Optimal hydrogels for fast and safe delivery of bioactive Compounds

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Dedications

I dedicate this thesis to my mother for her love and unconditional support in the endeavor I took.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The design of injectable, biocompatible hydrogels encapsulating bioactive substances and exhibiting minimal aerosol formation is an important problem and its optimal solution can lead to more effective delivery vehicles in various applications. The delivery rate from such hydrogels is generally slow if one targets minimal aerosol formation. This thesis explores different biomaterials as potential hydrogels for such applications. The design criteria are good extrusion consistency without phase separation, fast release rate of the encapsulated biomolecule and ability to maintain the functionality of the encapsulated entity. In vitro tests were developed to evaluate the hydrogels synthesized. We were able to develop a gel showing good extrusion consistency and fast release rate of an active virus.

1. Introduction

Hydrogels have been used for various drug and viral delivery applications. But not much research has been put into injectable delivery systems for safe delivery of bioactive substances that if delivered in an aerosol or free form, may have an adverse effect on the subject and people in vicinity, such as live vaccine. In order to address this setback, we have tried to prepare a hydrogel formulation that can potentially be used to encapsulate these special bioactive agents (exemplified by Influenza virus) and inject them without aerosolization.

Ideally a hydrogel to be used in such applications should be able to prevent aerosol formation when extruded through 26G needle, while also exhibiting appropriate release kinetics and degradation characteristics. Compatibility with the encapsulated bioactive substance is a prominent parameter that should be considered in design.

To find a formulation that has the desired properties, we tried three different types of hydrogels namely,

1. Alginate hydrogel prepared using internal setting technique with CaSO4 as the crosslinking catalyst
2. Supramolecular hydrogels based on alpha cyclodextrins and PEO
3. Alginate hydrogel prepared using reverse addition technique with $CaCl_2$ as the crosslinking catalyst

Different formulations of the three types of hydrogels were tested for their extrusion consistency and phase separation, as phase separation can cause aerosolization. The formulations with a consistency score higher than four (maximum was five) were selected for the release study of 200 nm amine modified fluorescent nanoparticles. The nanoparticle release study was performed for better understanding of the release and degradation properties of the hydrogel. The formulations that showed more than 1% release of nanoparticles in 24 hours were selected for the Virus release study / hemagglutination assay. The hemagglutination assay was used to assess if the hydrogel was compatible for bioactive substances and that it had no effect on the surface integrity of the bioactive agent.

The results obtained by performing the three in-vitro tests on all the three types of hydrogels are presented.

2. Background and Literature Survey

2.1 Alginates

First described in 1881 by British chemist E. C. C. Stanford [1], alginates are water soluble biopolymers and are quite abundant in nature. They are present in marine brown algae (Phaeophyceae) as structural components and in soil bacteria as capsular polysaccharides [2].

2.1.1 Structure

After being discovered in 1881, the final structure of alginate as we know it today came after continuous research and a number of revisions of the originally proposed structure. At first Stanford believed that alginic acid has nitrogen [3]. In 1926, some research groups showed that uronic acid was a constituent of alginic acid [4]. Later several research groups working independently found out that the uronic acid present in alginate was D-mannuronic acid [5]. Hirst et.al proved that the uronic acid molecules were linked by β1, 4 bonds [6]. This simple structure of alginate went through a major revision in 1955 when Fisher and Dorfel showed the presence of L-Guluronic acid along with D-mannuronic acid in the hydrolysates of alginic acid. From then on, alginate was regarded as a binary polymer comprising of α-L-Guluronic acid and β-D-mannuronic acid residues. The sequential structure of alginate was established later when Haug et al [7,

8]performed partial acidic hydrolysis and fractionation Studies. Their studies established that alginate is a true block copolymer composed of homopolymeric regions of Mannuronic (M) and Guluronic (G) acids, termed M- and G-blocks, respectively, interspersed with regions of alternating structure (MG-blocks) (Figure 1).

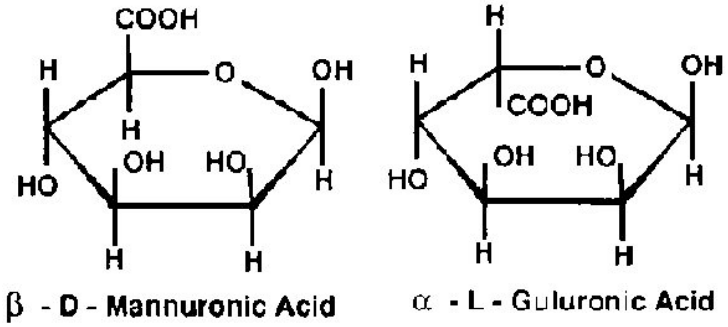
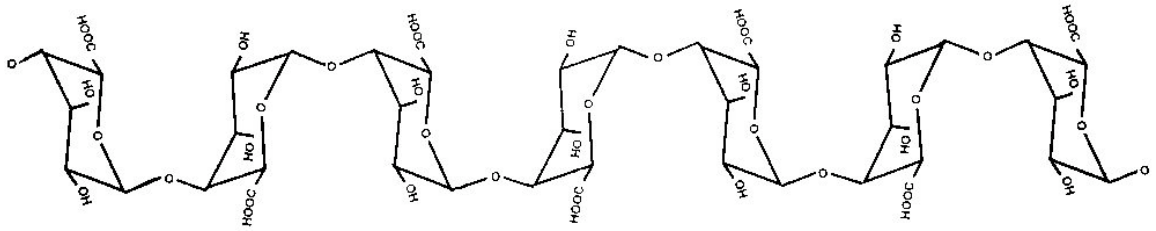
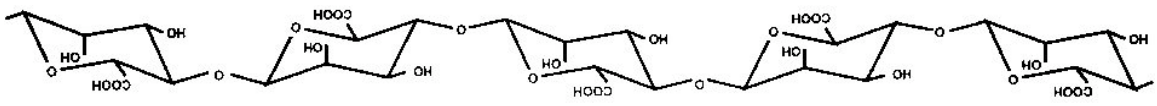


Figure 1: Classical formulae of the two alginate monomers

The G blocks contain only units derived from L-guluronic acid (Figure 2), the M blocks are based entirely on D-mannuronic acid (Figure 2) and the MG blocks consist of alternating units from D-mannuronic acid and L-guluronic acid [2].



G Block



M Block

Figure 2: G and M Blocks in Alginate

The M and G blocks have different shapes. Because an M block is formed from equatorial groups at C-1 and C-4, it is a relatively straight polymer, like a flat ribbon. However the G block is formed from axial groups at both C-1 and C-4 so the resulting chain is buckled [2] ; the importance of this buckled shape will be apparent later when the formation of gels from alginate solutions is discussed.

So an alginate molecule can be regarded as a block copolymer containing M, G, and MG blocks, the proportion of these blocks varying with the seaweed source [2]. It has been shown that the physical properties of alginates depend on the relative proportion of the three types of blocks. For example formation of gels, by addition of calcium ions, involves the G blocks so the higher the proportion of these, the greater the gel strength; solubility of alginate in acid depends on the proportion of MG blocks present. The industrial utilization of any particular alginate will depend on its properties and therefore on its uronic acid composition so it has become important to have some measure of the relative proportions of the uronic acids.

2.1.2 Unique arrangement of the Uronic residues in alginate

The classical Haworth formulas for β-D-mannuronic acid and α-L-Guluronic acid are shown in Figure 1.

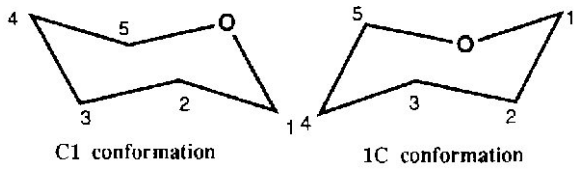


Figure 3: C1 and 1C forms of the tetrahydropyran ring

The basic structure of each monomer is the tetrahydropyran ring and this has two possible chair forms, C1 and 1C (Figure 3). β -D-mannuronic acid assumes the C1 form; in the other form, 1C, there would be steric interaction between the axial -COOH on C-5 and the axial -OH on C-3; the C1 form has these groups in the equatorial positions and so is

more stable. For similar reasons, α-L-guluronic acid assumes the 1C form rather than the C1 form [9-11].

The alginate polymer is formed by joining these monomers at the C-1 and C-4 positions. An ether-oxygen bridge joins the carbon at the 1-position in one molecule to the 4-position of another molecule. It has been shown that the polymer chain is made up of three kinds of regions or blocks, the M blocks, the G blocks and the MG blocks.

2.1.3 Sources

All the major commercial alginates are produced mainly from Laminaria hyperborea, Macrocystis pyrifera, Laminaria digitata, Ascophyllum nodosum, Laminaria japonica, Eclonia maxima, Lessonia nigrescens, Durvillea antarctica and Sargassum spp. Table 1 gives some sequential parameters (determined by high field NMR-spectroscopy) for samples of these alginates. Physical properties of alginates have been shown to be dependent on the relative proportion of the M, G and MG blocks [11].

Source	F_G	F_M	F_{GG}	F_{MM}	$F_{GM,MG}$
Laminaria japonica	0.35	0.65	0.18	0.48	0.17
L. digitata	0.41	0.59	0.25	0.43	0.16
L. hyperborea, leaf	0.55	0.45	0.38	0.28	0.17
L. hyperborea, stipe	0.68	0.32	0.56	0.20	0.12
L. hyperborea, outer cortex	0.75	0.25	0.66	0.16	0.09
Lessonia nigrescens	0.38	0.62	0.19	0.43	0.19
Ecklonia maxima	0.45	0.55	0.22	0.32	0.32
Macrocystis pyrifera	0.39	0.61	0.16	0.38	0.23
Durvillea antarctica	0.29	0.71	0.15	0.57	0.14
Ascophyllum nodosum, fruiting body	0.10	0.90	0.04	0.84	0.06
Ascophyllum nodosum, old tissue	0.36	0.64	0.16	0.44	0.20

Table 1: Composition of algal alginate [12] (F_X = % of x in the polymer)

2.1.4 Alginate Hydrogels

Hydrogels are superabsorbent (they can contain over 99% water) hydrophilic polymer networks of natural or synthetic polymers [13]. Most of the polysaccharides derived from seaweeds such as alginates, agars, carrageenans and furcelleran - can all be induced to form hydrogels under certain conditions [13].

Alginates form hydrogels by the virtue of their selective ion binding properties. Solutions of alginate will form gels in the vicinity of many di- and trivalent cations. Because of the particular shapes of the monomers and their modes of linkage in the polymer (figure 1), the geometries of the G-block regions, M-block regions, and alternating regions are substantially different. Specifically, the G-blocks are buckled while the M-blocks have a shape referred to as an extended ribbon, as shown in Figure 2. If two G-block regions are aligned side by side, a diamond shaped hole results. This hole has dimensions that are ideal for the cooperative binding of calcium ions. When calcium ions are added to a sodium alginate solution, such an alignment of the G-blocks occurs; and the calcium ions are bound between the two chains like eggs in an egg box, as shown in Figure 4. Thus the calcium reactivity of alginate is the result of calcium-induced dimeric association of the G-block regions [14]. Depending on the amount of calcium present in the system, these inter-chain associations can be either temporary or permanent. With low levels of calcium, temporary associations are obtained, giving rise to highly viscous, thixotropic solutions. At higher calcium levels, precipitation or gelation results from permanent associations of the chains. Alginate's ability to form a gel is determined by the proportion

and length of G-blocks in its molecular structure[15]. The affinity of alginates for alkaline earth metals increases in the order Mg<<Ca<Sr<Ba; a property unique for alginates compared to other polyanions.

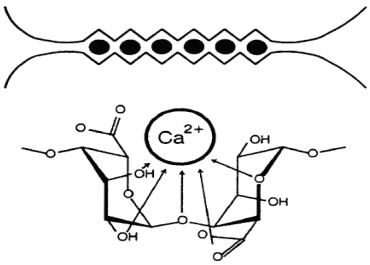


Figure 4: The egg-box model for alginate gelation [14]

Many groups have suggested more accurate models explaining alginate gelation supported by NMR spectroscopy [16, 17] and X-ray diffraction [18, 19], the simple egg-box model is still used most widely to explain alginate gelation, as it may be regarded as giving an intuitive understanding of the characteristic chelate-type ion-binding properties of alginates.

2.1.5 Factors controlling alginate gelation

Alginate gelation can be mainly controlled by three major factors, type of alginate used, type of calcium salt used and modulator or sequestrant [2]. The choice of these components is determined by the final application of the alginate gel produced. The properties of the resulting gel are fit to the final specifications of the gel. These properties include modulus, elasticity, brittleness and syneresis ('ageing' of the gel).

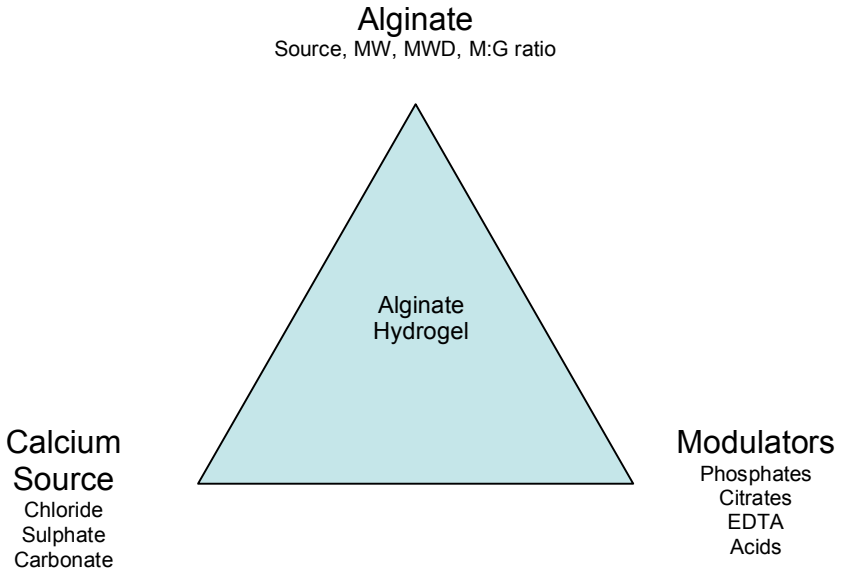


Figure 5: Factors controlling the kinetics and final properties of alginate gel[12]

2.1.6 Gelling Techniques

While alginates naturally lend themselves to multiple gelling methods, in practice, alginate gels are obtained using two major methods; namely, diffusion setting and internal setting [2]. Alginate in the presence of multivalent cations reacts very rapidly and irreversibly to form a gel, and thus direct mixing of these two components will rarely produce homogeneous gels. The ability to control the introduction of crosslinking ions hence becomes essential, to form smooth gels.

2.1.6.1 Diffusion Setting: Diffusion setting is the simplest technique and, as the term implies, the gel is set by allowing calcium ions to diffuse into an alginate solution. This method is mainly used for immobilization purposes as it involves rapid gelling kinetics. During immobilization each droplet of alginate solution can encapsulate bioactive agents, in one single bead. An important feature of the diffusion gelling is that the final gel may exhibit an inhomogeneous alginate distribution, as the alginate concentration gradually decreases towards the center of gel. The homogeneity of a diffusion gel can partly be controlled. A high degree of inhomogeneity is obtained by using a low concentration of gelling ions in the absence of non-gelling ions. A more homogeneous gel is obtained when gelling occurs in the presence of high concentrations of both gelling and non-gelling ions.

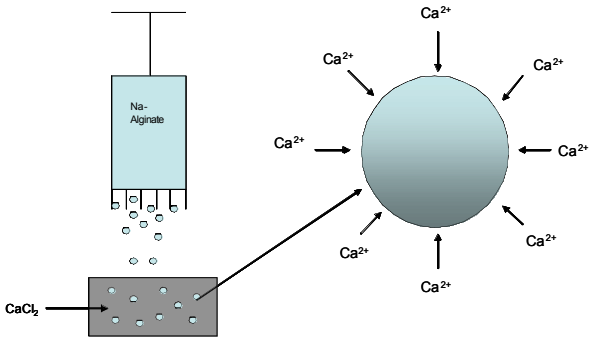


Figure 6: Diffusion Setting exemplified by immobilization technique

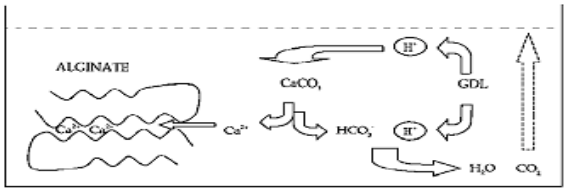


Figure 7: Internal Setting exemplified by $CaCO_3$/GDL technique

2.1.6.2 Internal setting: In internal or bulk setting, the calcium is released under controlled conditions from within the system. Calcium sulfate (usually as the dihydrate), gypsum, and dicalcium phosphate (calcium hydrogen orthophosphate) are the sources of calcium most commonly used. The rate at which the calcium is made available to the alginate molecules depends primarily on pH and the amount, particle size and intrinsic solubility characteristics of the calcium salt. Small particle size and low pH favor rapid release of calcium. In most situations, calcium release during the mixing of the ingredients is so rapid that a calcium sequestrant is required to control the reaction by competing with the alginate for calcium ions. The main difference between internal and diffusion setting is the gelling kinetics, which are not diffusion-controlled in the former case. With internal setting, it is possible to tailor a manufacturing process to produce a desired gel system due to the controlled, internal release of cross-linking ions [20].

2.2 Supramolecular hydrogels

2.2.1 Supramolecular Chemistry

Supramolecular chemistry as defined by Jean-Marie Lehn, is a highly interdisciplinary field of science covering chemical, physical and biological features of chemical species with greater complexity than molecules themselves, that are held together and organized by means of intermolecular (non-covalent) binding interactions [21, 22]. Supramolecular chemistry studies the interactions between molecules rather than within them. In contrast to molecular chemistry where strong binding forces such as covalent and ionic bonds are used to build molecules from individual atoms, supramolecular assemblies are held together by weak non-covalent interactions, such as hydrogen bonding, polar attractions, Van der Waals forces, and hydrophilic-hydrophobic interactions [23].

Supramolecular chemistry is divided into five major sub-types, which include molecular self-assembly, molecular recognition, host-guest chemistry, mechanically interlocked molecular architectures, and dynamic covalent chemistry.

Out of the five subtypes, the host-guest chemistry has been exploited the most in the formation of supramolecular hydrogels. Host-Guest Chemistry studies such interactions between a 'Host' (receptor) and a target 'Guest'. Two or more molecules are held together in unique structural relationships by weak interactive forces.

2.2.2 Supramolecular Hydrogels

Supramolecular chemistry and especially host guest chemistry has been extensively studied in the past for the macromolecular self-assembly between polymers and cyclodextrins [24-26]. These unique complexes formed by non-covalent host-guest interactions are known as polymer inclusion complexes (PIC) or polyrotaxanes. Cyclodextrins (CDs) have been the most popular candidates to be used as host molecules, because they are water-soluble and capable of selectively including a wide range of guest molecules.

Harada et al. have studied the combinations of CDs, usually α, β and ϒ-CD, which consist of 6,7 and 8 glucose units, respectively and linear polymers such as poly (ethylene glycol) [24-30]. Li et al reported hydrogel preparation using inclusion complex formation between high molecular weight poly(ethylene oxide) and α-CD[25]. Kang Moo Huh et al prepared a thermoreversible hydrogel network with a supramolecular structure that consisted of biodegradable and biocompatible components, PEG grafted dextrans and CD molecules, using host -guest interactions. The unique thermoreversible gel-sol transition based on supramolecular assembly and dissociation, and the transition temperature range was between 20 to 55 °C [31].

Recently Yang et al have prepared a novel hydrogel made of anti inflammatory molecules (N-(Fluorenyl-9-methoxycarbonyl)-L-leucine and Ne-(fluorenyl- 9-methoxycarbonyl)-L-lysine) and a uranyl ion chelating ligand (pamidronate), for topical treatment of simulated uranium wounds [32].

Li et al have reported better and easy to prepare supramolecular hydrogels using cyclodextrins as host and high-molecular weight PEOs [32, 33][36] or triblock copolymer PEO-PHB-PEO[34], as guest polymers. These hydrogels were found to be thixotropic, reversible, and injectable through needles. Both of them can be used as injectable formulations for the sustained controlled delivery of encapsulated bio-active agents. The properties of the second (Triblock copolymer and Cyclodextrin) hydrogel can be controlled by using different length and types of the middle hydrophobic poly[(R)-3-hydroxybutyrate] PHB segment, thus allowing design of a hydrogel with different functions for a wide range of biomedical applications.

The unique property of these gels to flow like a liquid upon application of sideways force (thixotropic behavior) affords us to use them as an injectable hydrogel drug delivery system. Various bioactive agents (drugs, proteins, vaccines or plasmid DNAs) can be encapsulated inside these hydrogels, which can then be loaded in a syringe and stored. Owing to the thixotropic nature of these hydrogels, the drug-loaded formulation can then be injected into the tissue under pressure.

2.3 Polymers in Drug Delivery

The interest in preparing novel drug delivery systems has increased steadily during the last 50 years. The global drug delivery market generated total revenues of $426.9 billion in 2005 and is expected to reach $543.8 billion by the end of 2010 [35]. In most cases the purpose is to make a product that allows maximum encapsulation efficiency and controlled release of the encapsulated drug. Providing control over the drug delivery is the most important factor at times where traditional drug delivery formulations cannot be used. These include situations requiring the sustained release of certain drugs to obtain a prolonged effect or fast release of specific drugs at specific target site to prevent the drug from losing its potency with time, drug delivery to specific sites, drug delivery using nanoparticulate systems, delivery of two or more agents with the same formulation, and systems based on carriers that can dissolve or degrade and be readily eliminated. The ideal drug delivery system must not react with the encapsulated agent, should be biocompatible, should allow high drug loading, be safe for the people handling it, simple to prepare, and simple and safe to administer.

