drying time it was possible to decrease the initial burst release by approximately 13%. In this study, among various processing conditions tested, nanospheres prepared using the 5% alginate, 10-minute cross-linking time and 1.5-hour drying time showed the least initial burst release. Among various process parameters tested, varying the alginate concentration and the relative number of nanospheres (by varying the alginate concentration, cross-linking time or drying time) were found to be the major contributors to control the initial burst release. Evaluating the effect of this process parameters opens a new avenue to consider what can be done in the future.

4.2 Future Directions

In this study, by changing the polymer concentration, cross-linking time and drying time it was possible to reduce the burst effect to some extent. However, more investigation is needed to further reduce the initial burst release of alginate nanospheres for their intended application in cartilage defect repair.

The effect of other cross-linkers, such as $BaCl_2$ and $Al_2(SO_4)_3$ can also be tested in future to evaluate their effects on the burst release. For example, in one study it was found that compared to alginate microspheres cross-linked with $CaCl_2$, the alginate microspheres cross-linking with either $BaCl_2$ or $Al_2(SO_4)_3$ resulted in almost nonporous alginate beads due to an increased degree of cross-linking. The use of those cross-linkers also decreased the swelling and degradation of alginate beads in a release medium [130]. However, at the same time, compatibility of using the $BaCl_2$ and $Al_2(SO_4)_3$ cross-linked alginate nanospheres on cell viability also needs to be confirmed.

In this study, the effect of drying time on the protein release kinetics and initial burst release was not well understood. Even if nanospheres prepared using the 24-hour drying time decreased the initial burst release by 7%, the reason why the 24-hour drying time showed an incomplete protein release is still unclear. More study is needed to determine the optimal drying time necessary to reduce the burst effect. It might be interesting to see what effect of increasing the drying time more than 4.5-hour and less than 24-hour might have on the initial burst release.

Based on earlier studies, the distribution pattern of encapsulating molecules within microsphere plays a significant role in the burst release. For example, Lee *et. al.* showed that instead of having a uniform distribution, if the distribution of bioactive molecules is such that the highest concentration of a bioactive molecule is present near the core of microspheres then the burst release can be significantly reduced [169]. The confocal laser scanning microscopy (CLSM) can be used to investigate the protein distribution around alginate nanospheres [124, 133].

The degree of swelling has a profound impact on the protein release kinetics and initial burst release. In this study, the influence of various processing parameters on the degree of swelling was not tested. Therefore, the effect of various processing conditions on the degree of swelling needs to be evaluated. The degree of swelling can be determined following one of the previous studies [170]. Briefly, dried nanospheres can be placed into a reservoir containing a release buffer and incubated in a shaking incubator. At different times, the weight of each swollen sample can be determined using an electrical balance. More swelling means more weight of wet nanospheres. By determining the weight of dry nanospheres and the weight of wet nanospheres, the degree of swelling (DS) can be evaluated using following equation:

$$\% \, DS = (W_2\text{-}W_1) \, / \, W_1 \, x \, 100 \, \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots(4.1)$$

where W_1 and W_2 represents weight of dry microsphere and weight of wet microspheres respectively.

Several studies have confirmed that with the increase of the free Ca^{2+} ion in the release medium the degree of swelling of alginate microspheres reduces which results in less protein release [171, 172, 173]. Therefore, the effect of various amount of free Ca^{2+} ion in release medium needs to be examined in future. However, increasing the free Ca^{2+} ion might hamper cell viability. Therefore, the effect of various amount of free Ca^{2+} ion on the cell viability needs to be tested at first to determine the maximum amount of free Ca^{2+} can be used in a release medium.

The bioactivity of the released protein has not been evaluated in this study. Bioactivity test is very important to confirm if the processing conditions used to fabricate nanospheres are denaturing the protein. The bioactivity of a protein can be tested by the Immunocytochemistry [84].

For cartilage defect repair, BMP-7 is required to incorporate in nanogram level in order to release the BMP-7 within a physiological range. Therefore, use of more sensitive protein detection methods is needed. For example, using the Fluorometric analysis it is possible to detect the protein concentration in the nanogram level, thereby, decreasing the protein loading in each sample. This decrease in the protein loading will also decrease the initial burst release. In order to perform fluorometric analysis, a protein can be labeled by FITC. Afterwards, the protein release at different timepoints can be determined by measuring the fluorescent intensities using a Fluorometer.