Drugs are mostly administered to a patient in a formulated state. A dosage form generally consists of one or more active principles together with a varying number of other substances (excipients) that have been added to the formulation in order to facilitate the preparation and administration, promote the consistent release and bioavailability of the drug, and protect it from degradation. These excipients strongly influence the physicochemical characteristics of the final products. Excipients were considered to be inert in that they should not exert any therapeutical or biological action or modify the

biological action of the drug substance. It is now recognized that excipients can potentially influence the rate and/or extent of absorption of a drug (e.g., by complex formation). The successful formulation of a stable and effective dosage form therefore depends on the careful selection of excipients.

In this context, the use of polymers as a formulation aid in drug delivery systems has been an important area of research and development over the years. Polymeric materials are playing increasingly important roles in the development of novel drug delivery systems. For more than three decades, the delivery of drugs/bioactive agents from polymeric materials has attracted considerable attention of polymer chemists; chemical engineers; pharmaceutical scientists and entomologists. All these scientists have been working on designing predictable or controlled as well as burst or fast release delivery systems [36-39]. Polymer science and engineering has enabled the explosive growth in the therapeutic-device combination products.

Even the earliest drug delivery systems, first introduced in the 1970s, were based on polymers formed from lactic acid. Since the field of controlled drug delivery emerged, continuously increasing numbers of scientists in academia and industry have adopted the challenge of designing polymeric systems for the controlled, systematic, or site-specific release of pharmaceutical agents. Many approaches have been adopted in attempts to obtain the optimal drug delivery device [40], including diffusion-controlled membranes (depot and monolithic systems), osmotic pumps, resorbable implants, hydrogels, ion-exchange materials, polymeric pro-drugs, and slowly dissolving matrices [40-43].

Today, polymeric materials still provide the most important avenues for research, primarily because of their ease of processing and the ability of researchers to readily control their chemical and physical properties via molecular synthesis.

The types of polymer used for controlled release can be biodegradable, non-biodegradable, non-biodegradable and soluble As drug-carriers, these polymers exist in the form of mlcrospheres, matrices and membranes They can be administered via the parenteral, mucosal, oral, tropical and transdermal routes (Figure 8).

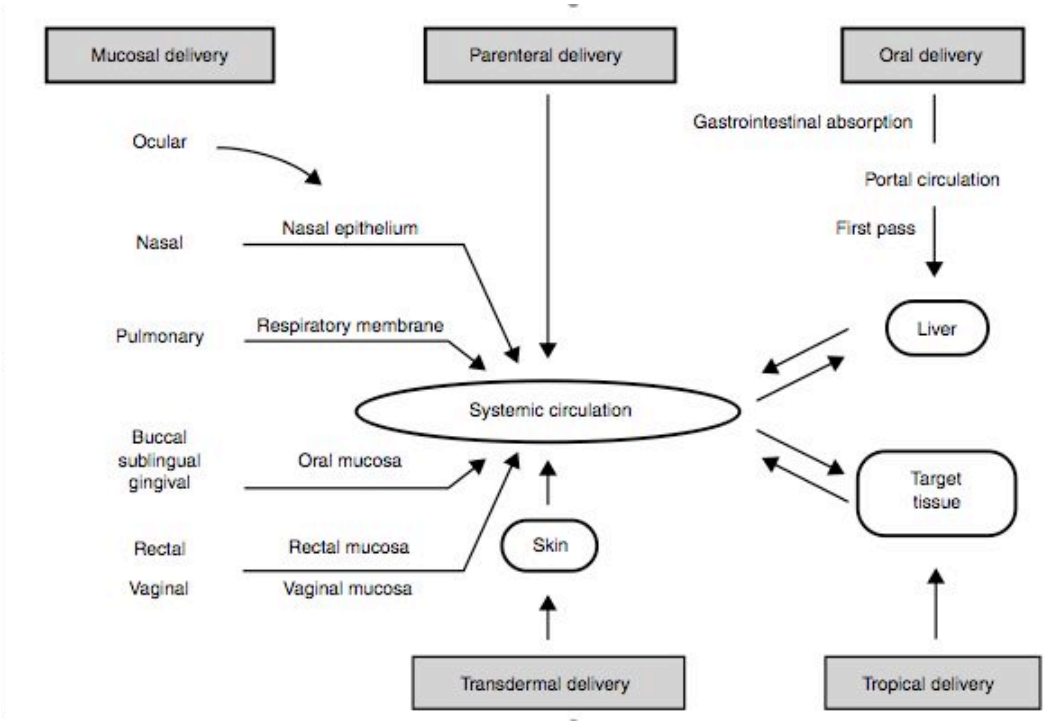


Figure 8: Different routes of Drug delivery

Several mechanisms may be responsible for the overall release of a therapeutic agent

dispersed in a degradable polymer matrix [40, 44, 45]:

– Erosion of the polymer;

– Diffusion of the drug particles through the matrix;

– Dissolution of the drug in the surrounding medium.

Drug molecules entrapped within a degradable polymer matrix will be liberated and released, as degraded material is lost from the matrix by erosion. Concomitantly, the concentration gradient is a driving force for the diffusion of drug molecules from the matrix to the surrounding medium. The third mechanism is most significant in the initial stages of incubation when drug molecules deposited on or near the matrix surface are lost by dissolution in the surrounding medium.

The relative importance of these mechanisms for the overall release rate varies considerably from one system to another, depending on the polymer hydrophilicity, flexibility, degradation rate, molecular weight, crystallinity, and the matrix size, shape, and porosity [46]. For the vast majority of drug delivery systems, all mechanisms contribute to the overall release of drug, albeit with varying proportions [44]. The kinetics of drug release are also influenced by the physical properties of the drug, particularly its molecular weight and solubility in water. Diffusivity of a drug through a polymer barrier is dependent upon the solubility of the drug in the polymer, the size of the drug molecule, and its distribution throughout the matrix.

The trend in polymeric drug delivery technology has been toward biodegradable polymer excipients requiring no follow up surgical removal once the drug supply is depleted. Biodegradable systems have garnered much of the recent attention and development in drug delivery systems because nonbiodegradable systems need retrieval or further

manipulation after introduction into the body. The use of biodegradable polymers has generally been favored over biostable polymers, since degradation of the matrices eliminates the need for surgical removal of the device after depletion [47].

Although the number of biodegradable polymers is large, only a limited number of polymers are suitable for drug delivery applications. Suitable candidates must not only be biodegradable but also fit the high prerequisites of biocompatibility. In addition, a polymer should ideally offer processability, sterilizability, and storage stability if it is to be useful for biomedical applications [48].

Polymers mainly investigated for drug delivery applications are of either natural or synthetic origin. The former group includes:

– Polysaccharides, e.g., alginate, dextran or cellulose [49-51];

– Chitin [52];

– Chitosan [52, 53];

– Proteins (e.g., collagen, fibrin, gelatin, albumin) [54, 55].

Synthetic degradable polymers investigated for controlled drug delivery applications include:

– Aliphatic polyesters;

– Poly(glycolide), PGA [56]

– Poly(lactide), PLA [57];

– Poly(glycolideco-lactide), PLGA [56-60];

– Poly(e-caprolactone), PCL [47, 61];

– Poly(3-hydroxybutyrate), PHB [62, 63];

– Poly(3-hydroxybutyrateco-3-hydroxyvalerate), P(HBco-HV); [62-64],

– Polyanhydrides [65-68];

– Aliphatic polycarbonates [69, 70];

– Poly(orthoesters) [71, 72];

– Poly(amino acids) [73];

– Poly(ethylene oxide) [74];

– Polyphosphazenes [75].

Many efforts have been made to obtain new polymer systems having the desired mechanical and physicochemical properties for a specific medical application.

2.3.1 Poly (lactic acid), Poly (glycolic acid), and Their Copolymers

The aliphatic polyesters based on lactic and glycolic acids are the most widely investigated biodegradable excipients for controlled drug delivery. Poly (esters) based on poly (lactic acid) (PLA), poly- (glycolic acid) (PGA), and their copolymers, poly(lactic acid-co-glycolic acid) (PLGA), offer a wide range of rates and duration of drug release, which makes them the most versatile polymers in drug delivery applications. A broad spectrum of characteristics with the polylactides can be obtained by careful manipulation of four key variables: monomer stereochemistry, comonomer ratio, polymer chain linearity and polymer molecular weight. Biodegradation of these aliphatic polyesters

occurs by bulk erosion. The lactide/glycolide polymer chains are cleaved by hydrolysis to the monomeric acids and are eliminated from the body through the Krebs cycle, primarily as carbon dioxide and in urine. Crystallinity and water uptake are the key factors in determining the rates of in vivo degradation [76].

PGA is commonly obtained by ring-opening polymerization of the cyclic diester of glycolic acid, glycolide [77, 78]. PGA is a hard, tough, crystalline polymer with a melting temperature of 225 °C and a glass transition temperature, Tg, of 36 °C [77]. PLA has gained widespread application in the medical field, for use in sutures [79], drug delivery devices [26-30], prosthetics, scaffolds, vascular grafts, and bone screws, pins and plates for temporary internal fracture fixation. Good mechanical properties and the fact that it degrades into non-toxic products explain the popularity of PLA [57, 80].

PLA has been investigated for the systematic delivery of a broad variety of therapeutic agents. Early reports include the use of PLA for the delivery of contraceptive steroids [81], narcotic antagonists [82], and antimalarial agents [83]. Zero-order release of L-methadone (a narcotic antagonist) was obtained when using a mixed matrix composed of PLA, P(LA-co-CL), and PLGA [84]. Macromolecular bioactive substances may also be encapsulated into and released from PLA matrices. The delivery of proteins from PLLA and PDLLA microspheres [85] and the delivery of DNA from PLLA microspheres have been presented [86].

Copolymerization of glycolide and lactide has been widely utilized to engineer the properties of PGA and PLA (PLLA or PDLLA) [57] . PLGA is less stiff than the original components, since the crystallinity decreases with an increase in the content of either comonomer. Non-steroidal anti-inflammatory drugs, e.g., diflunisal [87] and diclofenac sodium [88], have been incorporated into PLGA microspheres and investigated for the treatment of rheumatoid arthritis, osteoarthritis, and related diseases. Several peptides, including vapreotide and rismorelin porcine, have been successfully incorporated and released from PLGA microspheres [89, 90]. PLGA has also been investigated for the treatment of schizophrenia. Microspheres prepared from PLGA and PLGA/PCL blends were loaded with nerve-growth factors and ovalbulmin, intended for the treatment of central nervous system injuries [91]. PLGA was also used as a component in a system intended for the prolonged release of doxycyclinehyclate to periodontal pockets [92] .

2.3.2 Polycaprolactone (PCL)

The successful use of polymers of lactic acid and glycolic acid as biodegradable drug delivery systems led naturally to an evaluation of related polyesters in the search for new degradable polymers in similar applications. PCL was recognized as a biodegradable and nontoxic material.

PCL is obtained by ring-opening polymerization of the 6-membered lactone, e-caprolactone (e-CL). The homopolymer itself is degraded very slowly when compared with PGA and PLGA, and is most suitable for long-term delivery systems such as

Capronot, a 1-year contraceptive. The range of PCL properties can be extended by copolymerization with many other lactones, such as glycolide, lactide, d-valerolactone, e-decalactone, poly(ethylene oxide), and alkyl-substituted e-CL [93, 94] .

Nitrofurantoin, an antibacterial agent used in the treatment of urinary tract infections, has been incorporated into PCL microspheres [61]. PCL copolymers have been considered useful for androgen replacement therapy in the treatment of aging men with a testosterone deficiency.

2.3.3 Polyanhydrides

To maximize control over the release process,, it has generally been considered to have a polymeric system which degrades only from the surface. To obtain a device that erodes heterogeneously, the polymer should be hydrophobic yet contain water sensitive linkages. One type of polymer system that meets this requirement is the poly(anhydrides). Aliphatic polyanhydrides degrade within days or weeks while the erosion of aromatic polyanhydrides ranges from several months to years. The erosion time can thus be varied over a broad range by changes in the polymer backbone [95]. Poly (anhydrides) are best formed into drug-loaded devices by compression-molding or microencapsulation because of their high melting temperatures. A wide variety of drug and proteins, such as insulin, enzymes, and growth factors, have been incorporated into poly (anhydride) matrixes and their in vitro and in vivo release characteristics evaluated. [65-68].

Pharmaceutical research has to date been focused on polyanhydrides derived from sebacic acid (SA) and its copolymers with bis(p-carboxyphenoxy)propane (CPP) [96]. More recently, a new class of polyanhydrides was presented, containing fatty acid dimers (FAD) [97]. Erosion characteristics, microsphere preparation, pH-dependence, release rates, morphology, and in vivo performance of polyanhydrides from SA, CPP, and FAD have been intensely studied [95, 97, 98]. Polyanhydride devices for controlled delivery of local anesthetics [67] and chemotherapeutic agents [99] have been investigated.

Natural polymers are increasingly being studied for novel drug delivery systems because of their biocompatibility and biodegradability.

2.3.4 Collagen

Collagen is a potentially useful biomaterial since it is a major constituent of connective tissues. It is the most abundant mammalian protein accounting for around 30% of all body proteins [100]. Biomaterial made of collagen offers several advantages - it is biocompatible and non-toxic to most tissues; it has well-documented structural, physical, chemical, and immunological properties; and it can be readily isolated and purified in large quantities.

The attractiveness of collagen as a drug delivery system rests largely on the view that it is a natural material of low immunogenicity and is therefore seen by the body as a normal

constituent rather than foreign matter [101]. Collagen can be processed into a number of forms such as sheets, tubes, sponges, powders, fleeces, injectable solutions and dispersions, all of which have found use in medical practice [102]. Attempts have been made to apply these systems for drug delivery in a variety of applications such as ophthalmology, wound and burn dressing, tumor treatment, and tissue engineering (Table 2).

Application form	Drug	Indications
Ophthalmology		
Inserts	Pilocarpine, erythromycin, gentamicin	Infection, glaucoma
Shields	Gentamicin, vancomycin, tobramycin, netilmycin, polymyxin B sulfate, trimethoprim, amphotericin B, 5-FU, pilocarpine, steroids, flurbiprofene	Infection, mycosis, glaucoma, inflammation
	Model	N/A
Particles	Cyclosporine	Allograft implantation
Gels	Keterolac	Inflammation
Aqueous injection	Vinblastin, cisplatin, Tc, 5-FU, ^{111}In or ^{90}Y labeled monoclonal antibodies, TGF-β, fibroblast growth factor Insulin, growth hormone	Local cancer treatment, wound repair
Solid parenteral application		
Sponge	Gentamicin, cefotaxim, fusidic acid, clindamycin, all-*trans* retinoic acid	Infection, cervical dysplasia
	Growth factors, bone morphogenetic proteins	Tissue regeneration
Films	Medroxyprogesterone acetate, human growth hormone, immunostimulants, tetracycline, growth factors	
Monolithic devices	Minocycline, lysozyme, interleukin-2, interferon	
Microparticles	Retinol, tretinoin, tetracain, lidocain, ethacridine lactate,	Local anaesthesia, dermal

Table 2: Collagen drug delivery applications [103]

2.3.5 Chitosan

Chitosan is a polysaccharide comprising copolymers of glucosamine and N-acetylglucosamine and can be derived by partial deactylation of chitin from crustacean shells. Chitosan has been extensively examined in the drug delivery systems. This is due to it's unique polymeric cationic character and it's unique gel and film forming properties.

The susceptibility of chitosan to lysozyme makes it biodegradable and an ideal drug carrier [104]. Molecules such as bovine serum albumin, diphtheria toxoid (DT) [105]and bisphosphonates [106] have been successfully incorporated into Chitosan microspheres. Yao et al. [107] reported a procedure for the preparation of semi-IPN hydrogel based on glutaraldehyde-crosslinked chitosan with an interpenetrating polyether polymer network.

Thacharodi and Rao [108-110] reported permeation-controlled transdermal drug delivery systems (TDS) using chitosan. Hoffman et al synthesized hybrid copolymers by grafting temperature-responsive polymers (Pluronic®) to chitosan backbones. Modified chitosans were reported to display a growthinhibitory effect on tumor cells [111]. This property was employed by Ouchi et al. [112] by conjugating chitosan or chitosaminooligosaccharide (COS) to 5-fluorouracil (5FU) in order to provide a macromolecular system with strong antitumor activities and reduced side effects.

2.3.6 Alginate

A detailed description on alginate structure and properties has been covered in the previous sections.

The successful formulation of a stable and effective dosage form depends on the careful selection of excipients. The present trend points to an increasing interest in the use of natural ingredients in food, drugs, and cosmetics. The naturally occurring alginate polymers have a wide potential in drug formulation due to their extensive application as food additives and their recognized lack of toxicity. Alginates can be tailor-made to suit

the demands of applicants in both the pharmaceutical and biomedical areas. This group of polymers possesses a number of characteristics that makes it useful as a formulation aid, both as a conventional excipient and more specifically as a tool in polymeric drug delivery.

Alginate gel beads have been found to be most effective in retarding drugs at higher alginate concentrations and when the alginates are rich in guluronic acid [113]. The guluronic acid conformation gives a high degree of coordination of the calcium, and thereby forms more rigid gels that are less prone to swelling and erosion. By increasing the mannuronic acid content the gels become softer, more elastic, but less porous and they dissolve more easily. The situation may be different for drug molecules that strongly interact with alginate. Gentamicin sulfate was found to interact selectively on the mannuronic residues of alginate without competition with calcium ions involved in the polymer gelation. In this case a higher mannuronic acid content would lead to a higher binding capacity for drug molecules, and mannuronic-rich alginates may be preferred [114]. The drug: alginate ratio and calcium chloride concentration affect the drug release. The release of nicardipine from alginate particles prepared in a ratio of 1:1 was delayed more than that from 1:2 particles [115].

Encapsulation of cells or DNA in the alginate matrix is another field of growing interest. Alginate has several unique properties that have enabled it to be used as a matrix for the entrapment and/or delivery of biomolecules like DNA, proteins, and cells. A relatively mild gelation process free of organic solvents enables biomolecules and cells to be

incorporated into the matrices with the retention of the three-dimensional structure (i.e., full biological activity). The aqueous environment within the matrix is quite inert, and may consist of distilled water or sucrose solutions [116, 117]. The porosity of the gel allows for acceptable diffusion rates of macromolecules or low molecular weight drugs bound to macromolecules [118].

A large number of proteins have been encapsulated in alginate microbeads [119-122]. Positively charged proteins can potentially compete with calcium ion for available carboxylic acid sites on the alginates, resulting in a reduction in diffusion rate or protein inactivation.

Vascular Endothelial Growth Factor (VEGF) has been incorporated into ionically cross-linked alginate hydrogels [123-126]. It has been pointed out that the bioactivity of VEGF delivered from alginate microspheres was greater than that obtained when VEGF was administered without the microspheres, the effect being due to the stabilization of the growth factor via an alginate interaction. The efficacy of this system in driving the angiogenesis around the implant site has been demonstrated both in vitro and in vivo [127].

2.4 Influenza

Influenza, commonly known as flu, is a highly infectious respiratory viral infection caused by influenza viruses, which are RNA virus of the family Orthomyxoviridae. The influenza virus was first isolated and identified in the UK in 1933 [128]. There are three types of influenza virus: A, B and C. Influenza A and B viruses cause virtually all of the clinical illness. The symptoms of influenza C infection are usually mild. The three types differ in the antigenic makeup of their nuclear and matrix proteins. Influenza A and C infect multiple species, while influenza B almost exclusively infects humans [129]. The type A viruses are the most virulent human pathogens and are responsible for major pandemics and outbreaks of disease, whereas type B viruses cause outbreaks of more limited scope and severity

Each year in winter epidemics about 10% to 20% of the US population is infected with flu, responsible for >200,000 annual hospitalizations and 20,000 to 40,000 infleunza associated deaths annually, principally in the elderly [130-134]. Morbidity and mortality rates go very high during pandemics. Records show that the infection rates have risen to about 70% during pandemics [135].

2.4.1 Structure of Influenza A Virus

On initial isolation, influenza A viruses are small (80 to 120 nm in diameter), pleomorphic particles that later become generally spherical [136].

The genome of influenza A viruses consists of eight unique segments of single-stranded RNA, which are of negative polarity (i.e., complementary to the mRNA sense). The RNA is loosely encapsidated by multiple NP molecules. Complexes containing the three viral polymerase proteins (PB1, PB2, and PA) are situated at the ends of the nucleocapsids. To be infectious, a single virus particle must contain each of the eight unique RNA segments [137, 138].

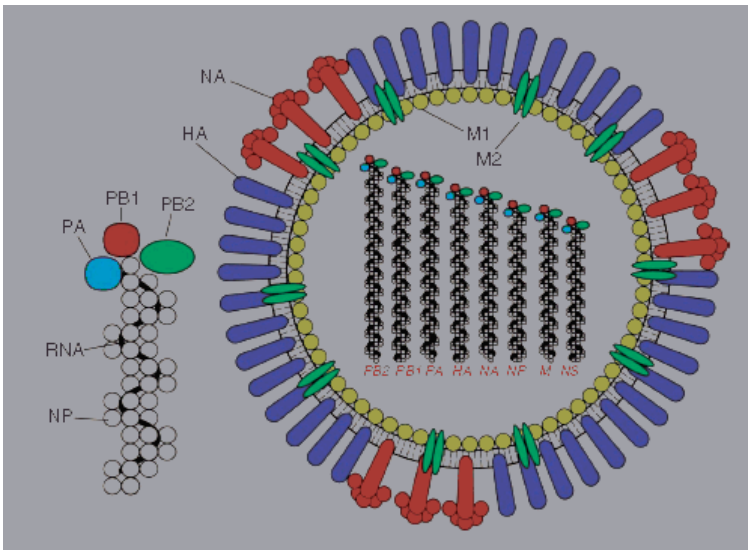


Figure 9: A diagrammatic representation of influenza A virus [137]

The eight influenza A viral RNA segments encode 10 recognized gene products. These are PB1, PB2, and PA polymerases, HA, NP, NA, Ml and M2 proteins, and NS1 and NS2 proteins [137].

HA is the most important antigen of the virus and is made up of two polypeptides, HAl and HA2. These two constituents of HA were separated and studied by peptide mapping, when it was discovered that antigenic change occurred in the HAl molecule almost entirely. It was also found out that the peptide maps of the HAs of different subtypes of influenza A were completely different; each was independent and could not transform into other subtypes by mutation [139]. Gene shuffling is probably the technique adopted by influenza A virus to reinvent itself as a new pandemic virus, utilizing the reservoir of 15 HAs and 9 NAs residing in birds, horses, pigs, seals and whales [140].

2.5 Hemagglutination assay

Hemagglutination (HA) assay is a well-known method for the quantification of viruses [140]. This assay takes advantage of the fact that many viruses contain proteins that can agglutinate (stick to) red blood cells (erythrocytes) and bind to its N-acetylneuraminic acid. Normally, red blood cells will fall to the bottom of a culture well, forming a sharp dot. However, if viruses are present, the red cells become bound to the virus particles in a lattice or network (this happens because a single virus can bind multiple red blood cells [141]. This lattice then coats the well. The assay is very simple and does not require any special equipment. It basically involves; serial dilution of a virus suspension into a 96 Well plate and then addition of a standard amount of red blood cells, and allowing them to sit for approx. 30 min, often at $4^{O}C$, because else viruses with neuraminidase activity

will detach the virus from the RBCs. Then the lattice forming parts are counted and an estimation of the quantity of virus present is made [142].

Hemagglutination assay has been used in the past for the quantification of influenza virus [143-147]. Hemagglutinin, a special surface protein of the influenza virus, causes red blood cells to clump together or hemagglutinate [142]. HA assay is only dependent on the amount of □emagglutinin on the surface of influenza viruses and not the ability of virus to replicate; therefore this assay quantifies viral particles regardless of their infectivity. Hemagglutination test depends on virus surface integrity, and thus if a virus which has been released from an encapsulated hydrogel shows hemagglutination, it will suggest that the virus integrity was preserved in the hydrogel.

Therefore we will be using hemagglutination assay, to test the interaction between the internal environment of the different hydrogels and the influenza virus, to see if hydrogel causes any change in its surface integrity.

3. Aim and Scope

3.1 Aim

The goal of the thesis is to prepare a fast release hydrogel with thixotropic properties. The hydrogel formed should be injectable from a 26G needle, without any phase separation during extrusion. This hydrogel should be able to encapsulate a biomolecule such as a live virus without affecting the properties of that molecule and enable release demonstrating the stability of the biomolecule. In my thesis I have encapsulated Live Influenza Virus and virus was tested for potency using an in vitro assay, the hemagglutination test.

3.2 Challenges

As discussed in the aim, this project involves preparation of a fast release hydrogel with good extrusion consistency from a 26G needle. The major challenge is to incorporate two antagonistic properties in the same gel; liquid – like gels tend to release encapsulated materials faster due to low crosslink density, however have poor extrusion consistency through syringe needles. High crosslinking density materials extrude with high consistency and exhibit no phase separation, but demonstrate slow release. Slow release is undesirable as the active biomolecule, such as the virus, becomes inactive with time.

One condition to make sure that the gel comes out of the syringe with good consistency without phase separation necessitates making a firm gel with high crosslinking density.

However, this very same property will reduce the pore size of the hydrogel and thus decrease or inhibit the release of virus from the gel after extrusion. On the other hand, if we try to prepare a fast release hydrogel, we must decrease the amount of crosslinking and this would lead to poor extrusion consistency and phase separation while extrusion.

So the objective is to select an optimum hydrogel, which is firm enough to be extruded from the 26G needle with good consistency (comes out without bleeding) and porous enough to release the virus. Another approach could be to find a hydrogel which can be extruded from a 26G needle without bleeding and which can dissolve/degrade fast in a limited quantity of fluid (water). As ultimately the gel needs to be extruded transdermally under the skin, where they is very limited quantity of body fluid available and if we manage to prepare a fast degradable hydrogel, it will as well satisfy the fast release condition.

The model biomolecule we have chosen in this project is the Influenza virus. Our goal is to release enough virus particles so as to produce equal hemagglutination as that obtained by adding Direct Virus.

This project was carried out in three stages for selecting the optimum hydrogel formulation to finally progress to an animal study. The extrusion characteristics of the hydrogel will be tested by the Extrusion consistency Test. The general release characteristics will be tested by studying the release of 200 nm fluorescence particles. Finally to prove that the gel causes no harm to biomolecules i.e. they don't loose their potency when encapsulated, we will perform the Virus release/Hemagglutination Study.

These stages are described in the flow chart on Figure 10. The details of each stage will be discussed in detail in further sections.

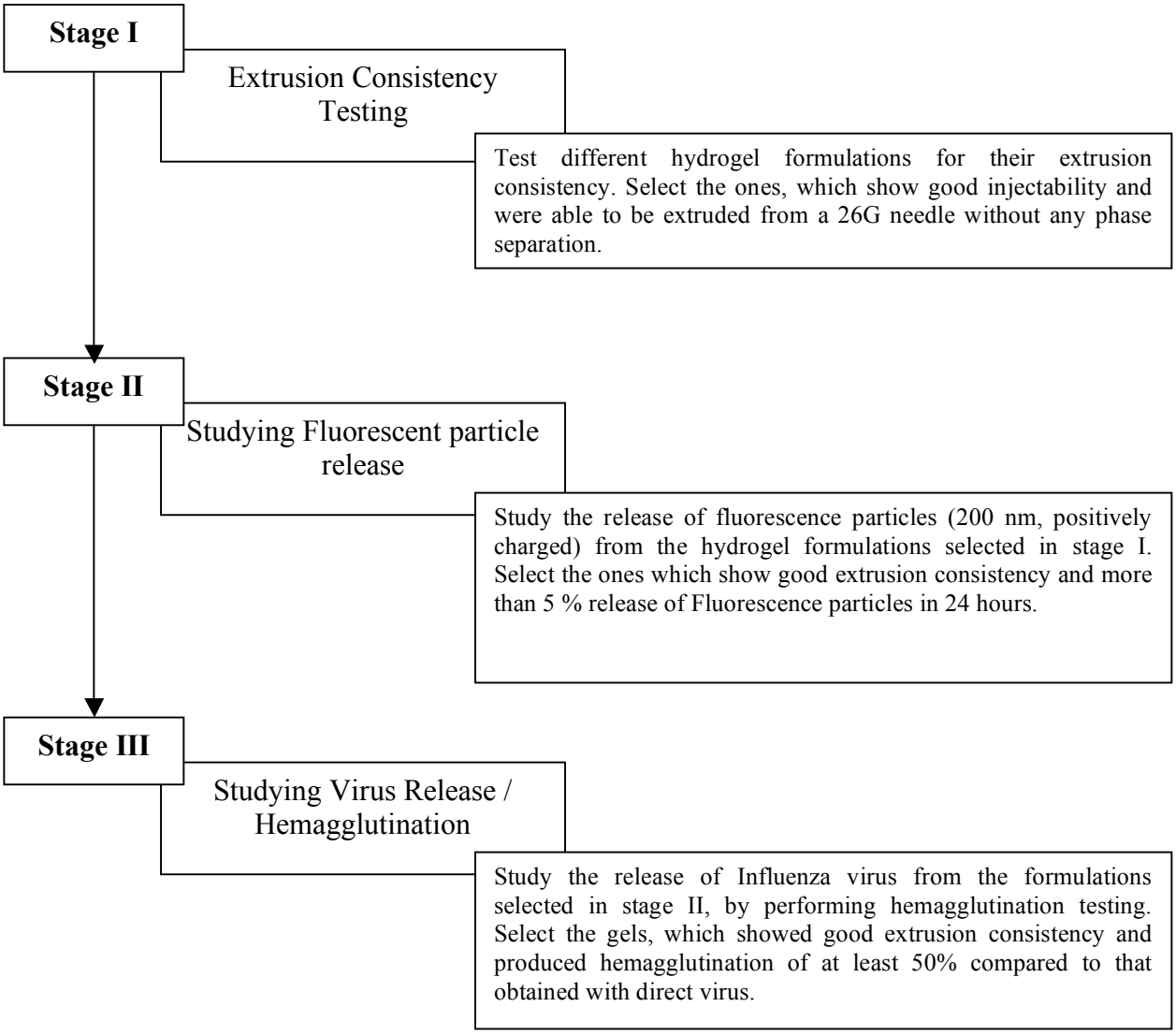


Figure 10: Flowchart explaining the different stages of selection of hydrogels

3.3 Three different approaches to obtain the desired hydrogel

Many different hydrogels are being used for the encapsulation of immunizing agents [148-153]. However it must be pointed out that the mechanisms involved in antigen uptake and presentation after the injection of the hydrogel are not fully understood. Therefore, the selection of the appropriate characteristics of the hydrogel designed to perform the desired task is not very simple.

In this particular project, we need a fast release thixotropic hydrogel. Towards achieving this aim we have tried three different approaches, two of them involve alginates, but with different sources of calcium salt, and the third one is a supramolecular hydrogel based on PEO and cyclodextrins.

3.3.1. First Approach

Use of alginate hydrogels using CaSO4 as crosslinking catalyst

Alginate is one of the most widely used hydrogels for the encapsulation of different types of bioactive agents [154-157]. Alginate is a polysaccharide obtained from brown algae and is composed of guluronic and mannuronic acid residues varying in proportions and molecular weight [158].

Among many interesting characteristics which turns alginate very attractive as a polymer for encapsulating biologically active materials, its mucoadhesive properties, stability to proteolytic and acid conditions, biodegradability, low toxicity, availability and relatively low cost when compared to other hydrogel matrixes can be pointed out [15, 159, 160].

In solution alginate behaves like a viscous liquid, however in the presence of divalent cations (i.e., calcium) alginate is crosslinked and forms a gel [14]. Mild gel formation conditions are involved, consisting of cross-linking the guluronic acid units from alginate with divalent metallic ions such as calcium. The relatively mild gelation process enables proteins, cells, DNA and virus to be incorporated into alginate matrices with retention of complete biological activity [156, 157]. All the above characteristics of alginate made it our first choice for encapsulating virus.

3.3.1.1. Selection of gelation method

Alginate hydrogels can be formed by two different methods, diffusion setting (external gelation) or internal setting (internal gelation).

External Gelation is characterized by fast gelation, and usually involves extrusion of droplets of sodium alginate-active agent solution into a calcium salt solution. The calcium ions diffuse into the mix containing alginate forming a calcium alginate gel when the calcium ions react with the alginate. The calcium salt used in this case must be highly soluble in water, and thus calcium chloride is used most often. This method mainly leads to the formation of alginate microspheres with encapsulated bioactive agent. The microspheres are not usually thixotropic i.e. if extruded through a needle they usually burst out with the release of water inside.

Internal gelation on the other hand involves slow diffusion of calcium ions into the alginate molecules. A very sparsely soluble calcium salt is used, and the calcium ions are made available for gelation in a very controlled fashion. The rate at which the calcium is made available to the alginate molecules depends primarily on pH and the amount, particle size and intrinsic solubility characteristics of the calcium salt. . Calcium sulfate (usually as the dihydrate), gypsum, and dicalcium phosphate (calcium hydrogen orthophosphate) are the sources of calcium most commonly used. In most cases a calcium sequestrant is used to control the reaction by competing with the alginate for calcium ions. This method finally leads to the formation of homogenous, thixotropic hydrogel, which can thus be injected through a 26G needle.

Comparing the characteristics of the two different gelation techniques, we decided to use the internal gelation method, since it produces a homogenous, thixotropic calcium alginate hydrogel, which promises to be injectable through a 26G needle with excellent consistency and no bleeding.

3.3.1.2. Using a mixture of High M and High G Alginate polymers to form hydrogels

Gelling occurs when the divalent cations take part in the interchain ionic binding between guluronic acids blocks (G-blocks) in the polymer chain giving rise to a three dimensional network. The high affinity of polyguluronic acid regions for Ca 2+ is attributed to the presence of electronegative cavities formed by adjacent guluronic acid residues in the polymer chain which are of a suitable size for the chelation of Ca 2+. The Ca 2+ are held in the junction zones between alginate molecules in solution [14] and these junction zones are essentially formed by a dimerisation of polyguluronic acid blocks [161]. Thus alginates with a high content of G-blocks induce stronger gels.

Gels made of M-rich alginate are softer and more fragile, and may also have a lower porosity. This is due to the lower binding strength between the polymer chains and to the higher flexibilities of the molecules [162, 163].

Thus we see that the physical properties of alginate hydrogels vary widely depending on their composition i.e. the proportion of guluronic to mannuronic acid residues.

We can summarize it as; alginates with a high % of guluronic acid develop firm, more porous gels, which can maintain their integrity for longer periods of time. This property helps the alginate to be injected through a very small diameter needle without losing its integrity or without phase separation. On the other hand, alginates with a high % of mannuronic acid develop softer and fragile gels that tend to disintegrate easily. This property will help the alginate with high M to dissolve quickly in a solution as it forms a very loosely bonded gel. Alginates with a high mannuronic acid content are also plagued by a high degree of swelling and shrinking during cationic cross-linking [164].

Based on the above knowledge, we decided to prepare hydrogels made from a mixture of High G and High M alginate polymers. This new mixture hydrogel will allow us to reach the desired properties required in our hydrogel. The High G alginate will make sure the gel formed is firm enough to be extruded through a needle without losing its integrity, where as the High M alginate will help in making the gel more fragile and thus disintegrate or dissolve quickly in limited quantity of fluid and also induce a high degree of swelling, both of which will help in faster release of the encapsulated bio-active agent.

3.3.2. Second Approach

Use of Supramolecular Hydrogels

Li et al have reported a supramolecular hydrogel based on PEO and α-cyclodextrins [33]. α-Cyclodextrin is a cyclic oligosaccharide composed of 6 D (+) glucose units linked by α-1,4-linkages. When PEO; a water-soluble biocompatible synthetic polymer, is brought in contact with α-Cyclodextrin it penetrates the inner cavity of cyclodextrins to form gelatinous complexes where the PEO chains are partially included by α-CD cavities. The gelation is induced by the supramolecular self-assembly of the partially included PEO chains and α-CD molecules in aqueous solution. The hydrogel formation only involves physical crosslinking [24, 165, 166]. The formation of inclusion complex between PEO and α cyclodextrins and their supramolecular self-assembly in the hydrogels were confirmed with wide-angle X-ray diffraction studies [33].

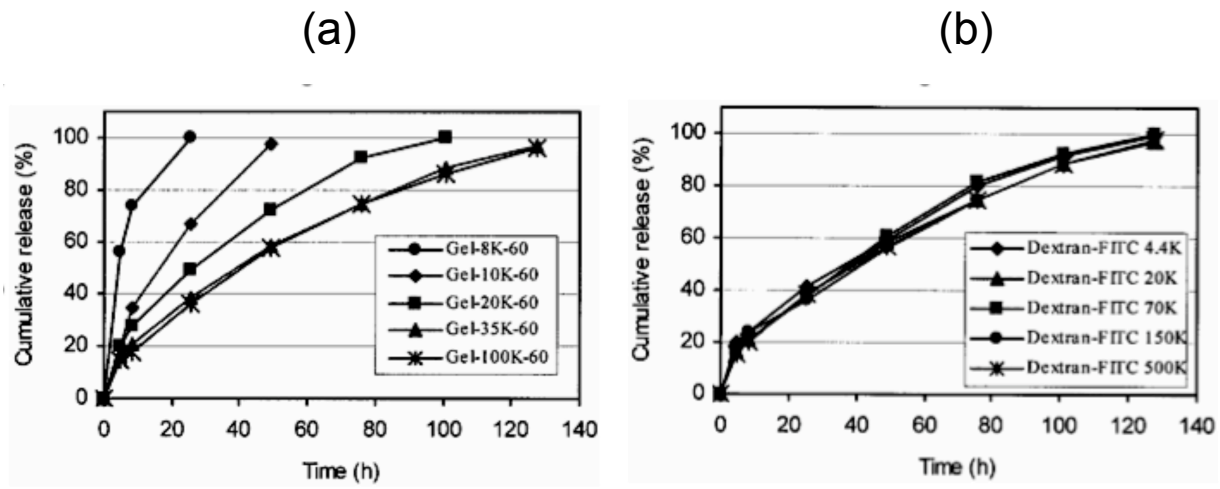


Figure 11: (a) In vitro release profiles for dextran-FITC (MW 20,000) released from _-CD–PEO hydrogels formed from PEO of different molecular weights. (b) In vitro release profiles for dextran-FITC of different molecular weights between 4400 and 500,000 released from Gel-35K-60. (33)

These supramolecular hydrogels as reported by Li et al were found to have thixotropic and reversible properties. This particular property induced the hydrogels to be injectable through fine needles. They have been successfully shown to release through needle diameter as small as 27G [33].

Release studies of Fluorescein isothiocyanate-labeled dextran (dextran-FITC) of different molecular weights between 4400 and 500,000 (Figure 11), suggested that the diffusion of dextran-FITC through the gel was not the dominant factor in controlling the release, but it was basically the degradation of gel via the de-threading of the PEO chains from the cyclodextrin cavities which was affecting the release. This particular property is very helpful as the release kinetics of any encapsulated bioactive agent would be very less dependent on its own physical or chemical properties [33].

All the above mentioned properties including physical crosslinking, simple process for encapsulation of bioactive agent without any contact with organic solvents, thixotropic and reversible properties, inducing injectability through fine needles and erosion controlled release of agents, made them ideal for the encapsulation and release of virus in our project.

3.3.3. Third Approach

Use of reverse addition crosslinking method for Alginate hydrogel with CaCL2 as the crosslinking catalyst

Alginate gel preparation usually involves addition of calcium salt to a preformed or already existing solution of alginate. In internal setting method of alginate gelation (which is used in the first approach), a very slightly soluble calcium salt is used. The reactions proceeds very slowly and the final product is a jelly-like hydrogel. A major problem with the methods using slightly soluble calcium salts is that the crosslinking continues for days, which thereby causes the viscosity to increase continuously.

One approach to solve this problem can be, to use a highly soluble calcium salt such as CaCL2 in the gel preparation. Steiner et al have reported a unique method for alginate hydrogel preparation that involves CaCl2 [167]. Their method of gel preparation is different from the internal setting method in two ways; first they have used a readily soluble rather than slightly soluble calcium salt and second in this method we have to add the alginate in dry form to the salt solution instead of adding salt to the alginate solution.

The gel is formed as rapidly as the alginate can pass into the solution, and the gel achieves complete crosslinking immediately, in the brief time required for the alginate to pass into the solution [167]. The final gel formed is free from lumps and granularity. One very important property of this gel is that, it does not bleed after spreading which will be very helpful in our project, as this gel will not show phase separation (release water)

when extruded through fine needles. Also this gel can be used to encapsulate bioactive agents by adding the agent to the calcium salt solution before adding the alginate dry powder to it. Once all the alginate molecules have passed into the solution, the materials will be encapsulated in the polymer matrix of the hydrogel.

The above mentioned special characteristics of the gel prepared using the unique method of preparation proposed by Steiner et al, made it a very suitable candidate for our project and therefore we decided to test the consistency and release properties of this gel as our third approach.

4. Materials and Methods

4.1. Synthesis of the hydrogel

4.1.1. First Approach: - Alginate hydrogel prepared by internal setting method using CaSO4

As discussed in the background, internal setting method of alginate hydrogel preparation involves controlled release of Calcium ions into alginate molecules [22]. Release was controlled in two ways, first by using a low solubility calcium salt, CaSO4 and second by using a calcium sequestrant, Trisodium Metaphosphate (TSMP). Table below compares the solubility values of the four most commonly used calcium salts in alginate hydrogel preparation.

Calcium Salt	**Solubility** g/100 mL of H_20
Calcium Chloride	81.3
Calcium Sulphate	0.205
Calcium Citrate	0.096
Calcium Carbonate	0.00066

The general protocol for calcium alginate hydrogel preparation is as follows:

i. Alginate solution of required concentration is prepared in distilled water

ii. 500 uL of the alginate solution is transferred to a 1.5 ml eppendorf tube

iii. 10 uL of 10% TSMP is added to the alginate solution in the eppendorf tube

iv. The mixture is vortexed thoroughly on a vortexer.

v. Calculated amount of known concentration CaSO4 is added to the eppendorf tube solution to achieve a desired final CaSO4 concentration

vi. The whole mixture is again vortexed thoroughly for about 1 minute, soon after addition of CaSO4

vii. The solution from the eppendorf tube was transferred to a 1 ml syringe, fitted with a 26G ½ needle.

viii. The gel in the syringe was then allowed to cure at $4^{O}C$ for 24, 48 or 72 hours.

Table 3 shows the general Outer diameter (O.D) and Inner Diameter (I.D) of needles of different gauge.

Needles of 26G are generally preferred in vaccination.

Gauge	**O.D**	**I.D**
30	0.31	0.15
27	0.41	0.2
26	0.46	0.25
25	0.51	0.25
23	0.64	0.33
22	0.71	0.41
21	0.81	0.51
20	0.91	0.58
19	1.07	0.69
18	1.27	0.84

Table 3: Inner and outer diameter for needles of different Gauge

4.1.2. Second approach: - Supramolecular Hydrogels made of PEO and Cyclodextrins

Cyclodextrin is a cyclic polymer of alpha-D-glucopyranose composed of 6, 7, and 8 d (+)-glucose units linked by α-1, 4-linkages, and named α -, β and γ-CD, respectively. [21,22] It has been in shown that linear polymers, such as poly (ethylene oxide) (PEO), can penetrate the inner cavity of cyclodextrins to form inclusion complexes with necklace-like supramolecular structures [23-25] and this unique arrangement results in the formation of a hydrogel. The hydrogel formation is based on physical crosslinking induced by supramolecular self-assembling with no chemical crosslinking reagent involved [28].

The general protocol for supramolecular hydrogel preparation goes in this order:

i. Required concentrations of α-CD and PEO solutions are prepared in distilled water.

ii. Known amounts of aqueous α-CD and PEO solutions are mixed to achieve the desired final α-CD and PEO concentrations followed by vigorous stirring and then sonication for 1 minute.

iii. The mixture is then incubated in a 40°C water bath for 1 hour.

iv. The solution is transferred to a 1 ml syringe, fitted with a 26G needle

v. The hydrogel is allowed to cure at 4^{O}C for 12 hours.