Once the protein release kinetics and burst effect is optimized, the next step will be loading alginate nanospheres with BMP-7, necessary for cartilage defect repair. BMP-7 is a high pI protein with the pI value greater than 7.4. Therefore, in the release medium having a neutral pH it will be positively charged. Because of attractive electrostatic interactions between the positively charged BMP-7 and negatively charged alginate, presumably BMP-7 will be physically cross-linked with alginate resulting in the prolonged release and less burst effect compared to BSA. However, it was not possible to use BMP-7 in this study, as it is very expensive, and more studies are required in future to reduce the initial burst release of alginate nanospheres for their intended application in CTE. During the use of a high pI protein to prepare protein loaded alginate nanospheres, it should be kept in mind that sometimes the alginate protein interaction using a high pI protein might cause protein denaturation [138]. Therefore, additives need to be incorporated in a protein to minimize its denaturation.

Since the main objective of this study was to prepare protein loaded alginate nanospheres for their intended application in CTE, therefore, it is very important to see how this BMP-7 loaded alginate nanospheres can regulate the middle zone-specific gene expression when incorporated in a scaffold along with cells and cultured *in vitro*.

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INTERFERENCE OF ALGINATE DURING BRADFORD ASSAY

To test the interference of alginate during Bradford protein assay, two groups of nanospheres were prepared. Group 1 was nanosphere prepared with alginate alone and group 2 was nanospheres prepared with BSA containing alginate. The concentration of alginate and cross-linking time used for nanospheres fabrication was 5% and 10-minute respectively. The supernatant was collected from both group at different timepoints and the Bradford assay was performed to determine the optical density (OD) value of both groups. It was found that alginate alone without any BSA was also showing a comparatively high OD value during the Bradford assay (see Figure A.1). Therefore, it was decided to use the alginate nanospheres without BSA as a negative control.
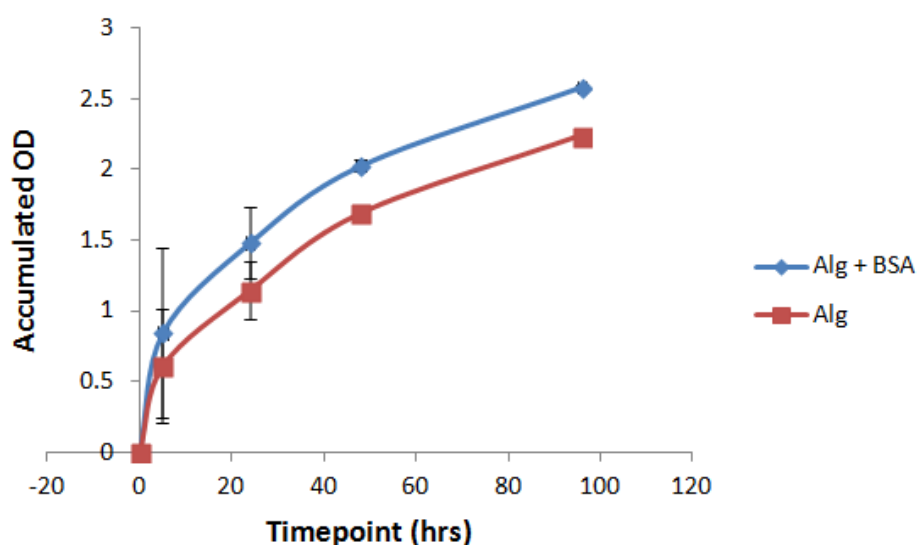

Figure A.1: Interference of alginate during the Bradford assay.

REPRODUCIBILITY ISSUE USING INDIVIDUAL TUBE PROTOCOL FOR LOW VISCOSITY ALGINATE

Unlike the medium viscosity alginate, the 3% low viscosity alginate was showing protein release at different timepoints when re-suspended during washing. However, like before the protein release kinetics using the individual tube protocol was not showing an expected trend. An expected trend of the protein release kinetics means the increase of the protein release with time. However, using the individual tube protocol the protein release was increasing for some of the timepoints and then again was decreasing for some other timepoints. Also, under the same processing condition the release patterns between batches were different. Figure B.1 shows the reproducibility issue using the individual tube protocol. In order to determine the effect of 3% alginate on the protein release kinetics, the experiment was repeated 2 times. The release pattern using the 3% alginate was quite different between these 2 experiments (Figure B.1). At that point it was assumed that this might be because of using different batches of nanospheres at different timepoints for the protein release study that added lots of variability during the process. Therefore, it was decided to use the supernatant protocol to determine the protein release kinetics of alginate nanospheres.
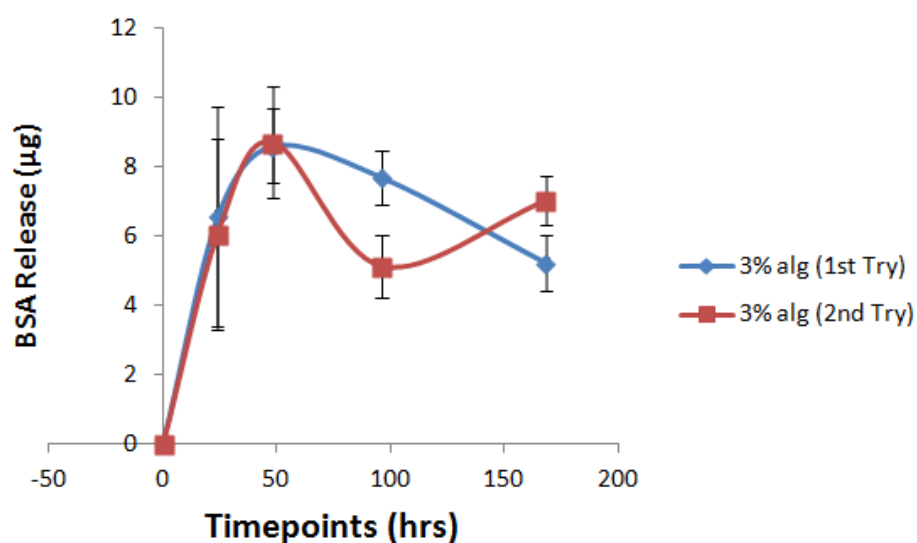

Figure B.1: Reproducibility issue using the individual tube protocol.

APPENDIX C

TROUBLESHOOTING SUPERNATANT PROTOCOL FOR LOW VISCOSITY ALGINATE

The individual tube protocol showed reproducibility issue and couldn't show an expected trend. Therefore, it was decided to use the supernatant protocol i.e, using the same batch of nanospheres to collect the supernatant at different timepoints. Using the supernatant protocol, it was possible to show an expected trend in the protein release kinetics with the protein release increasing with time. However, one of the limitations of supernatant protocol was that to collect supernatant the same batch of nanospheres was needed to be centrifuged at different timepoints. Since alginate has a very poor mechanical strength, high centrifugation speed can damage alginate nanospheres, thereby, affecting the protein release kinetics. Therefore, to reduce the damage of alginate nanospheres, they were centrifuged at 1983 x g to collect the supernatant. However, supernatant collection at 1983 x g resulted in loss (probably, approximately 5 %) of some nanospheres. To prevent nanospheres' loss during centrifugation, after collecting the majority of nanospheres by centrifuging at 1983 x g, the resulting supernatant was centrifuged again at 16873 x g for 15 minutes to recover the remaining nanospheres which were then added back into the original tube to determine the protein release at later timepoints. However, the supernatant protocol was still showing reproducibility issues. Figure C.1 shows the difference in the protein release kinetics between 2 batches of nanospheres when they were dried for 2 hours. At this point a correct way of the normalization of protein release at different time point was not determined. Therefore, the protein release at different timepoints was used without normalization. Each time the protein release kinetics using the 2-hour drying time was quite different.
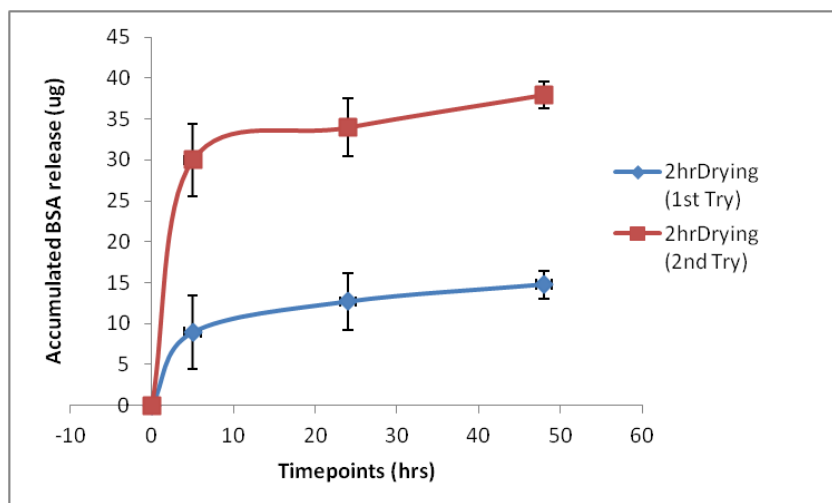
Figure C.1: Reproducibility issue of using the supernatant protocol. The concentration and cross-linking time used to prepare nanospheres were 5% and 10 minutes respectively.