4.1.3. Third approach: - Use of reverse addition crosslinking method for Alginate hydrogel with CaCL2 as the crosslinking catalyst

Fast and complete crosslinking with no bleeding while extrusion through 26G needles are the important properties of the gel, we selected for our third approach. This gel is prepared by using Alginate and Calcium Chloride via a unique method proposed by Steiner et al [73].

The general protocol for our third hydrogel called as alginate hydrogel paste goes like this,

i. Calcium chloride solution of a desired concentration is prepared.
ii. A required amount of pre-weighed alginate powder is slowly added to a known volume of CaCL2 solution prepared in step 1, to achieve a final desired concentration.
iii. The whole mixture is vortexed thoroughly for about 5 minutes
iv. The mixture is then incubated for 2 hours at $4^{O}C$.
v. The gel is ready after the two hours curing period.

4.2. Qualitative Extrusion Consistency Test

The extrusion consistency test was performed to make sure the hydrogels we select for the nanoparticle release studies and finally the Virus release studies are injectable from a 26G needle without bleeding (release of water / Phase separation).

The test involved manual extrusion of the different hydrogels prepared, and we assigned a specific consistency score based on visual observation. The scores ranged from 1 to 5, representing bad to excellent consistency.

The criterion for each particular score is explained in Table 4 below,

Consistency Score	Observation after manual extrusion from a 26G needle
1	No Gelation at all. Bad Consistency.
2	Partial Gelation and lot of bleeding.
3	Water in the system though completely gelled and showed good consistency for the rest of the part.
4	Only about 3-4 drops of water, with good consistency for the rest of the part.
5	No bleeding at all. Excellent consistency

Table 4: Scoring criterion for Extrusion Consistency

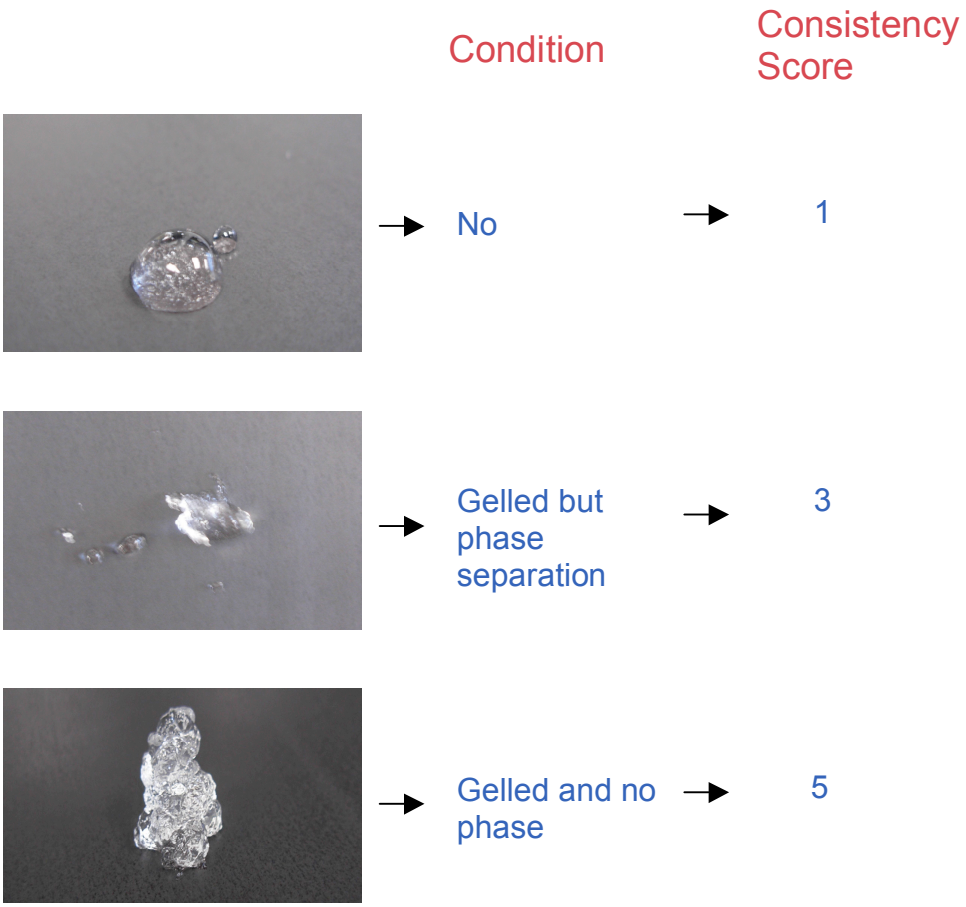


Figure 12: Pictures of three different alginate hydrogel formulations after extrusion through 26 G needles.

Figure 12 shows the pictures of three alginate-CaSO4 formulations, after extrusion through 26G needles. The first picture represents a formulation, which can be assigned a consistency score of 1, as there was no gel formation and the formulation came out like a solution. The second picture represents a formulation, which was gelled but still showed some phase separation during extrusion. Hence it was assigned a score of 3. The third picture represents a formulation, which was completely gelled and showed absolutely no phase separation or bleeding during extrusion.

4.3. Release of Fluorescent Nanoparticles

This study was designed to test the release of 200 nm fluorescent particles from the hydrogels which show an extrusion consistency score better than "4". For this nanoparticle release study we specifically chose the 200 nm amine modified fluorescent particles because its size and charge closely match to the Influenza virus, which was used as a model biomolecule in the virus release studies. Nanoparticle release study involves encapsulation of nanoparticles and then release study in HBSS 1X, medium.

4.3.1. Encapsulation of fluorescent nanoparticles (FNP)

The nanoparticle release study first involves the encapsulation of nanoparticles in the hydrogels. The encapsulation process varies slightly for each approach and is individually explained in the following section.

4.3.1.1. First Approach

The gels were prepared exactly in the same fashion as explained in the synthesis section. The only modification in that protocol was the addition of 200nm amine modified fluorescent nanoparticles to the alginate solution. After the addition of required amount of nanoparticles, TSMP was added followed by the addition of CaSO4. The mixture was then transferred to a syringe and allowed to cure for 24, 48 or 72 hours at $4^{O}C$.

4.3.1.2. Second Approach

The gels were prepared exactly in the same fashion as explained in the synthesis section. The only modification in that protocol was the addition of 200nm amine modified fluorescent nanoparticles to the mixture of aqueous solutions of α-CD and PEO. The whole mixture is then thoroughly and the regular procedure is followed for hydrogel preparation.

4.3.1.3. Third Approach

The gels were prepared exactly in the same fashion as explained in the synthesis section. The only modification in that protocol was the addition of 200nm amine modified fluorescent nanoparticles to the $CaCl_2$ solution. The mixture was then vortexed thoroughly, followed by the addition of required amount of alginate powder.

4.3.2. General Protocol for Release study

Once the gel was cured, 200 uL of the gel was extruded in a 1.5 ml centrifuge tube filled with 1 ml of HBSS, 1X buffer. The centrifuge tubes were incubated for 24 hours at 37 ^{o}C. After the incubation period, the tubes were centrifuged for 10 min at 2000 RPM, to allow all the undissolved gel to settle down at the bottom. Three 150-uL aliquots were sampled out from the supernatant and their fluorescence intensity was measured on the FLx800 Fluorescence spectrophotometer. You may need to show the calibration curve for this spectrophotometer.

Below is a schematic representation of the Nanoparticle release study showing each step one at time, using an example of alginate hydrogel, of approach 1. It starts with Encapsulation of Nanoparticles in the hydrogel followed by Extrusion in HBSS medium, Incubation at 37OC for 24 hours, Centrifugation and finally Measurement of Fluorescence intensity.

4.4. Virus Release Study and Hemagglutination test

This study was designed to test the release of virus from the hydrogels. The released virus was then tested for its surface integrity and ability to infect red blood cells by performing a hemagglutination assay. Hemagglutination assay (HA) is a well-known method for the quantification of viruses.

This study involves encapsulation of virus, followed by extrusion of gel on DMEM medium and finally the HA. A summary of the hemagglutination test is included in Background and Literature survey Section. For the HA study we will only select the gels which have achieved release of the amine modified 200 nm fluorescent nanoparticles in 24 hours. Since the nanoparticles closely match the influenza viral particles in their dimension and charge, we expect a similar release profile for the virus. Thus if the DMEM medium shows good hemagglutination it will suggest that the viral particles can be released from the encapsulated hydrogel in time to infect the cells and maintained their surface integrity. This would further suggest that the internal environment of the hydrogel is compatible with the virus at least to some degree. Comment: If you observe 10% HA

versus naked virus you do not know if 20% of virus is released but it is 50% as affective. What you are actually testing is a combined effect of release and degradation.

This test is performed in two stages, first the preparation of virus encapsulated hydrogels and second, the virus release study in DMEM medium which is followed by a hemagglutination test of the medium.

4.4.1. Encapsulation of Virus

The encapsulation process varies slightly for each approach and is individually explained in the following sections.

4.4.1.1. First Approach

During the preparation of the gel, the influenza virus was added to the alginate solution before adding the other ingredients to it. After the addition of required amount of virus, TSMP was added followed by the addition of CaSO4. The mixture was then transferred to a syringe and allowed to cure for 24, 48 or 72 hours at $4^{O}C$.

4.4.1.2. Second Approach

To encapsulate the influenza virus in the supramolecular hydrogel, the virus was addedto the mixture of aqueous solutions of α-CD and PEO. The whole mixture is then vortexed thoroughly and the regular procedure is followed for hydrogel preparation as mentioned in the synthesis section.

4.4.1.3. Third Approach

To encapsulate the influenza virus in the alginate-CaCl2 hydrogel, the virus was added to the $CaCl_2$ solution. The mixture was then vortexed thoroughly, followed by the addition of required amount of alginate powder.

4.4.2. General Protocol for Release study and Hemagglutination assay

This whole study is divided into two important parts, which are explained individually in the following sections

Part A:

In part A, we prepare MDCK cell suspension of concentration 0.25 million cells/mL. MDCK cells are an adherent cell line that supports the growth of various viruses including influenza viruses

The cell suspension is prepared in the following steps,

1. Trypsin-EDTA is removed from the freezer and placed in a water bath at 37OC.
2. One T-cell culture flask of MDCK cell suspension (which has cells in the late log phase of their growth cycle) is removed from the incubator and placed inside the hood.
3. Media is removed from the T-flask and disposed in a waste beaker
4. The cells are then washed with PBS, 1X (without Ca++ and Mg++)
5. PBS, 1X is removed from the T-Flask and disposed in a waste beaker
6. About 2.5 ml of Trypsin-EDTA is added to the T-Flask

7. The Flask is rocked gently to ensure that the entire monolayer of cells is covered with the trypsin solution.
8. The T-Flask is then incubated for 3-5 minutes at $37^{O}C$, where the cells begin to detach.
9. The Flask is banged with the hand to aid the removal of cells from the substrate
10. About 7-10 ml of DMEM medium (with 5% FBS) is added to stop the action of trypsin.
11. Steps 2-10 are repeated for one more T-Flask with MDCK cell suspension.
12. The cell suspensions from both the T-flask are then transferred to a 50 mL Falcon tube, and the suspension volume is made up to 50 mL by adding more DMEM medium (5 % FBS).
13. The Falcon tube is then centrifuged at 1500 RPM for 5 minutes
14. After centrifugation the supernatant is discarded in a waste beaker
15. About 5 mL of DMEM medium (5% FBS) is added on the cell pallet inside the falcon tube.
16. The cells are suspended using a pipette
17. 10mL of more medium is added to the falcon tube and the cells are suspended again, to make sure there are no lumps of cells
18. 50 uL of the medium in the falcon tube is transferred into one well of a 96 well plate
19. 50uL of 1X, cell counting solution (Ethidium bromide-acridine orange) is pipetted out in the 96 well plate, in the same well with 50uL medium
20. The two solutions are mixed thoroughly with a pipette

21. 20 uL aliquot is extracted from the cell suspension prepared in step 20 and immediately transferred to a hemocytometer
22. The hemocytometer consists of nine 1 mm squares divided into smaller squares. One of the 1 mm squares represents a volume of 0.1 mm^3 or 10^-4 ml.
23. The hemocytometer is placed on a light microscope stage. Using the 10X objective, the number of cells are counted in a 1 mm square area
24. The total number of cells per mL is calculated using the formula

$$c=2*n*10^4$$

where: c = cell concentration in cells/ml

n = avg. number of cells/mm^2 area

25. Based on the above concentration dilute the cell suspension in the falcon tube to achieve a final cell concentration of 0.25 million cells/mL

Part B:

1. The crosslinked gels with encapsulated virus inside were removed from the refrigerator after their respective periods of incubation.
2. The DMEM medium of final cell concentration 0.25 million cell/mL is added to the required number of well of a 24 well plate with 1mL of medium per well.
3. The hydrogels are removed from the refrigerator after their respective periods of incubation.
4. 100uL each well, of the gel is extruded on three adjacent wells of the 24 well plate, over the DMEM medium.
5. The well plate is then incubated at 37^oC for 24 hours.

6. After 24 hours of incubation 1uL of TPCK trypsin was added to each well to remove the adhered MDCK cells from the wells.
7. The well plate was incubated at 37^{o}C for another 48 hours.
8. After 48 hours the standard hemagglutination assay was performed on the supernatant medium from each well.
9. Briefly, 200 uL of the supernatant from each well was transferred to a well on the top row of a U-bottom 96 well plate, where it was diluted 50% each time till the 8th row.
10. On each of the eight wells 50 uL of 0.5 % Chicken Red Blood Cell (CRBC) solution was added.
11. The well plate was then read for hemagglutination after 1.5-2 hours.

The results of the hemagglutination experiment are based on visual observation of the 96 well plate. The results are classified into three types, Complete Hemagglutination (HA), Partial Hemagglutination (HA ±) and No Hemagglutination or complete Pellet formation (P) (Figure 13). These visual observations were quantified by assigning numerical Hemagglutination (HA) Scores depending on the row till where we observe Complete Hemagglutination.

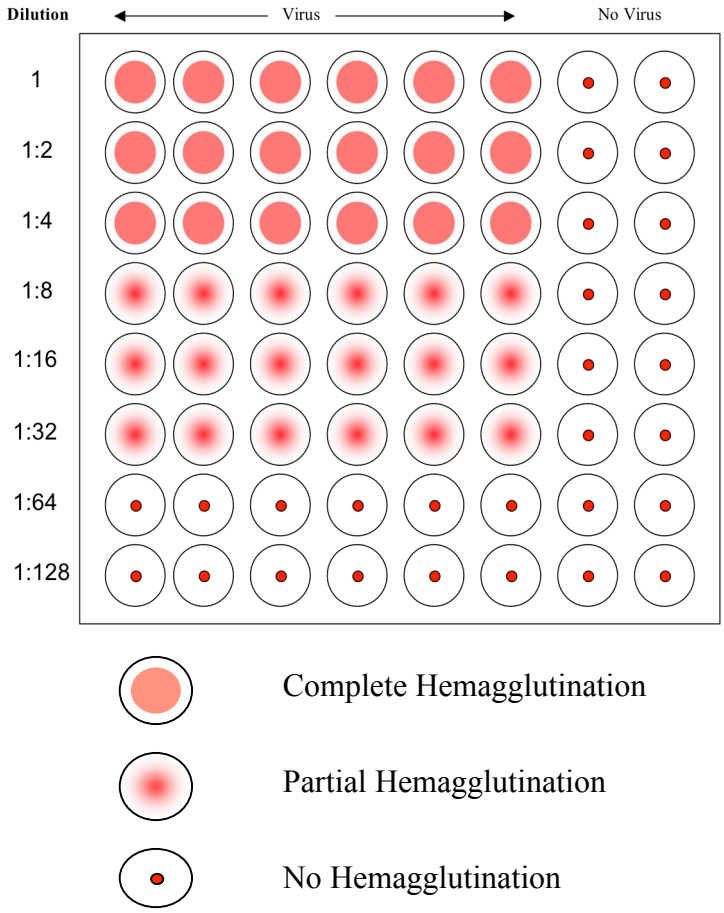


Figure 13: Example of Hemagglutination assay final result

Any particular sample was given an HA score equal to the reciprocal of the number of dilutions till where it showed Complete Hemagglutination. For example if a sample showed Complete Hemagglutination till the 4^{th} row i.e. till $1/8^{th}$ dilution, it will have an HA score equal to 8. Regarding the partial hemagglutination, we made a provision that the HA score will be raised by 1 unit if we observe partial hemagglutination for at least 2 more rows after the complete hemagglutination. So in the above example if the sample showed a complete hemagglutination till the 4^{th} row but also showed partial hemagglutination till the 7^{th} row, then the HA score for this sample will be 9 and not 8.

5. RESULTS AND DISCUSSION

5.1. First Approach: Alginate-CaSO4 hydrogels

5.1.1. LVG Alginate

This approach was implemented with 1% Low viscosity, High Guluronic acid (LVG) alginates. We tested nine different formulations with varying final CaSO4 and TSMP concentrations. Three different final CaSO4 concentrations (3650, 4550 & 5550 ug/mL) were selected and each of them was tested for three different final TSMP concentrations (0.2, 0.45 & 0.7 %).

5.1.1.1. Stage 1: Extrusion Consistency Test

All nine gels were prepared in triplicates as per the protocol explained in the methods section and the extrusion consistency test was performed after 24 hours of curing. The details of the nine formulations and their average extrusion consistency scores obtained after the test are shown in table 5.

A plot of average consistency score versus final TSMP Concentration, for the three different hydrogels (with different final CaSO4 concentrations) is shown in figure 14.

Conditions	Alginate Composition			1% LVG alginate			
	Incubation Temperature			$4^{O}C$			
	Incubation Time			24 hours			
Sample #	Final CaSO4 Concentration		Final TSMP Concentration (%)	Extrusion Consistency values of triplicates			Average Consistency Score
	mg/mL	mM					
1	3.65	21.2	0.2	4	4	5	4.3
2			0.45	3	4	5	4.0
3			0.7	2	2	3	2.3
4	4.55	26.4	0.2	4	4	4	4.0
5			0.45	4	4	4	4.0
6			0.7	4	5	4	4.3
7	5.55	32.2	0.2	3	2	2	2.3
8			0.45	3	3	3	3.0
9			0.7	4	5	5	4.7

Table 5: Extrusion Consistency results obtained with 1% LVG alginates

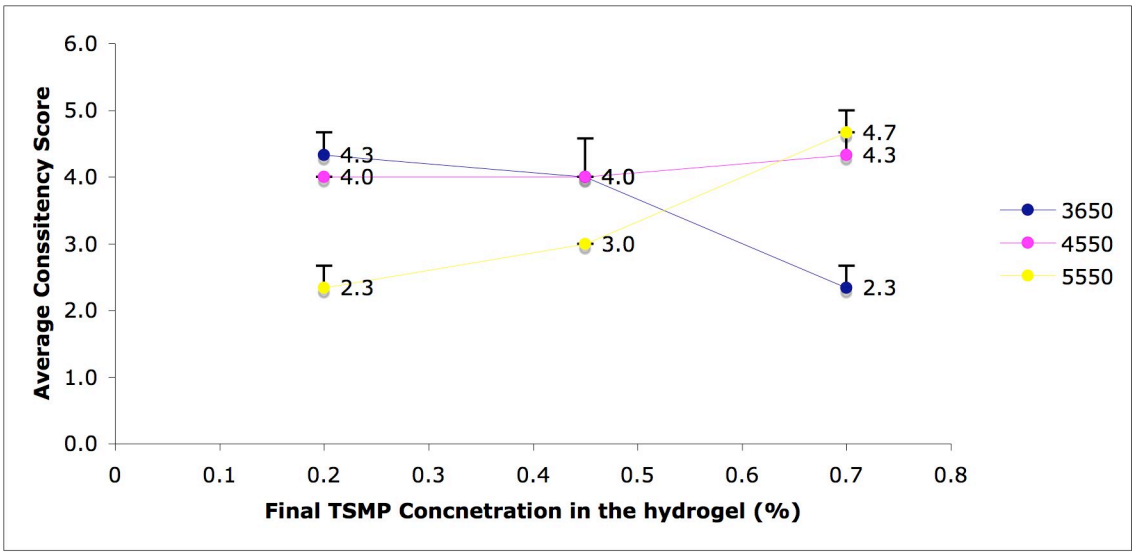


Figure 14: Average consistency scores vs final TSMP concentration for three different final CaSO4 concentrations in 1% LVG alginates

The results of the Extrusion Consistency Test on different formulations prepared using 1% LVG alginates, gave us three hydrogel compositions which had an average consistency score greater than 4 (Table 6). From the result we can see that for the lowest CaSO4 concentration i.e. 3650 ug/mL the consistency scores decreases with increasing TSMP concentration. However for gels with CaSO4 concentrations of 4550 and 5550 ug/mL the consistency score improves with increase in TSMP concentration. This behavior can be explained by understanding the nature of TSMP. TSMP is a calcium sequestrant. Therefore if we add more TSMP in a solution with low amount of Calcium, it will bind all the available calcium and leave much less for crosslinking. This is the very reason why we saw poor consistency for gels with CaSO4 concentration of 3650 ug/mL and more than 0.2% TSMP concentration. A score better than 4, denotes that these three hydrogels can be extruded out through a 26G needle very smoothly with minimum phase separation or bleeding. The closer the score is to 5, the better its consistency.

Conditions	**Alginate Composition**		**1% LVG alginate**	
	Incubation Temperature		**4^{O}C**	
	Incubation Time		**24 hours**	
Sample #	**Final CaSO4 Concentration**		**Final TSMP Concentration (%)**	**Average Consistency Score**
	mg/mL	**mM**		
1	**3.65**	**21.2**	**0.2**	**4.3**
2	**4.55**	**26.4**	**0.7**	**4.3**
3	**5.55**	**32.2**	**0.7**	**4.7**

Table 6: Hydrogel compositions for 1% LVG alginate, with consistency score better than 4

5.1.1.2. Stage 2: Nanoparticle Release Study

The three-hydrogel compositions of Table 4 were selected for the Nanoparticle Release study. The study was performed as per the protocol explained in the Methods section. The details of the release study are shown in Table 7 and the result obtained is shown in figure 15.