At first it was assumed that the issue might be because BSA was not mixing uniformly with the alginate solution. Therefore, mixing time of BSA with alginate was increased from 30 minutes to 2 hours. However, still the BSA release between batches was quite different. This might be because at this centrifugation condition the collected supernatant was not clear enough interfering the result. Therefore, after collecting majority of nanospheres at 1983 x g centrifugation speed, instead of centrifuging the collected supernatant 1X at 16873 x g, the supernatant was centrifuged 2X at 16873 x g for 15 minutes. This way it was possible to get almost similar amounts of BSA release using different batches. Table C.1 shows the differences in the BSA release between batches when the collected supernatant was centrifuged either 1X at 16873 x g or 2X at 16873 x g.

As can be seen in Table C.1, for 1X centrifugation, batch 2 showed almost twice BSA release compared to batch 1. Even if, using centrifugation variation within samples in same batch was found more compared to the one centrifuged 1X, still 2X centrifugation was used since using this, the average BSA release between batches was almost similar.

Table C.1. Effect of centrifugation condition on the reproducibility of protein release between batches

BSA	1X centrifugation of collected supernatant at 16863 x g for 15 minutes		2X centrifugation of collected supernatant at 16873 x g for 15 minutes	
	Batch 1	Batch 2	Batch 1	Batch 2
BSA-1	17.24	32.5	10.68	27.88
BSA-2	20.26	30.83	44.34	36.77
BSA-3	16.47	36.73	46.67	33.07
BSA-Avg	17.99	33.35	33.9	32.57

NO RELEASE OF BSA AFTER 96 HOURS

In order to determine the release kinetics of alginate nanospheres, 5 timepoints, i.e. 5-hour, 24-hour, 48-hour, 96-hour and 168-hour were used in the beginning. However, it was found that no release of BSA was found after 96 hours, as shown in Figure D.1. Therefore, the 96-hour time point was assumed to be the maximum time required to release all the encapsulated BSA from alginate nanospheres.
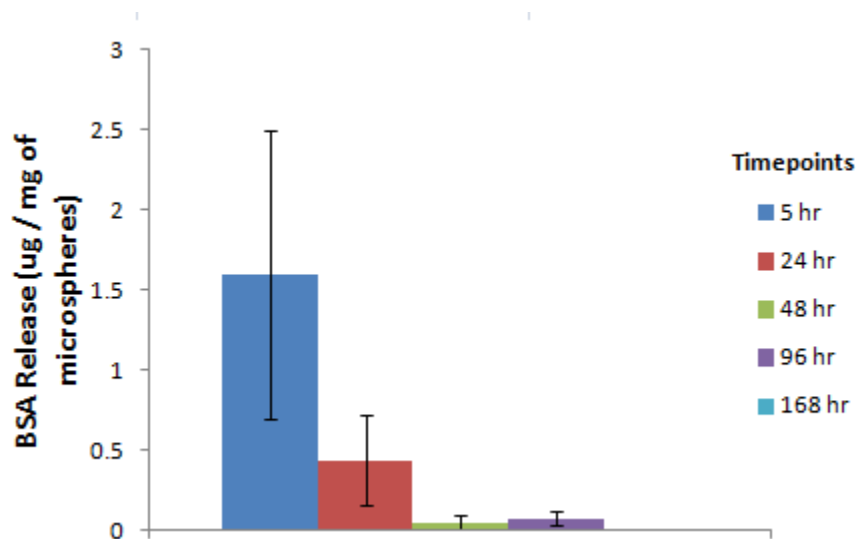