Conditions	**Alginate Composition**		**1% LVG alginate**	
	Nanoparticles (NP) Composition		**2% solids, 200 nm amine modified fluorescent nanoparticles**	
	Incubation Temperature		**4^{O}C**	
	Incubation Time		**24 hours**	
	Release Medium		**HBSS, 1X**	
	Release Study performed for		**24 hours**	
Sample #	**Final CaSO4 Concentration**		**Final TSMP Concentration (%)**	**Concentration of NP (ug/uL)**
	mg/mL	**mM**		
1	**3.65**	**21.2**	**0.2**	**0.25**
2	**4.55**	**26.4**	**0.7**	**0.25**
3	**5.55**	**32.2**	**0.7**	**0.25**

Table 7: Details of the Nanoparticle release study performed on 1% LVG alginate hydrogels

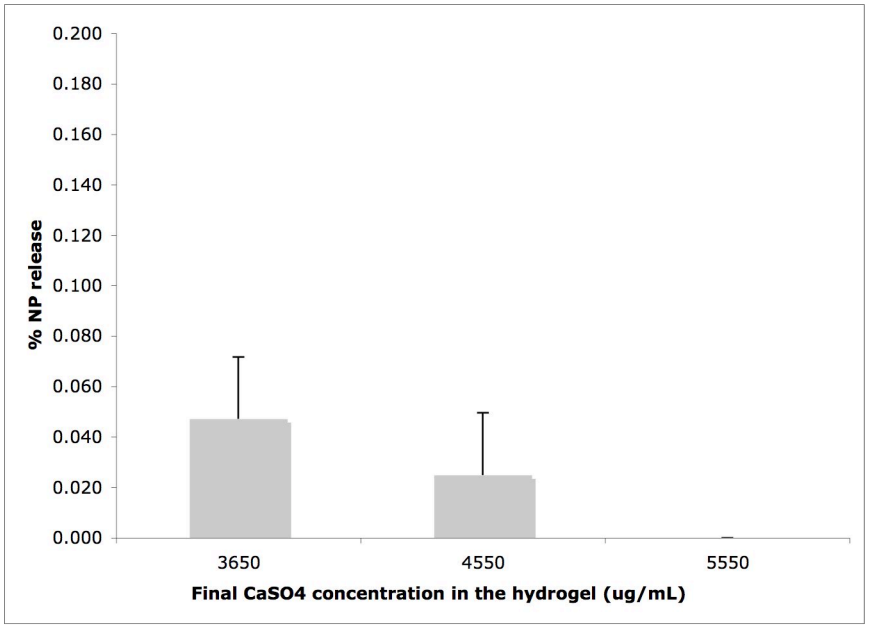


Figure 15: 24 hour release (%) of 200 nm fluorescent nanoparticles from 1% LVG alginate hydrogels crosslinked with three different final CaSO4 concentrations.

The results obtained from the Nanoparticle Release Study performed on the three formulations composed of 1% LVG alginate, demonstrated very slow release. As can be seen in figure 2, none of the hydrogels exhibited more than 0.1% release in 24 hours. This suggests that the hydrogels formed with these LVG alginates were highly crosslinked with very small pores. These hydrogels were also very stable and exhibited no degradation in the surrounding medium. Visual observation of the gels after 24 hours shows them to be intact. Such a high degree of crosslinking can be attributed to the high guluronic acid content, as these are the molecules in alginate, which are responsible for the gelation.

Thus even though the LVG alginate hydrogels showed good extrusion consistency we didn't perform virus release studies (HA) on them, considering their poor performance in the nanoparticle release study.

5.1.2. LVM Alginate hydrogels

Since the LVG alginates did not produce the desired gels, we proceeded to use Low viscosity High Mannuronic acid (LVM) alginates to form hydrogels and check their extrusion consistency.

5.1.2.1. Stage 1: Extrusion Consistency Test

Five different gel formulations with 1% LVM alginate and varying final CaSO4 concentrations were tested. The details of the five samples and their results obtained from the extrusion consistency test are given in Table 8. A plot of the results obtained for the extrusion consistency versus Final CaSO4 concentration is shown in figure 16.

Conditions	**Alginate Composition**		**1% LVM alginate**				
	Incubation Temperature		**4^{O}C**				
	Incubation Time		**24 hours**				
Sample #	**Final CaSO4 Concentration**		**Final TSMP Concentration (%)**	**Extrusion Consistency values of triplicates**			**Average Consistency Score**
	mg/mL	**mM**					
1	**2.35**	**13.6**	**0.2**	**2**	**2**	**3**	**2.3**
2	**2.85**	**16.6**	**0.2**	**3**	**4**	**4**	**3.7**
3	**3.2**	**18.6**	**0.2**	**4**	**4**	**4**	**4.0**
4	**3.65**	**21.2**	**0.2**	**2**	**3**	**2**	**2.3**
5	**3.57**	**20.7**	**0.4**	**3**	**2**	**3**	**2.7**

Table 8: Extrusion Consistency results obtained with 1% LVM alginates

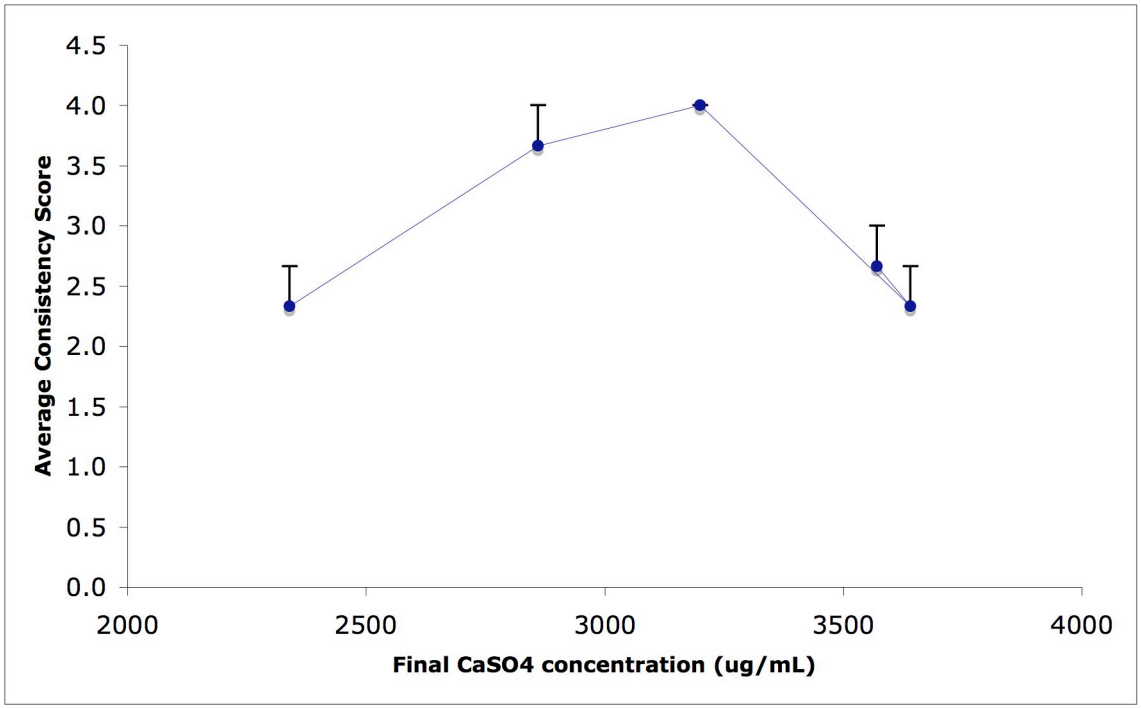


Figure 16: Plot of average consistency score vs final CaSO4 Concentration for 1% LVM Alginate hydrogels

The extrusion consistency test results (table 8 and figure 16) for 1% LVM alginate hydrogels show that the consistency increases with increase in CaSO4 concentration up to a certain point and then decreases with further addition of CaSO4. As can be seen in figure 3, the average consistency score for a gel with final CaSO4 concentration equal to 2350 ug/ml is 2.3, and it increases to 3.7 and 4.0 for gels with final CaSO4 concentrations of 2850 and 3200 ug/mL respectively. But with further increase in CaSO4 concentration causes a decrease in extrusion consistency because due to the high CaSO4 concentration a part of alginate solution binds to it very quickly and becomes a thick gel but a part remains in solution. Thus when these gels are extruded though a needle we observe a phase separation. Out of the five gels tested only one gel had a consistency score more than 4, thus we decided to select one more formulation with the next best score for Stage

2 (Nanoparticle release study). The two hydrogel formulations made of 1% LVM alginate, selected for stage 2 are given in Table 9.

Conditions	Alginate Composition		1% LVM alginate	
	Incubation Temperature		~ 4^{O}C	
	Incubation Time		24 hours	
Sample #	Final CaSO4 Concentration		Final TSMP Concentration (%)	Average Consistency Score
	mg/mL	mM		
1	2.85	16.6	0.2	3.7
2	3.2	18.6	0.2	4

Table 9: 1% LVM alginate hydrogel compositions selected for stage 2

5.1.2.2. Stage 2: Nanoparticle Release Study

The study was performed as per the protocol explained in the Methods section. The details of the release study are shown in Table 10 and the result obtained from the particle release experiment is shown in figure 17.

Conditions	Alginate Composition		1% LVM alginate	
	Nanoparticles Composition		2% solids, 200 nm amine modified fluorescent nanoparticles	
	Incubation Temperature		4^{O}C	
	Incubation Time		24 hours	
	Release Medium		HBSS, 1X	
	Release Study performed for		24 hours	
Sample #	Final CaSO4 Concentration		Final TSMP Concentration (%)	Concentration of NP (ug/uL)
	mg/mL	mM		
1	2.85	16.6	0.2	0.25
2	3.2	18.6	0.2	0.25

Table 10: Details of the Nanoparticle release study performed on 1% LVM alginate hydrogel

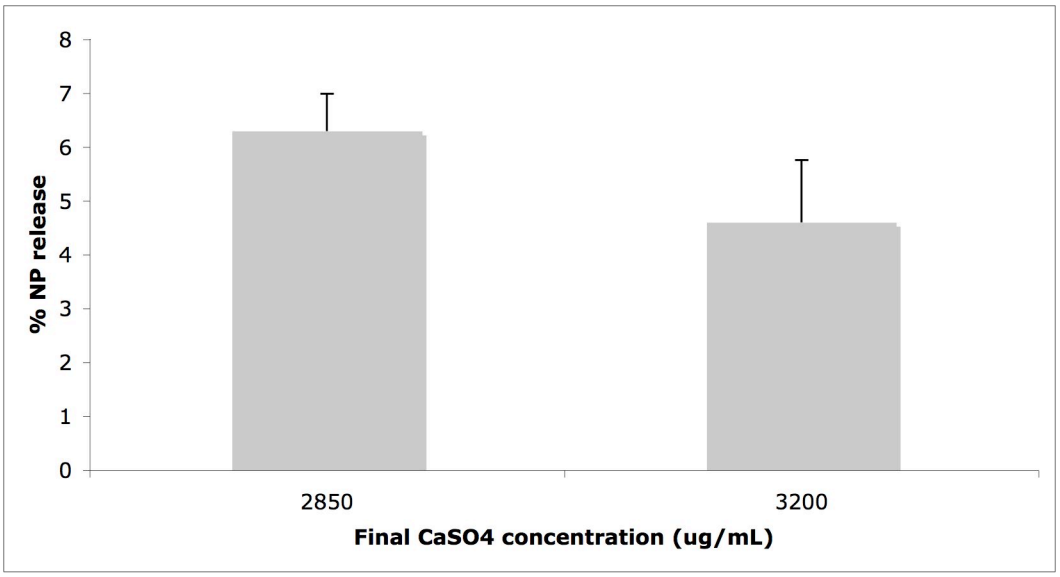


Figure 17: 24 hour release (percentage) of 200 nm Fluorescent nanoparticles from 1% LVM alginate hydrogels crosslinked with two different final CaSO4 concentrations

From the results (figure 17) we can see that the % release obtained from LVM alginate hydrogels in 24 hours is significantly greater than that obtained from LVG alginates. Also visual observation of the gels after 24 hours showed them to be broken down into small lumps. Thus the higher % release from these hydrogels can be attributed to both diffusion and degradation. Also we can see that the % release decreases with increasing final CaSO4 concentration, as is expected with higher degree of crosslinking achieved at higher CaSO4 concentrations. However, none of the above LVM hydrogels had an extrusion consistency score greater than 4, in fact for the first hydrogel (with final CaSO4 concentration of 2850 ug/mL) the consistency score was 3.7. Thus even though the LVM alginate hydrogels exhibited good release profile we did not continue with Virus release study with them due to their inconsistent extrusion performance.

5.1.3. LVM-LVG Alginates

The experiments with single component LVG and LVM alginates did not yield a gel with the desired properties. Thus we decided to prepare gels from a mixture of LVM and LVG alginates for reasons explained in the previous sections (Exact Section and Page).

5.1.3.1. Stage 1: Extrusion Consistency Test

We used two different combinations of LVM-LVG mixtures with 1:1 and 1:2 (w/w) ratio of LVM: LVG, keeping LVG concentration constant at 0.5% in each mixture. The details of the hydrogel compositions prepared using the two different mixtures of LVM-LVG alginates and their results from the extrusion consistency test are given in Table 11 and Table 12. Figure 18 and 19 represent the plots of average consistency score versus Final CaSO4 concentrations for the hydrogels made of two different mixtures of LVM-LVG alginates.

Conditions	Alginate Composition			LVM-LVG (0.5-0.5%) Alginate			
	Incubation Temperature			$4^{O}C$			
	Incubation Time			24 hours			
Sample #	Final CaSO4 Concentration		Final TSMP Concentration (%)	Extrusion Consistency values of triplicates			Average Consistency Score
	mg/mL	mM					
1	0.95	5.5	0.2	1	1	1	1
2	1.45	8.4	0.2	3	3	2	2.7
3	1.9	11.0	0.2	3	3	4	3.3
4	2.35	13.6	0.2	4	4	5	4.3
5	2.85	16.6	0.2	4	4	4	4
6	3.85	22.4	0.2	3	3	4	3.3

Table 11: Extrusion Consistency results obtained with LVM-LVG (0.5-0.5%) Alginate hydrogels

Conditions	Alginate Composition			LVM-LVG (1.0-0.5%) Alginate			
	Incubation Temperature			$4^{O}C$			
	Incubation Time			24 hours			
Sample #	Final CaSO4 Concentration		Final TSMP Concentration (%)	Extrusion Consistency values of triplicates			Average Consistency Score
	mg/mL	mM					
1	0.5	2.9	0.2	1	1	1	1.0
2	0.7	4.1	0.2	1	1	1	1.0
3	0.95	5.5	0.2	1	1	1	1.0
4	1.45	8.4	0.2	2	3	2	2.3
5	1.925	11.2	0.2	3	3	2	2.7
6	2.15	12.5	0.2	3	3	4	3.3
7	2.85	16.6	0.2	4	4	5	4.3

Table 12: Extrusion Consistency results obtained with LVM-LVG (1.0-0.5%) Alginate hydrogels

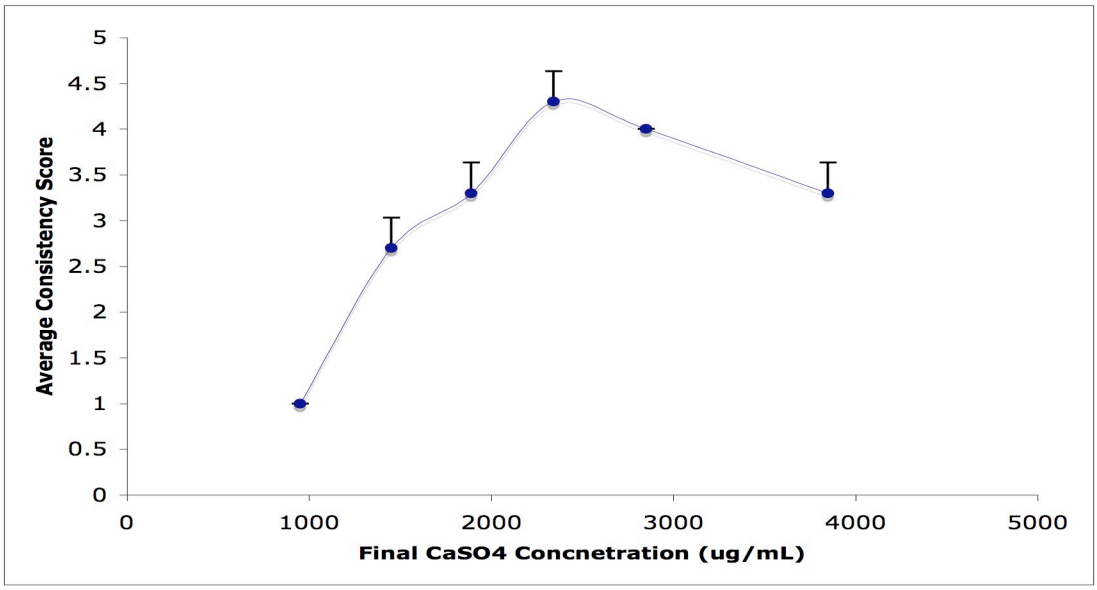


Figure 18: Plot of average consistency score vs final CaSO4 Concentration for LVM-LVG (0.5-0.5%) Alginate hydrogels

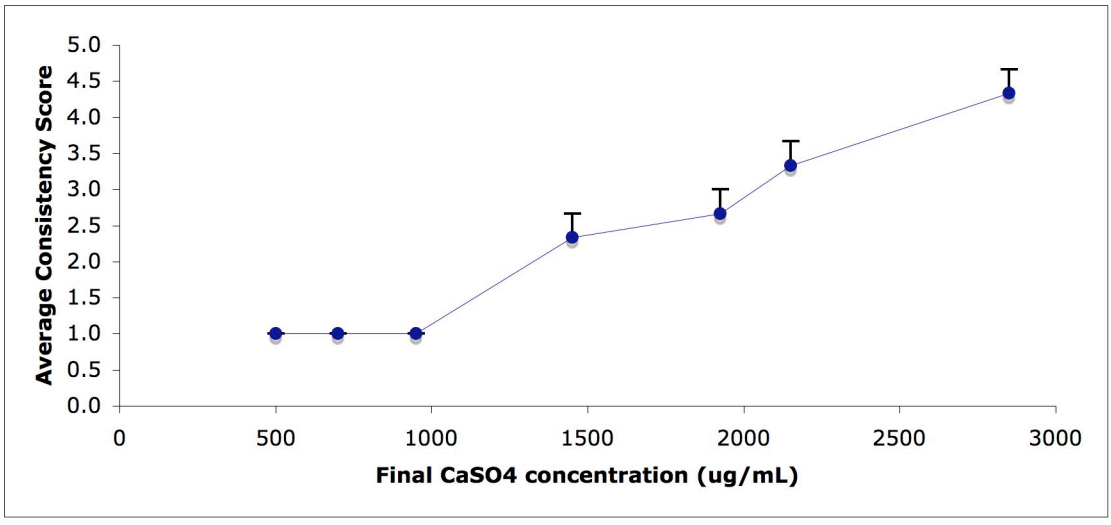


Figure 19: Plot of average consistency score vs final CaSO4 Concentration for LVM-LVG (1.0-0.5%) Alginate hydrogels

From the results obtained (figures 5 & 6) we can ascertain that the general gelling range for 0.5% LVG alginate solution is between 2000-3000 ug/mL of final CaSO4 concentration. Since we also have LVM alginate in the same system, the required CaSO4

also depends on the amount of LVM alginate present. The results show that LVM-LVG (0.5-0.5%) alginate hydrogel gives the best consistency at 2340 ug/mL final CaSO4 concentration whereas LVM-LVG (1.0-0.5%) alginate hydrogel gives the best consistency at 2850 ug/mL final CaSO4 concentration. This seems to be very logical as, the LVM-LVG (1.0-0.5%) alginate has more binding points than the LVM-LVG (0.5-0.5%) it requires more amount of Calcium to reach a desired consistency. Therefore we selected these two compositions, for our stage 2 i.e. particle release study (Table 13).

Conditions	**Incubation Temperature**		**$4^{O}C$**		
	Incubation Time		**24 hours**		
Sample #	**Alginate composition**	**Final CaSO4 Concentration**		**Final TSMP Concentration (%)**	**Average Consistency Score**
		mg/mL	**mM**		
1	**LVM-LVG (0.5-0.5%) Alginate**	**2.35**	**13.6**	**0.2**	**4.3**
2	**LVM-LVG (1.0-0.5%) Alginate**	**2.85**	**16.6**	**0.2**	**4.3**

Table 13: LVG-LVM alginate hydrogel compositions selected for stage 2

5.1.3.2. Stage 2: Nanoparticle Release Study

The study was performed as per the protocol explained in the Methods section. The details of the release study are shown in Table 14 and the result obtained from the particle release experiment is shown in figure 20.