Figure D.1: Release of BSA from alginate nanospheres at different timepoints. 5% alginate was used to prepare nanospheres. n=3 and data has been presented as mean ± S.D.

APPENDIX E

TROUBLESHOOTING INDIVIDUAL TUBE PROTOCOL USING MEDIUM VISCOSITY ALGINATE

At the beginning of this study the 5% medium viscosity alginate was used to prepare protein loaded alginate microspheres. To determine the protein release kinetics, the individual tube protocol i.e., using different batches of microspheres for measuring the protein release at different timepoints were used. Centrifugation speed that was used at the beginning to collect the supernatant was 1983 x g and for 10 minutes. However, preliminary results of collecting supernatant at 1983 x g centrifugation showed loss of some microspheres that is not desirable. Therefore, the supernatant was collected by centrifuging at 16873 x g for 15 minutes. At this point, during the washing of microspheres they were not re-suspended. This, in turn, was giving an unparticular trend of protein release kinetics when the medium viscosity alginate was used, as shown in Figure E.1. Therefore, it was decided to re-suspend the microspheres during washing. Surprisingly, even if the medium viscosity alginate was showing some release before, after re-suspending during washing it showed very little release or almost no release, as shown in Figure E.2. At this point, the correct way for normalization of the protein release at different timepoint was not determined. Therefore, the protein release at different timepoints was used without normalization. The reason of getting a comparatively high release when the microspheres were not re-suspended might be because of the presence of unreacted surfactants, oil residue, cross-linker and some of the un-complexed polymers giving artificial OD during Bradford assay. The reason of getting very little or almost no release after re-suspending during washing was assumed due to forming almost non-porous alginate microspheres using the medium viscosity alginate. This might be due to a very high degree of cross-linking using the 5% medium viscosity alginate. The very small amount of BSA that was released might be from the adsorbed BSA in the microspheres' surface via protein desorption. Therefore, decision has been made to use the low viscosity alginate in later studies.
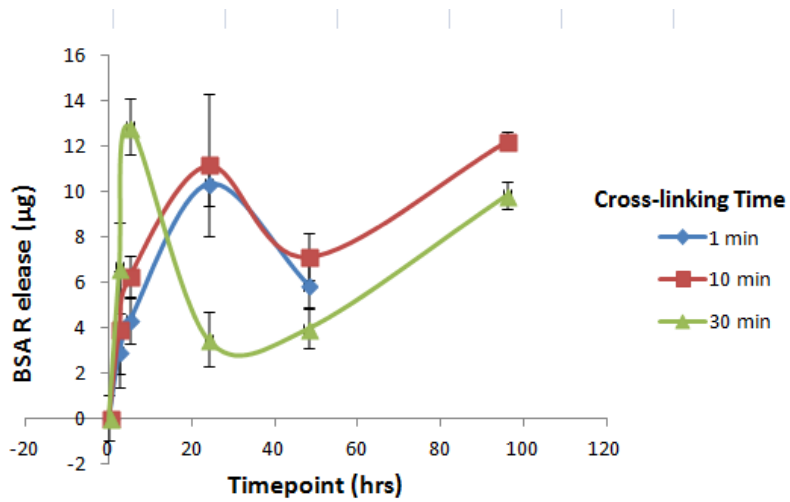
Figure E.1: An unparticular trend of the protein release kinetics of the medium viscosity alginate using the individual tube protocol.
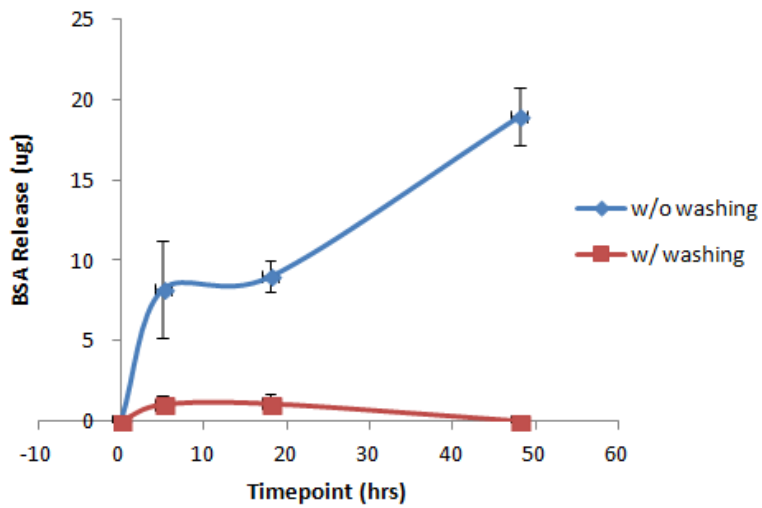