Conditions	Nanoparticles Composition		2% solids, 200 nm amine modified fluorescent nanoparticles		
	Incubation Temperature		$4^{O}C$		
	Incubation Time		24 hours		
	Release Medium		HBSS, 1X		
	Release Study performed for		24 hours		
Sample #	Alginate composition	Final CaSO4 Concentration		Final TSMP Concentration (%)	Concentration of NP (ug/uL)
		mg/mL	mM		
1	LVM-LVG (0.5-0.5%) Alginate	2.35	13.6	0.2	0.25
2	LVM-LVG (1.0-0.5%) Alginate	2.85	16.6	0.2	0.25

Table 14: Details of the Nanoparticle release study performed on the two different LVM-LVG alginate mixture hydrogel formulations

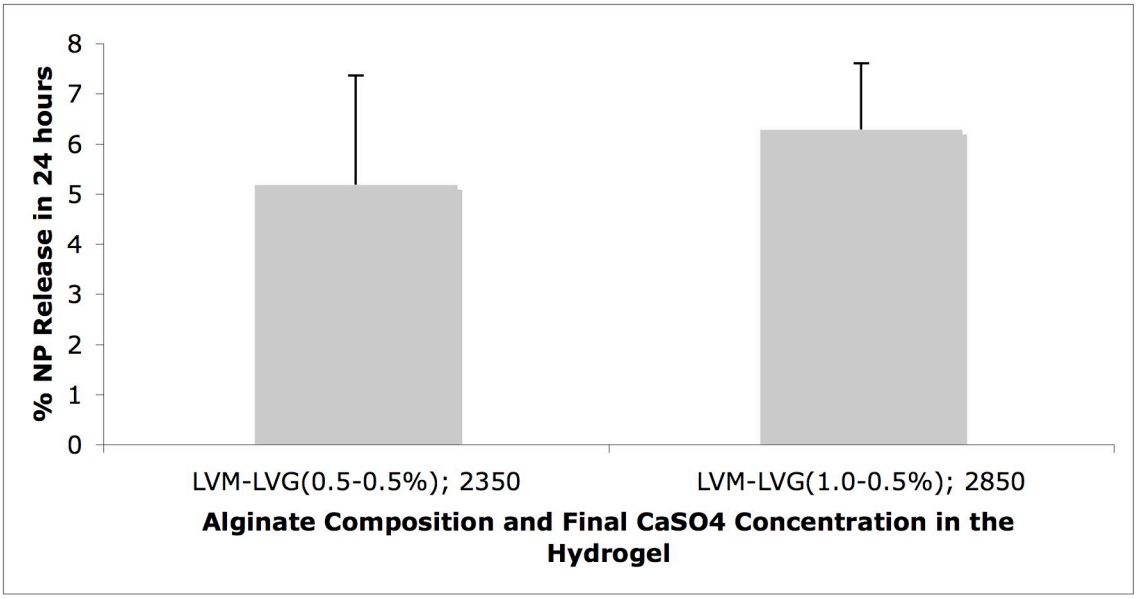


Figure 20: % 24 hour release of 200 nm fluorescence nanoparticles from LVM-LVG Alginate hydrogels

The nanoparticle release study of LVM-LVG alginate hydrogels showed about 6% release for both the formulations. We observe that although sample 1 has less final CaSO4 concentration it shows almost the same % release of nanoparticles. This can be attributed to the different total amount of alginate present in sample 1(1%) as compared to sample 2 (1.5%). Therefore a smaller amount of CaSO4 can be adequate in producing a very firm gel, in the case of sample 1. . Visual observation of the gels after 24 hours shows them to be broken down into small lumps. Thus the release of nanoparticles from these hydrogels can be attributed to both diffusion and degradation.

Along with the above two samples we also performed nanoparticle release experiments with the alginate formulation of LVM-LVG (1.0-0.5%) while keeping the final CaSO4 concentration equal to 2350 ug/ml. From these experiments we obtained a mean % NP release of about 11 % from this gel, but the extrusion consistency score was only 3.7.

Figure 21 shows a comparison of 24 hours % FP release from the alginate formulation LVM-LVG (1.0-0.5%) with different final CaSO4 concentrations.

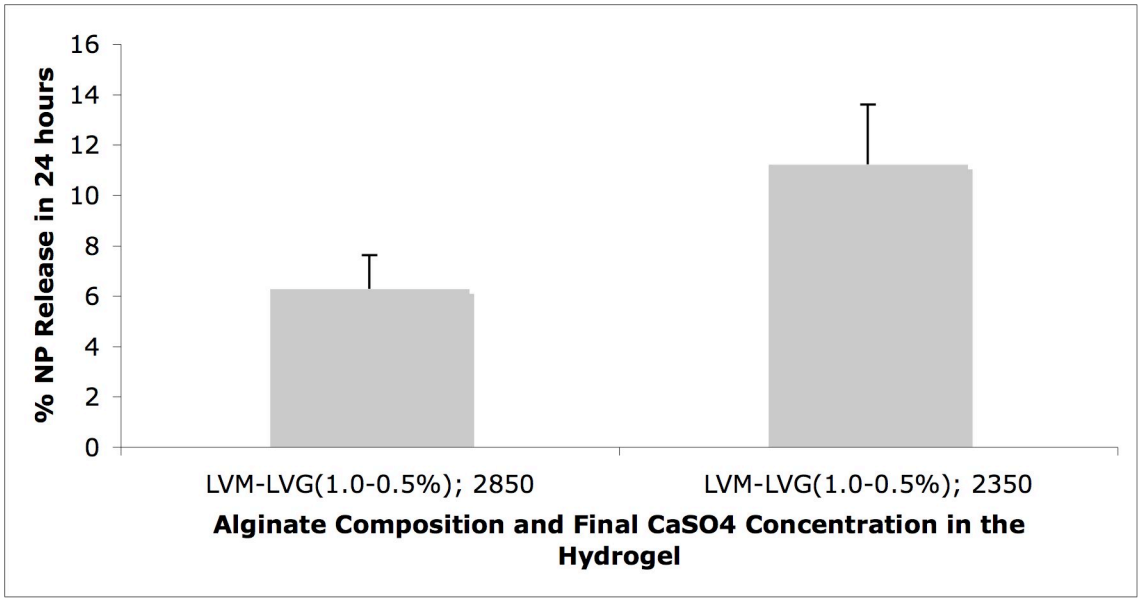


Figure 21: 24 hour Release of 200 nm fluorescence nanoparticles from LVM-LVG Alginate hydrogel s

The comparison of % NP release from the same alginate formulation LVM-LVG (1.0-0.5%) with two different final CaSO4 concentrations (figure 21) gave us a result as expected. We obtained a higher % release when we decreased the amount of crosslinker. But even though we obtained a better release from the formulation LVM-LVG (1.0-0.5%) when we decreased the final CaSO4 concentration to 2350 ug/mL from 2850 ug/mL, we had to compromise with the extrusion consistency. The consistency score for the gel with 2350 ug/mL Final CaSO4 concentration was only 3.7 as compared to 4.3 that obtained with the gel with 2850 ug/mL Final CaSO4 concentration.

5.1.3.3. Stage 3: Virus Release study/ Hemagglutination Experiment

The two LVG-LVM alginate formulations (table 14) had shown good extrusion consistency (had consistency score > 4) along with about 6% release of nanoparticles in 24 hours. Therefore we also performed the release of a chosen bioactive agent i.e. Influenza virus from these two formulations.

The Virus release study was performed as per the protocol described in the Methods section. The result of the hemagglutination study in terms of hemagglutination (HA) score is shown in figure 22.

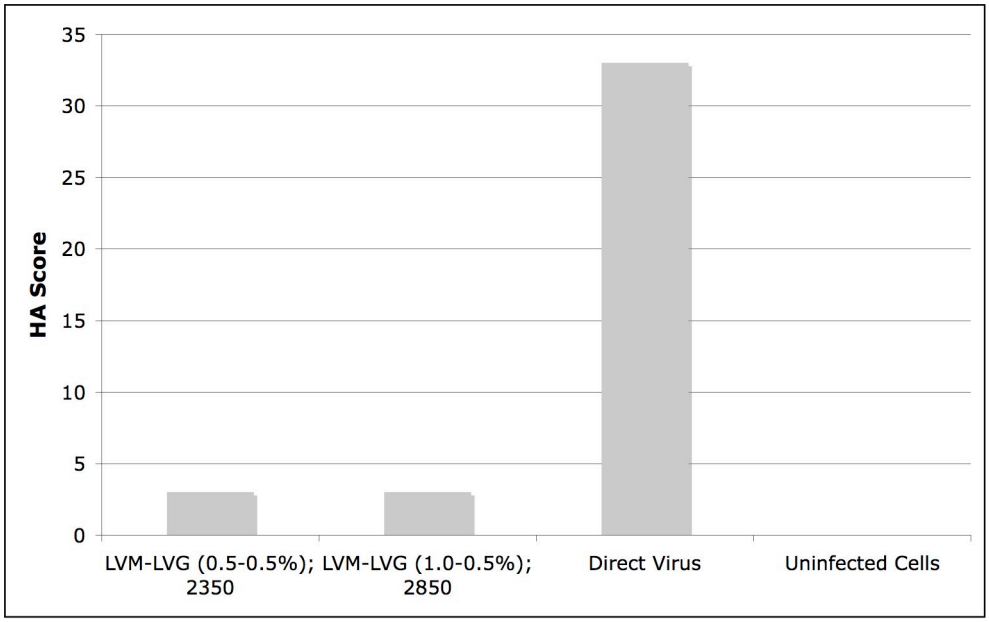


Figure 22: Result of Hemagglutination study performed on two different LVM-LVG hydrogel formulations

The result of the virus release study (figure 22) is shown in terms of Hemagglutination Scores. The hemagglutination score obtained from the medium in which the hydrogels were added has to be compared to the hemagglutination score obtained from the medium where the virus was added directly. This comparison can be considered to be an estimate

of % virus released from the hydrogel if we assume that the hydrogel has no effect on the surface integrity of the virus or in other words the internal environment of the hydrogel is compatible for the virus. The dimensions and charge of the nanoparticles match very closely to that of the Influenza virus particles. Therefore we expect a similar release profile for the virus. The % virus release considering the hemagglutination scores, should must be more than or at least equal to % release obtained for nanoparticles in 24 hours, since in the hemagglutination study the virus is allowed to release for 72 hours. In 72 hours we expect more % release than that obtained in 24 hours, since both diffusion of virus and degradation of gel are continuing for 72 hours. Therefore if we obtain less % release of virus (based on hemagglutination scores), it will indicate some loss in integrity of virus due to its encapsulation in the hydrogel. In such case we can conclude that the hydrogel environment is not compatible for biomolecules (in this case, influenza virus).

From the results of the hemagglutination study performed on the two different hydrogel formulations made of LVM-LVG alginates, we obtained about 10 % hemagglutination score for both the hydrogels compared to that of Direct Virus. In other words we obtained 10% release of virus from both hydrogels in 72 hours. As expected the % release of virus is greater than % release of nanoparticles from the same hydrogels. This indicates that the hydrogels made from the mixture of LVM and LVG alginates were found to be compatible with the virus and had no effect on the integrity of the virus. Therefore these two gels can be very useful to encapsulate biomolecules for applications where maintaining the integrity of the biomolecule is very important but does not require fast release.

5.1.4. LVM-LVG alginate hydrogels with Low amount of CaSO4

We had seen that even LVM-LVG alginate hydrogels didn't show hemagglutination at par with the Direct Virus. To increase the hemagglutination or say the HA we have to have more % release of virus. We can do this by decreasing the crosslinking density by lowering the final concentration of CaSO4 in the hydrogel, and allowing it to cure for a longer time. But using this method, we might not reach the desired extrusion consistency. Thus we were required to find an optimum concentration of CaSO4, which will give a higher % release as well as maintain a good consistency.

5.1.4.1. Stage 1: Extrusion Consistency Test

We decided to use the alginate composition (LVM-LVG (1.0-0.5%)) for this experiment, as the hydrogel prepared using LVM-LVG (1.0-0.5%) alginate solution gave a higher % release of nanoparticles (figure 24) compared to the hydrogel prepared using LVM-LVG (0.5-0.5%). We tested three formulations each with very low amount of crosslinking agent. The details of the three formulations used and their results obtained for the extrusion consistency test are given in Table 15.

Conditions	Alginate Composition			LVM-LVG (1.0-0.5%) Alginate			
	Incubation Temperature			$4^{O}C$			
	Incubation Time			24 hours			
Sample #	Final CaSO4 Concentration		Final TSMP Concentration (%)	Extrusion Consistency values of triplicates			Average Consistency Score
	mg/mL	mM					
1	1.4	8.1	0.2	2	3	3	2.67
2	1.6	9.3	0.2	3	3	3	3.00
3	1.9	11.0	0.2	4	4	4	4.00

Table 15: Extrusion Consistency results obtained with LVM-LVG (1.0-0.5%) Alginate hydrogels prepared with very low CaSO4 concentrations

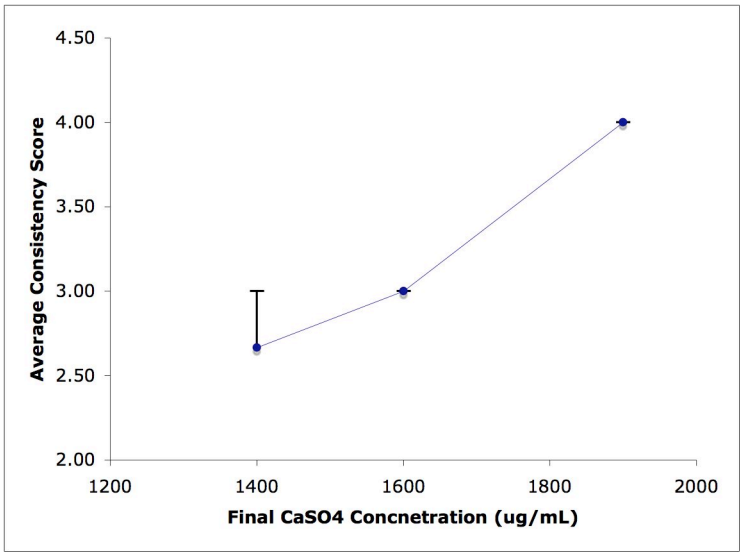


Figure 23: Plot of average consistency score vs final CaSO4 Concentration for LVM-LVG (1.0-0.5%) Alginate hydrogels prepared with very low CaSO4 concentrations

The results of the extrusion consistency test (figure 23) on the three formulations prepared with LVM-LVG (1.0-0.5%) alginates and low amount of crosslinking agent showed a linear increase in consistency score with increase in final CaSO4 concentration. But none of the three samples showed good consistency except for the third (with final CaSO4 concentration of 1900 ug/mL), which showed a consistency score of 4.

5.1.4.2. Stage 2: Nanoparticle release Study

Even though none of the above samples showed very good consistency we still decided to perform nanoparticle release study with these three, just as a proof of concept to see if we can obtain more % release if we decrease the $CaSO_4$ concentration. The result of the particle release experiment is shown in figure 24.

Conditions	**Alginate Composition**		**LVM-LVG (1.0-0.5%)**	
	Nanoparticles Composition		**2% solids, 200 nm amine modified fluorescent nanoparticles**	
	Incubation Temperature		**$4^{O}C$**	
	Incubation Time		**24 hours**	
	Release Medium		**HBSS, 1X**	
	Release Study performed for		**24 hours**	
Sample #	**Final $CaSO_4$ Concentration**		**Final TSMP Concentration (%)**	**Concentration of NP (ug/uL)**
	mg/mL	**mM**		
1	**1.4**	**8.1**	**0.2**	**0.25**
2	**1.6**	**9.3**	**0.2**	**0.25**
3	**1.9**	**11**	**0.2**	**0.25**

Table 16: Details of the Nanoparticle release study performed on LVM-LVG (1.0-0.5%) Alginate hydrogels prepared with very low CaSO4 concentrations

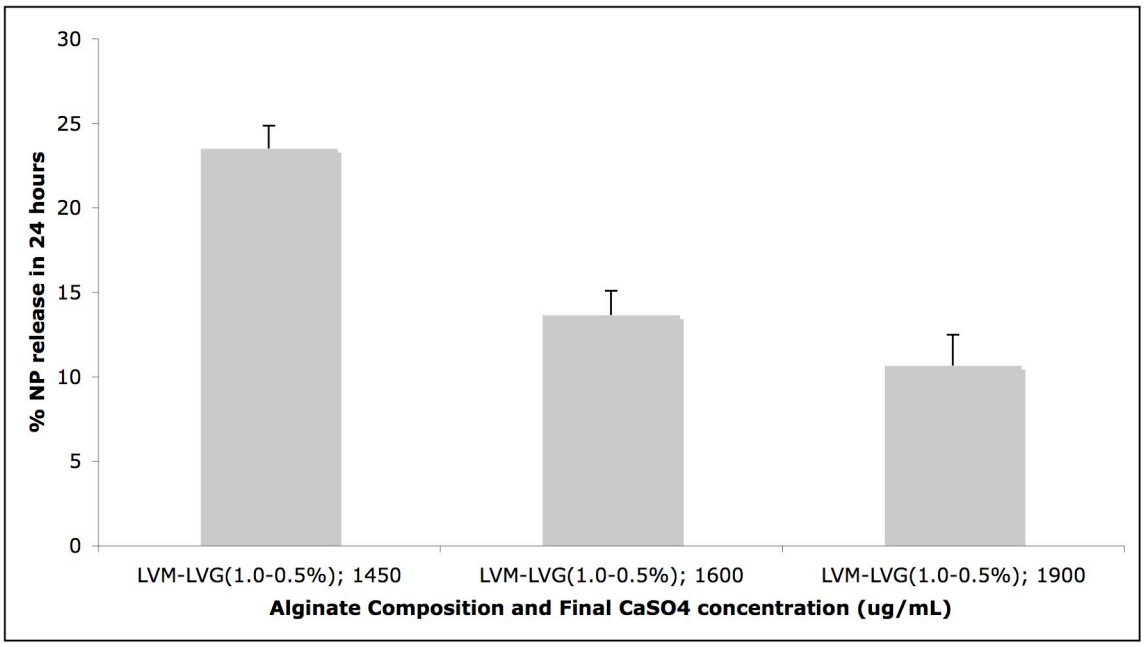


Figure 24: Release of 200 nm fluorescence nanoparticles from three different LVM-LVG Alginate hydrogel formulations in 72 hours

The results of the nanoparticle release experiment on the three formulations showed a good release. The first sample with final CaSO4 concentration equal to 1430 ug/ml showed the highest i.e. about 20% release, whereas the other two samples with 1610 and 1890 ug/ml final CaSO4 concentration showed about 11-12% release. The results came out to be as expected. We saw the highest release from the hydrogel formulation, which had the minimum final CaSO4 concentration. Visual observation of the gels after 24 hours shows complete dissolution of the gel into the HBSS medium. Thus degradation of hydrogels plays a major role in release from these hydrogels. The fast dissolution can be attributed to the low amount of crosslinking agent (CaSO4) in the gel.

5.1.4.3. Stage 3: Virus Release study/ Hemagglutination Experiment

We also performed a virus release study on three-alginate gel formulations with significantly lower amount of crosslinking agent, to see if we can achieve higher hemagglutination scores with such loose gels, which have shown a higher % release of nanoparticles. The final result of the virus release study in terms of the hemagglutination score is shown in figure 25.

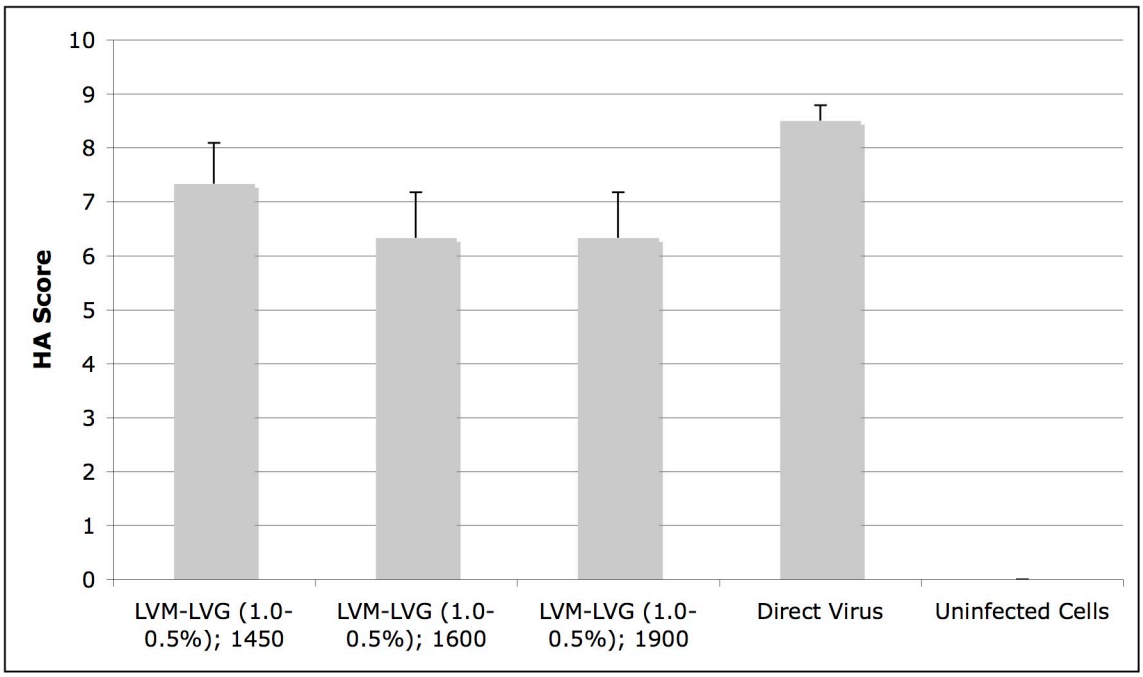


Figure 25: Result of the Hemagglutination study performed on different LVM-LVG alginate hydrogel formulations

All three hydrogels performed very well in the hemagglutination test, better than the gels of Figure 22. The first hydrogel sample (LVM-LVG, 1.0:0.5, 1430) showed almost 90% HA score compared to that of Direct Virus. The second and the third sample i.e. (LVM-LVG, 1.0:0.5, 1610) and (LVM-LVG, 1.0:0.5, 1890) both showed close to 70% HA

score. In other words first sample showed about 90% virus release in 72 hours whereas the other two showed about 70% virus release. Again as expected the % release of virus is greater than % release of nanoparticles from the same hydrogels. This indicates that the hydrogels made from the mixture of LVM and LVG alginates were found to be compatible with the virus and had no effect on the integrity of the virus. Therefore, all the above three formulations could be very effective in applications where one needs to encapsulate biomolecules and release them quickly while an extrusion consistency score of 3 or more is adequate. However, within the scope of this project we need hydrogels with very good extrusion consistency (consistency score > 4) with no bleeding in order to suppress aerosol formation.

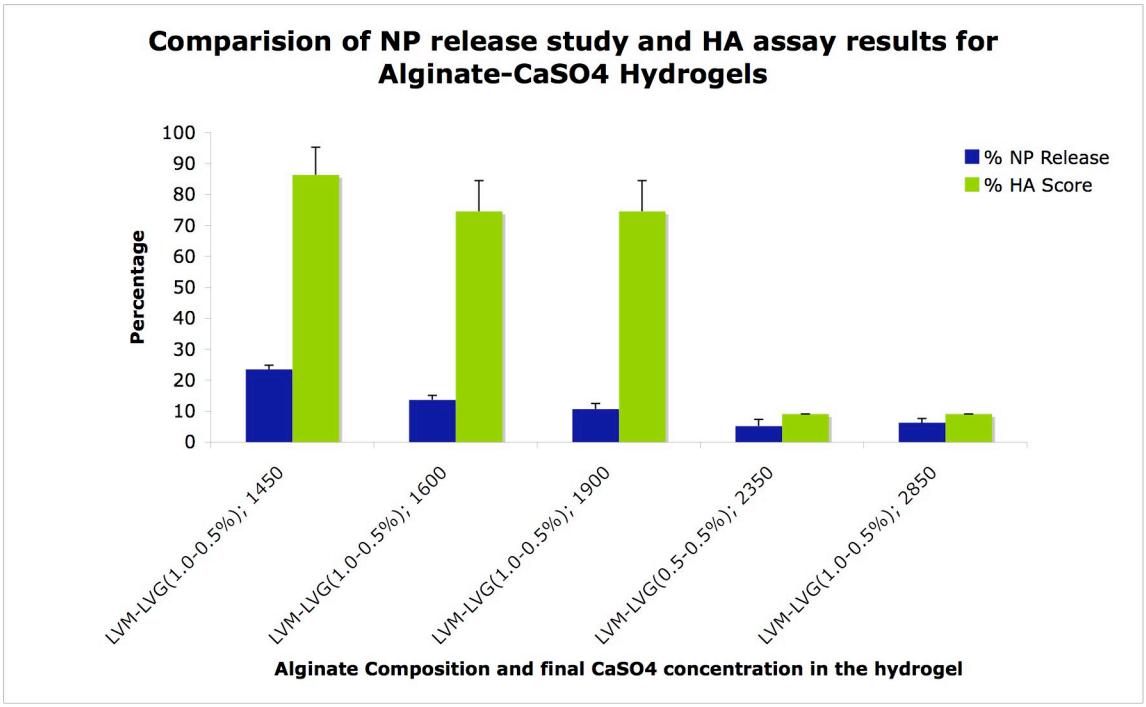


Figure 26: Comparison of nanoparticle release study and hemagglutination assay results for the first approach, i.e. alginate-CaSO4 hydrogels

5.2. Approach II: Supramolecular Hydrogels

Supramolecular hydrogels made of alpha cyclodextrins (Host) and PEO (Guest) have been reported to have good injectability through very fine needles and good release profiles. We have tested alpha cyclodextrin with PEO 10000 to form the desired hydrogels.

5.2.1. Stage 1: Extrusion Consistency Test

Twelve supramolecular hydrogels made of alpha cyclodextrin (CD) and PEO10000, having different ratios of PEO/CD were prepared as per the protocol mentioned in the methods section. These hydrogels were tested manually for their extrusion consistency and were given a particular consistency score based on a scale of 1 to 5.

The details of the 12 supramolecular hydrogels and their results obtained from the extrusion consistency test are given in table 17. A plot of the results obtained for the extrusion consistency versus ratio of PEO/CD is shown in figure 27.

Conditions	Incubation Temperature	$4^{O}C$			
	Incubation Time	12 hours			
Sample #	Ratio of PEO/ alpha CD (w/w)	Consistency Scores of Triplicates			Average Consistency Score
1	0.5	3	4	4	3.7
2	0.75	4	4	4	4.0
3	1	5	5	5	5.0
4	1.12	5	5	5	5.0
5	1.25	5	5	5	5.0
6	1.5	5	5	5	5.0
7	1.67	5	5	5	5.0
8	1.75	5	5	5	5.0
9	2	4	5	4	4.3
10	2.25	4	4	4	4.0
11	2.5	4	4	3	3.7
12	3	3	4	4	3.7

Table 17: Extrusion consistency results obtained for the supramolecular hydrogels

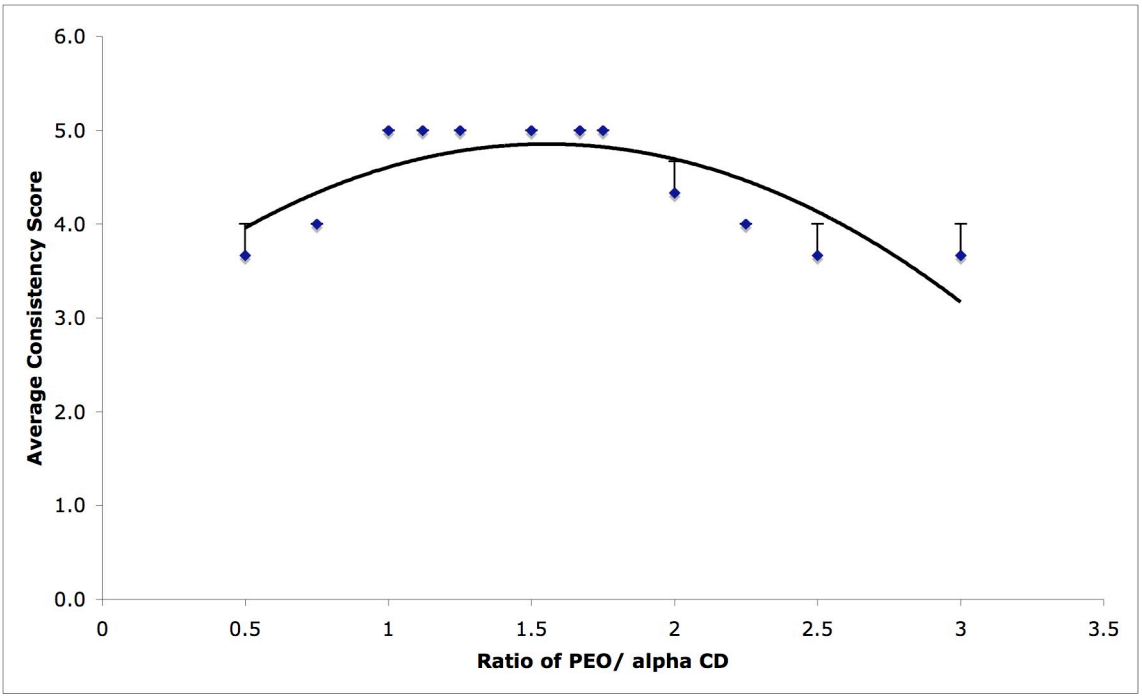


Figure 27: Plot of average consistency score vs ratio of PEO/CD for supramolecular hydrogels

From the results of extrusion consistency test obtained for supramolecular hydrogels, we saw that the amount of PEO added per gram of CD has a very distinct effect on the extrusion consistency. We obtained very thick hydrogels for ratios of PEO/CD less than 1. The first two samples with ratio 0.5 and 0.75 were extremely thick and were very difficult to inject through a 26G needle. The hydrogels became more and more easier to extrude with increasing ratio of PEO to CD. In other words they were becoming more and more thin. Therefore after increasing the ratio of PEO/CD to 2 and above, the hydrogels became so thin that they started to loose their extrusion consistency. They were coming out more like a liquid than a gel.

5.2.2. Stage 2: Nanoparticle Release Study

Out of the 12 hydrogels tested in the first stage six of them scored a consistency score of 5, which means they had the perfect extrusion consistency as desired for our application. Out the six we chose three hydrogels for stage 2 and tested the release of 200 nm fluorescent nanoparticles from them.

The details of the samples selected for the nanoparticle release study are given in table 18 and the result obtained from this study is shown in figure 28.

Conditions	**Nanoparticles Composition**	**2% solids, 200 nm amine modified fluorescent nanoparticles**
	Incubation Temperature	**$4^{O}C$**
	Incubation Time	**12 hours**
	Release Medium	**HBSS, 1X**
	Release Study performed for	**24 hours**
Sample #	**Ratio of PEO/alpha CD in the hydrogel (w/w)**	**Concentration of NP (ug/uL)**
1	**1**	**0.3**
2	**1.12**	**0.3**
3	**1.67**	**0.3**

Table 18: Details of the nanoparticle release study performed on three different supramolecular hydrogels

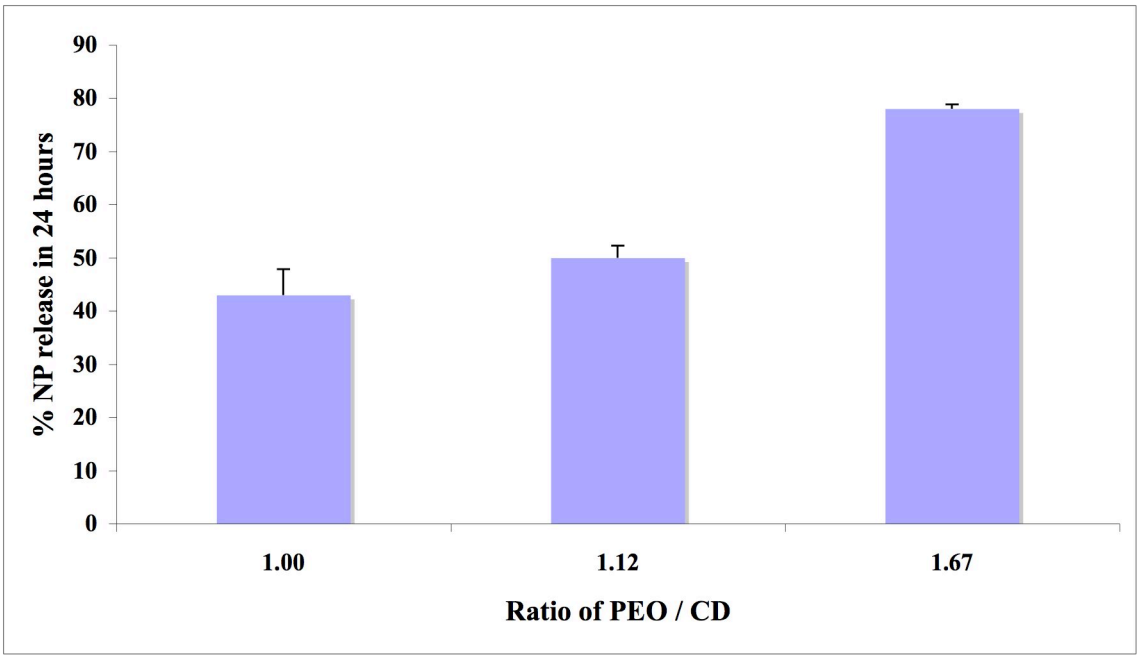


Figure 28: Result of nanoparticle release study on three different supramolecular hydrogels

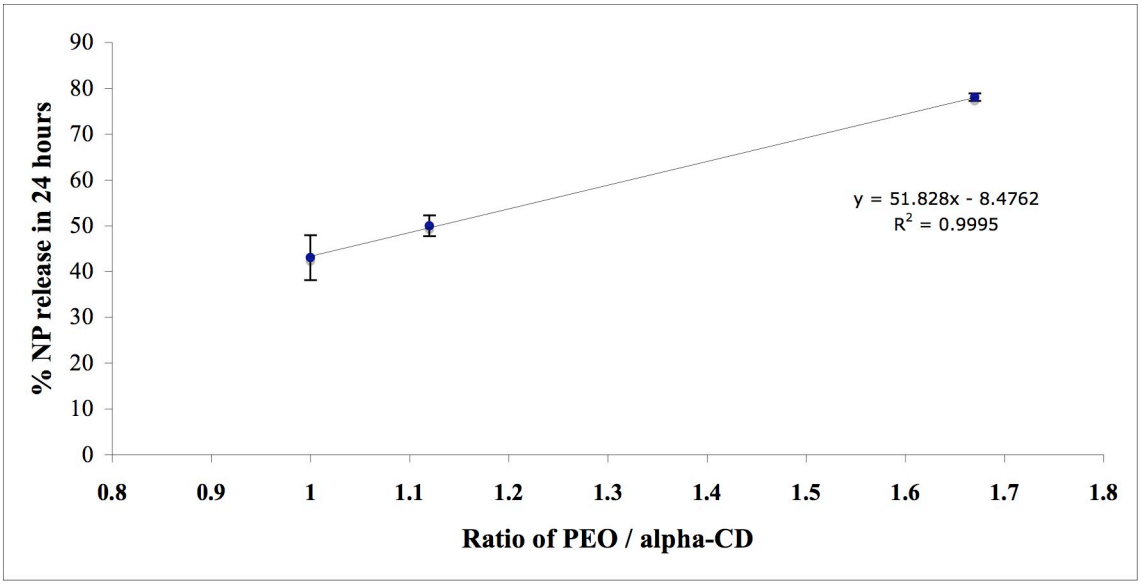


Figure 29: Scatter plot for % release of nanoparticles vs ratio of PEO/CD

The result of nanoparticle release is presented in the form of scatter plot in figure 29. The results obtained, showed a linear relationship between the % release of nanoparticles and ratio of PEO/CD. All the three hydrogels we selected for this release study had a consistency score of 5 but we see that the % release increases with increase in the ratio of PEO/CD. As we already mentioned that the gels become more and more thin and watery with the increase in the amount of PEO w.r.t. CD clearly explains why we see an increase in % release for higher ratios of PEO/CD.

5.2.3. Stage 3: Virus Release / Hemagglutination Study

Since all the three hydrogels chosen for nanoparticle release study showed very good release in 24 hours, we decided to use all three of them for our final test, to see if these are able to maintain the potency of biomolecules when encapsulated inside them.

The details of the virus release study are given in table 19 and the result obtained in terms of hemagglutination score is shown in figure 30.

Conditions	**Biomolecule Used**	**Influenza Virus**
	Incubation Temperature	**$4^{O}C$**
	Incubation Time	**12 hours**
Sample #	**Ratio of PEO/CD (w/w)**	**Volume of virus (per 100 uL hydrogel)**
1	**1**	**1**
2	**1.12**	**1**
3	**1.67**	**1**

Table 19: Details of the virus release study performed on supramolecular hydrogels

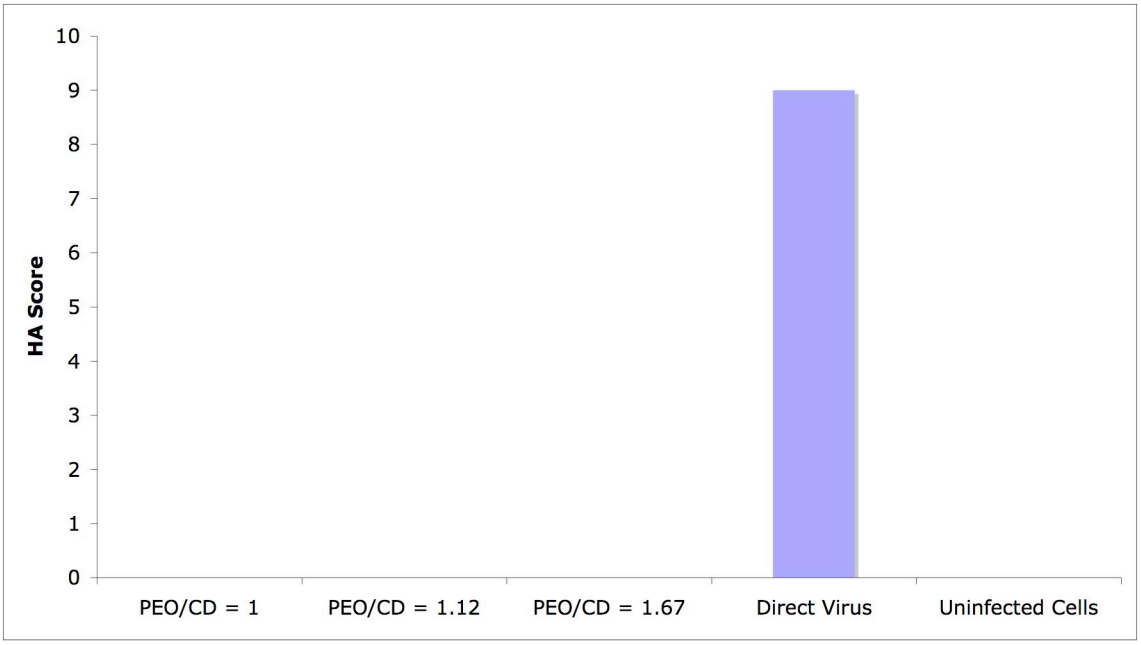


Figure 30: Result of hemagglutination study on supramolecular hydrogels

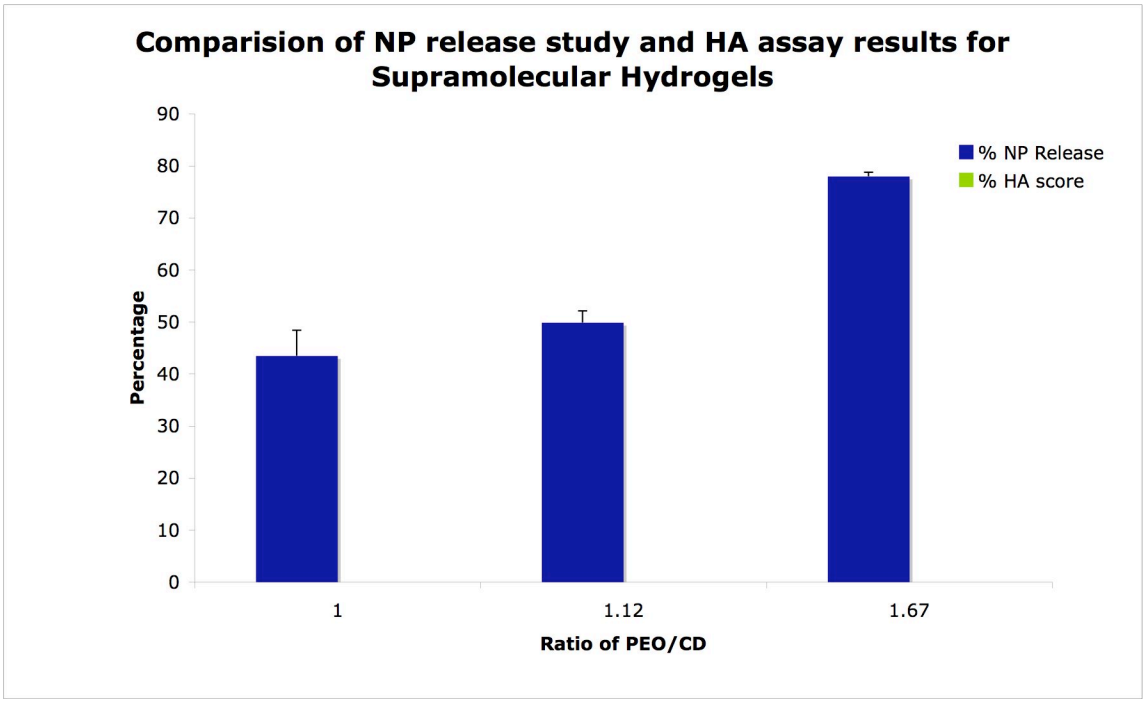


Figure 31: Comparison of nanoparticle release study and hemagglutination assay results for the second approach, i.e. supramolecular hydrogels

The results obtained from the hemagglutination study on the supramolecular hydrogels (as shown in figure 34) were very poor. None of the hydrogels showed complete hemagglutination even in the first row, where direct medium was used without any dilution. Figure 31 shows a comparison of nanoparticle release study and Hemagglutination assay results for the supramolecular hydrogels. The results show that the supramolecular hydrogels might be reacting with the encapsulated virus, which thereby leads to its loss of activity or inability to produce hemagglutination. There is no doubt that the virus does get released from the hydrogels since all these hydrogels had shown very good release of amine-modified nanoparticles (figure 32). Therefore such poor hemagglutination scores only prove that the supramolecular hydrogels are not compatible for encapsulating biomolecules at least not the influenza Virus.

5.3. Approach III: Alginate – CaCl2 hydrogels

5.3.1. Stage 1: Extrusion Consistency Test

Nine different alginate hydrogel paste formulations were prepared using LVG Alginate (150 mPa) and dehydrated CaCl2. These hydrogels were tested manually for their extrusion consistency and were given a particular consistency score based on a scale of 1 to 5.

The details of the nine, alginate hydrogel pastes and their results obtained from the extrusion consistency test are given in Table 20.

Conditions	**Incubation Temperature**		**4 ^{O}C**			
	Incubation Time		**12 hours**			
Sample #	**CaCL2 Concentration**		**LVG Alginate (150 mPa) Concentration (%)**	**Extrusion Consistency values of triplicates**		**Average Consistency Score**
	mg/mL	**mM**				
1	**0.1**	**0.6**	**4.5**	**5**	**5**	**5**	**5**
2	**0.1**	**0.6**	**5.5**	**5**	**5**	**5**	**5**
3	**0.1**	**0.6**	**6.5**	**5**	**5**	**5**	**5**
4	**0.15**	**0.9**	**5.5**	**5**	**5**	**5**	**5**
5	**0.15**	**0.9**	**6.5**	**5**	**5**	**5**	**5**
6	**0.15**	**0.9**	**7.5**	**5**	**5**	**5**	**5**
7	**0.2**	**1.2**	**6**	**5**	**5**	**5**	**5**
8	**0.2**	**1.2**	**7**	**5**	**5**	**5**	**5**
9	**0.2**	**1.2**	**8**	**5**	**5**	**5**	**5**

Table 20: Extrusion consistency results obtained for the different alginate-CaCl2 hydrogel

The extrusion consistency test on the nine different alginate hydrogel pastes gave very good results. As can be seen in Table 1, all the hydrogels prepared gave a consistency score of 5. A consistency score of 5 denotes, excellent extrusion consistency with

absolutely no bleeding while extrusion. Even though all the gels did earn a score of 5, we observed that the gel was getting thicker with increase in concentration of alginate for a given CaCL2 concentration.