Figure E.2: The effect of re-suspending of microspheres during washing on the protein release kinetics.

EFFECT OF WAITING TIME DURING IMAGING ON MICROSPHERE SIZE

Microphotographs needs to be taken using an optical microscope to determine the size of nanospheres. In order to take image, nanospheres were suspended in DMEM and 3 µL sample was used in the microscope slide to take image. Because of a low throughput of optical microscopes, it was time consuming to take multiple images to determine size of nanospheres. Moreover, sometime images of nanospheres were taken for more than one processing conditions. Therefore, the time required to take images varied from 3 to 4 hours. To test if the time required to take the image was affecting the result, a group of nanospheres image was taken immediately after fabrication and after 4 hours. Figure F.1 shows the effect of waiting time on the mean diameter of nanospheres. It was found that no significant change in the mean diameter of nanosphere was observed confirming the time required to take image didn't affect the result as no swelling of nanosphere occurred during this time.
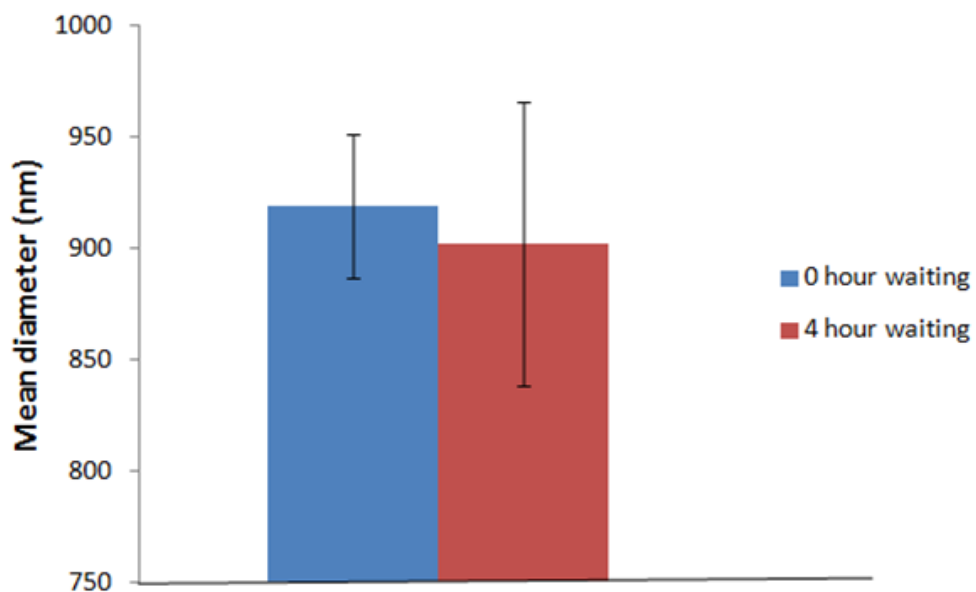

Figure F.1: The effect of waiting time during imaging on the nanospheres' size. The concentration and cross-linking time used to prepare nanospheres were 5% and 1 min respectively.