5.3.2. Stage 2: Nanoparticle Release Study

All the nine hydrogels tested in stage 1 (extrusion consistency test) scored a consistency score of 5. Since it was not feasible to perform the nanoparticle release study on all the nine formulations we chose two of them for stage 2.

The details of the samples selected for the nanoparticle release study are given in Table 21 and the result obtained from this study is shown in figure 32.

	Nanoparticles Composition		**2% solids, 200 nm amine modified fluorescent nanoparticles**	
	Incubation Temperature		**4^{O}C**	
	Incubation Time		**12 hours**	
	Release Medium		**HBSS, 1X**	
Conditions	**Release Study performed for**		**24 hours**	
Sample #	**CaCL2 Concentration**		**LVG Alginate Concentration (%)**	**Concentration of NP (ug/uL)**
	mg/mL	**mM**		
1	**0.1**	**0.6**	**5.5**	**0.25**
2	**0.1**	**0.6**	**6.5**	**0.25**

Table 21: Details of the nanoparticle release study performed on three different supramolecular hydrogels

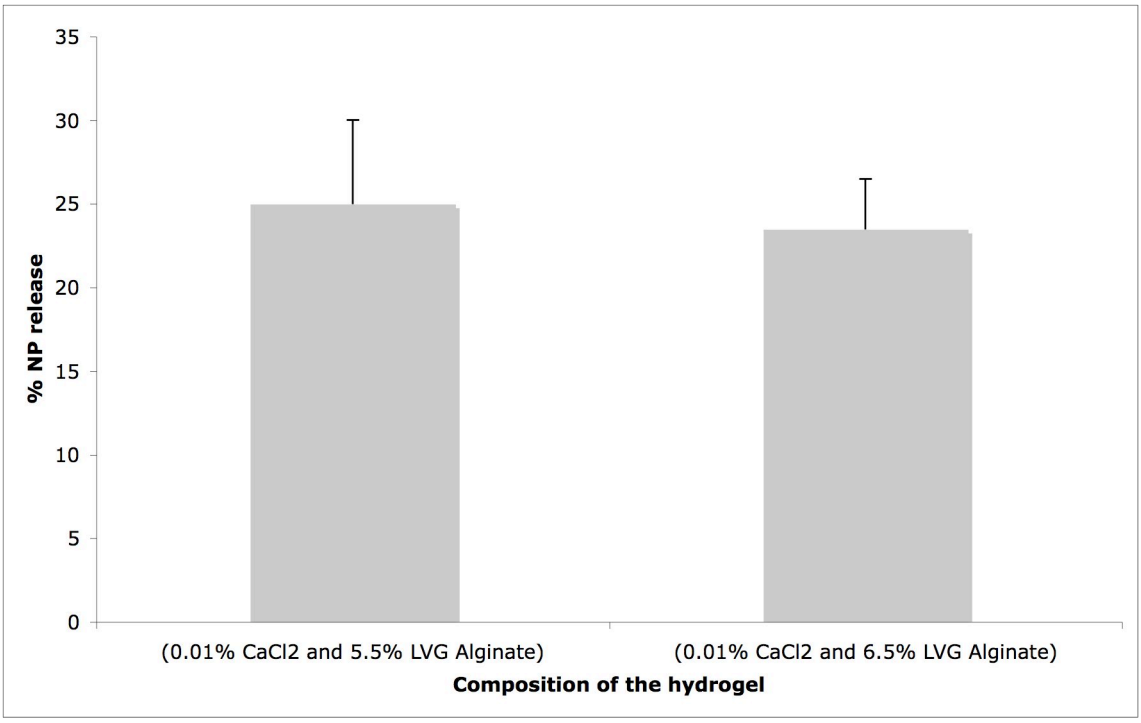


Figure 32: Result of nanoparticle release study on three different supramolecular hydrogels

The result obtained from the nanoparticle release study on the two selected alginate hydrogel paste formulations, showed almost 25% release of nanoparticles from both the formulations. Also, visual observation of the gels after 24 hours in the HBSS medium showed almost no sign of the gel in the medium, which indicates complete dissolution of the hydrogels in 24 hours.

5.3.3. Stage 3: Virus Release / Hemagglutination Study

Since both the hydrogels chosen for nanoparticle release study showed very good release in 24 hours, we decided to perform the virus release study on both of them, to see if their internal environment is compatible for biomolecules (in our case, Influenza Virus)

The details of the virus release study are given in table 22 and the result obtained in terms of hemagglutination score is shown in figure 33.

Conditions	**Biomolecule used**		**Influenza Virus**	
	Incubation Temperature		**4^{O}C**	
	Incubation Time		**12 hours**	
Sample #	**CaCL2 Concentration**		**LVG Alginate Concentration (%)**	**Volume of virus (per 100 uL hydrogel)**
	mg/mL	**mM**		
1	**0.1**	**0.6**	**5.5**	**1**
2	**0.1**	**0.6**	**6.5**	**1**

Table 22: Details of the virus release study performed on supramolecular hydrogels

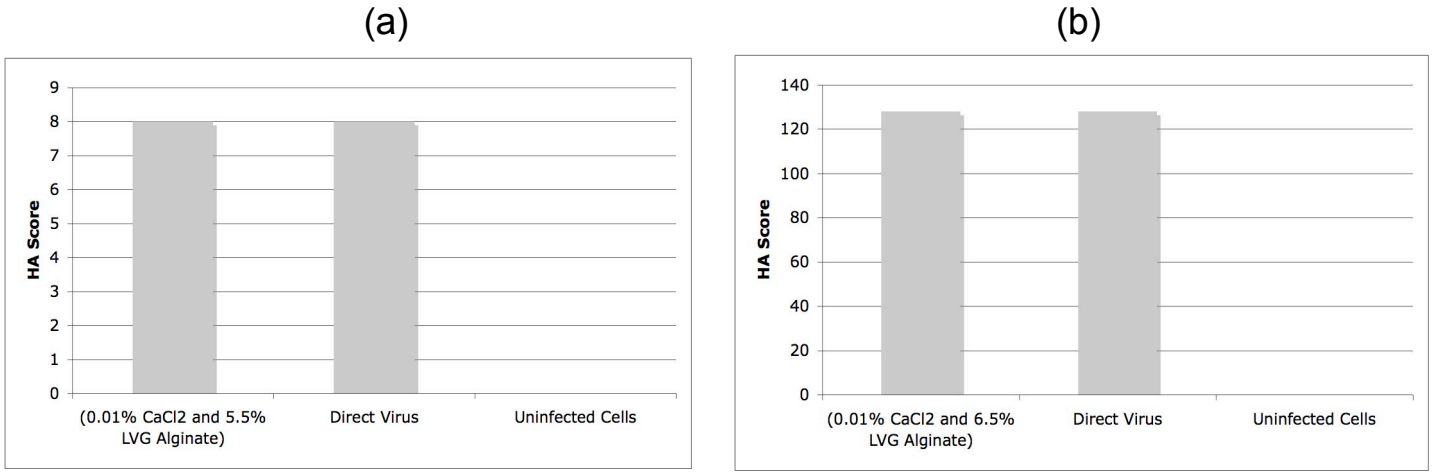


Figure 33: Hemagglutination study results for alginate hydrogel pastes. (a): Hemagglutination study result for (0.01% CaCl2 and 5.5% LVG Alginate) hydrogel. (b): Hemagglutination study result for (0.01% CaCl2 and 6.5% LVG Alginate) hydrogel.

As we can see in figure 33, we obtained very good hemagglutination results for both the alginate hydrogel pastes that were tested. Both of them showed equal hemagglutination as that obtained from the virus, which was added directly on the cells. If we see figure 33(a & b) we will find that the final hemagglutination score obtained for the two hydrogels is different, the first hydrogel which was prepared using 0.01% CaCl2 and 5.5% LVG Alginate, has a HA score equal to 8, whereas the second hydrogel which was prepared using 0.01% CaCl2 and 6.5% LVG Alginate, has a HA score equal to 128. But still both hydrogels are equally good since the HA score obtained for Direct virus in the respective experiments were equal to that obtained with the gels. The difference in HA scores obtained for direct virus in the two experiments may be due to the difference in potency of the two different batches that were used in these two experiments.

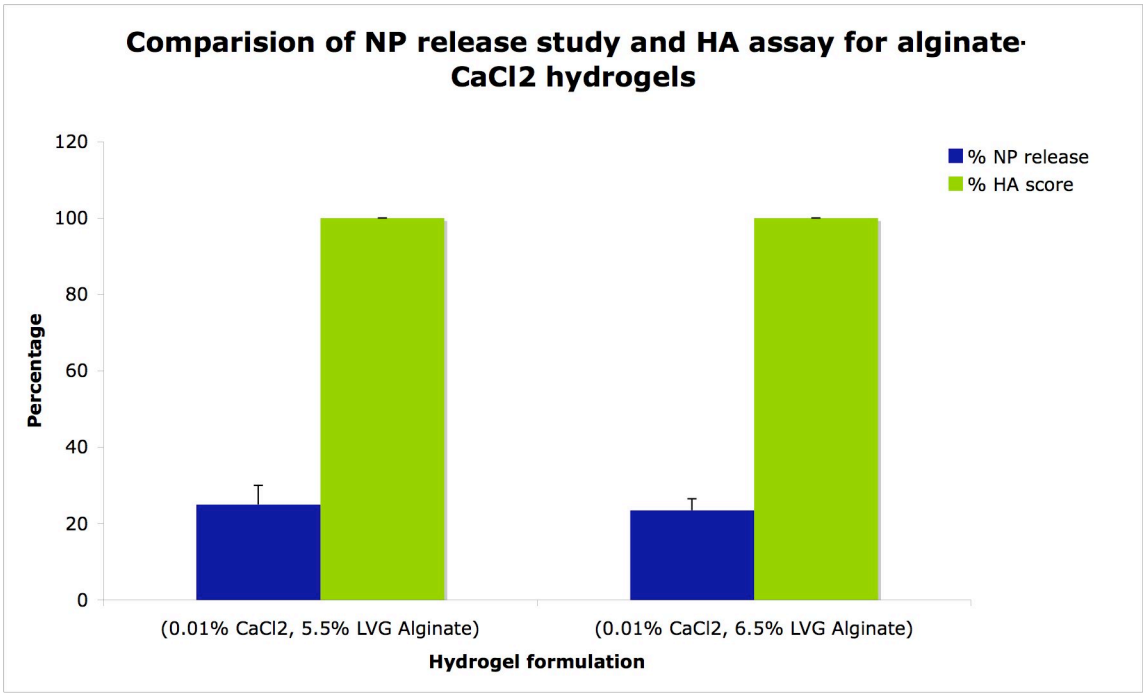


Figure 34: Comparison of nanoparticle release study and hemagglutination assay results for the third approach, i.e. alginate-CaCl2 hydrogels

Both of the alginate hydrogel paste samples showed 100% virus release (comparing the HA scores obtained from the medium which had gel and the medium where virus was added directly). As the two hydrogels showed equal hemagglutination compared to that of Direct virus we can conclude that encapsulation in the hydrogel had no adverse effect on the surface integrity of the virus and that the alginate hydrogels prepared in approach III are compatible for the influenza virus. Both these hydrogels also showed good extrusion consistency (consistency score of 5) along with almost 25% release of 200nm fluorescence nanoparticles in 24 hours. Therefore these alginate hydrogel pastes meet all the requirements of our desired hydrogel, which includes good injectability without bleeding through 26G needle, fast release of particles and good compatibility with biomolecules.

6. Discussion

6.1. First Approach: Alginate-CaSO4 Hydrogels

In general the results of extrusion consistency test for most of alginate-CaSO4 gels didn't came out very good. Most of the gels showed a lot of bleeding or leakage of water while extrusion through 26 G needles.

The results obtained with LVG alginates show that for the lowest CaSO4 concentration i.e. 3650 ug/mL the consistency score decreases with increasing TSMP concentration. However for gels with CaSO4 concentrations of 4550 and 5550 ug/mL the consistency score improves with increase in TSMP concentration. This behavior can be explained by understanding the nature of TSMP. TSMP is a calcium sequestrant. Therefore if we add more TSMP in a solution with low amount of Calcium, it will bind all the available calcium and leave much less for crosslinking. This is the very reason why we saw poor consistency for gels with CaSO4 concentration of 3650 ug/mL and more than 0.2% TSMP concentration. A score more than 4, denotes that these three hydrogels can be extruded out through a 26G needle very smoothly with minimum phase separation or bleeding. The closer the score is to 5, the better the consistency.

Increasing the concentration of CaSO4 increased the crosslinking density and hence a better extrusion consistency but this rule was followed only till a certain maximum limit of CaSO4 concentration, which depended on the percentage, viscosity and distribution of

alginate used. After the maximum limit of CaSO4 concentration is crossed, we found that a part of alginate solution binds to the high amount of Ca^{++} available and becomes a thick gel but a part remains in solution. Thus when these gels are extruded though a needle we observe a phase separation and we obtain poor consistency.

Among LVG alginate gels three formulations got a consistency score more than 4. These formulations were selected for the Nanoparticle release study. The result obtained was very unenthusiastic. None of the gels showed even more than 1% release. The unsubstantial release can be mainly ascribed to the presence of high % of Guluronic acid residues in the alginate used. The high % of G must have lead to the formation of highly crosslinked, less porous hydrogels. Since the hydrogels were so densely crosslinked, they could have limited both the diffusion and degradation processes, which help in the release. Since we obtained very poor results in the nanoparticle release study for gels prepared using LVG alginates we did not perform the Virus release study /Hemagglutination assay on these gels.After performing a few experiments with LVG alginates where we varied both the TSMP and CaSO4 concentration, we found that it would be very difficult to understand the system behavior if we had more than one parameter. So we decided to keep the TSMP concentration constant at 0.2%, for the following experiments.

For experiments with LVM alginates we maintained the TSMP concentration constant at 0.2% and varied the final CaSO4 concentration. The results showed a similar behavior in regard to consistency (Table 7). We managed to increase the consistency by increasing CaSO4 concentration but only up to CaSO4 concentration of 3.2 mg/mL. Increasing the

CaSO4 concentration beyond 3.2 mg/mL led to the formation of very thick gels, which thus reduced their consistency score. Out of the five different samples tested with LVM alginates none of them showed very good consistency. A reason for this can be the presence of large M-rich regions in the alginate used. High M and Less % of G-regions might have lead to the formation of softer and more fragile gels.

Nanoparticle release study was performed on two formulations of LVM alginates. As expected the LVM alginate hydrogels demonstrated a higher % release in 24 hours than that obtained using LVG alginates. Since the gels were more softer and fragile, we postulate that they would be degrading at a fairly faster rate than the highly crosslinked LVG alginate gels. Even though the LVM alginate gels showed good release they were not selected for the Virus release study /Hemagglutination assay due to their poor and inconsistent performance on the consistency test.

The LVM-LVG alginates performed in a similar fashion. One thing we observed was inclusion of LVG into LVM alginates increased the consistency when keeping all the other parameters same. The perfect example is gels prepared using 1% LVM alginate, 0.2% TSMP and 2.85 mg/mL CaSO4 gave a consistency of 3.7 whereas gels prepared using LVM-LVG (1.0-0.5%) alginate mixture, 0.2% TSMP and 2.85 mg/mL CaSO4 gave a consistency of 4.3. This example proves that our decision of preparing gels using a mixture of LVM-LVG alginates does work. Using LVM-LVG mixture we did manage to increase the consistency keeping the CaSO4 concentration constant. This property was very important in our project since along with getting a gel with good consistency we also require it to release the encapsulated agents faster. This was only possible by

keeping the final CaSO4 concentration to a lower value.

Out of the various LVM-LVG alginate gel formulations, only two showed a consistency score better than 4, which were thereby selected for the nanoparticle release study. We observed about 5-6% release of nanoparticles from these formulations. The % release was same as that obtained using only the LVM formulations keeping the TSMP and CaSO4 concentrations same (comparing figures 24 & 20). Thus we see that the addition of LVG alginates to LVM alginates helps in increasing the consistency, without compromising on the % release.

We tried to assess the effect of one more parameter i.e. amount of time allowed for crosslinking. To perform these tests we used the LVM-LVG (1.0-0.5%) alginate formulation. Till now we have been allowing a 24-hour time period for curing of the hydrogel. For these experiments we changed it to 72 hours and used three very low CaSO4 concentrations to check if the crosslinking is really complete by 24 hours or it still continues beyond that. We would know if the crosslinking continues, because this would be directly visible in the consistency test. From previous experiments we knew that, for LVM-LVG (1.0-0.5%) and 0.2% TSMP concentration the gels do not show consistency scores even better than 3 for CaSO4 concentrations below 2.0 mg/mL. The results obtained from this experiment (Table 14) showed a clear increase in consistency for similar CaSO4 concentrations performed previously with 24-hour curing period (Table 11). This experiment proved that all the gels we had studied previously were not completely crosslinked at the point they were tested. The continuous crosslinking can be

attributed to the low solubility of CaSO4 (2 mg/mL) in water. Because of the low solubility, CaSO4 ionizes at a very slow rate, providing a constant slow release of Ca++ ions for a long time. This continuous availability of Ca++ ions leads to the prolonged crosslinking of the hydrogel. This property is undesirable for our application, since we need a gel, which can release faster but since alginate-CaSO4 gels continue to crosslink they are actually creating more hindrances in the release of the encapsulated agent.

We also performed the nanoparticle release study with the above formulations, and obtained a higher % release than any of the previous formulations we tested before. The % release was inversely proportional the final CaSO4 concentration. The high % release obtained was expected since we used low final CaSO4 concentrations.

Out of all the various formulations of alginate-CaSO4 hydrogels, we selected five formulations for the final Virus release study/Hemagglutination assay. Out of the five, two were the LVM-LVG alginate formulations that showed consistency score better than 4, whereas the other three were the LVM-LVG formulations, with low CaSO4 concentrations and which were allowed to cure for 72 hours. The results of these studies are shown in terms Hemagglutination Scores. The hemagglutination score obtained from the medium in which the hydrogels were added, has to be compared to the hemagglutination score obtained from the medium where the virus was added directly. This comparison can be considered to be an estimate of % virus released from the hydrogel if we assume that the hydrogel has no effect on the surface integrity of the virus or in other words the internal environment of the hydrogel is compatible for the virus. The dimensions and charge of the nanoparticles match very closely to that of the

Influenza virus particles. Therefore we expect a similar release profile for the virus. The % virus release considering the hemagglutination scores, should must be more than or at least equal to % release obtained for nanoparticles in 24 hours, since in the hemagglutination study the virus is allowed to release for 72 hours. In 72 hours we expect more % release than that obtained in 24 hours, since both diffusion of virus and degradation of gel are continuing for 72 hours. Therefore if we obtain less % release of virus (based on hemagglutination scores), it will indicate some loss of functionality of the virus due to its encapsulation in the hydrogel. In such case we can conclude that the hydrogel environment is not compatible for biomolecules (in this case, influenza virus).

Out of the five the first two, hydrogel formulations showed very less hemagglutination (only about 10% compared to direct virus), whereas the last three formulations showed almost about 75% hemagglutination (compared to Direct Virus). But for all the LVM-LVG alginate hydrogels formulations the % virus release was greater than % nanoparticle release in 24 hours. This indicates that the hydrogels made from the mixture of LVM and LVG alginates were compatible with the virus and had no effect on its potency or functionality.

However, within the scope of this project we need hydrogels with very good extrusion consistency (consistency score > 4), fast release and compatible with biomolecules. We got all these properties in various different formulations of alginate-$CaSO_4$ hydrogels but no one formulation had combination of all three together.

6.2. Approach II: Supramolecular Hydrogels

Supramolecular hydrogels in general performed well on the extrusion consistency test. Consistency seemed to depend mostly on the ratio of PEO/α-CD (w/w) used, but there was no direct co-relation between the two. We tested 12 formulations with ratios varying from 0.5 to 3.0. Initially with increase in the ratio, the consistency increased but only up to ratio of 1. The hydrogels with ratios of PEO/α-CD less than 1 were very thick and were too difficult to extrude. This was probably due to excess amount of α-CD present compared to PEO. From ratio of 1 to 1.75, we obtained hydrogels with a perfect consistency score of 5, which signifies very good crosslinking and no bleeding during extrusion. All these ratios might be falling in the range that refers to the perfect number of PEO and α-CD molecules required to form the optimum hydrogels that are highly crosslinked and still injectable through 26 G needles. But hydrogels with ratio greater than 1.75, again lost their consistency. The gels formed for these ratios showed poor gelation and some phase separation during extrusion. The reason for this can be the excess amount of PEO present compared to α-CD molecules. Many of the PEO fibers would be moving around random with no α-CD molecules to bond with.

Three formulations with PEO/ α-CD ratios of 1, 1.12 and 1.67 were chosen for the nanoparticle release study. The % release of nanoparticles in 24 hours, obtained from these hydrogels was very promising. The % release increased with the increasing ratio of PEO/ α-CD. The least was about 40% obtained for ratio 1, whereas the maximum was about 70% obtained for hydrogels with ratio 1.67. As has been already mentioned the

gels become more and more thin and watery with the increase in the amount of PEO w.r.t. CD clearly explains why we see an increase in % release, for higher ratios of PEO/CD. The best supramolecular hydrogel from the first two in-vitro tests is the one with PEO/ α-CD ratios of 1.67. It releases the maximum amount of nanoparticles and still manages to keep very good consistency during extrusion.

The same three hydrogels that were used in nanoparticle release study were selected for the final in-vitro test i.e. Virus release study or Hemagglutination assay. No hemagglutination was obtained for any of the three gel formulations. We know that this condition was not because of impaired virus release (since these hydrogels had already shown very good release of nanoparticles, which match in size and surface charge with the virus). So the only other reason would be inactivity of the virus due to encapsulation in the hydrogel. These results show that the supramolecular hydrogels react with the encapsulated virus, which might be leading to its loss of potency or inability to produce hemagglutination. Thus supramolecular hydrogels are not compatible for encapsulating biomolecules at least not the influenza Virus.

6.3. Approach III: Alginate-CaCl2 hydrogel

Alginate-CaCl2 hydrogels prepared using the reverse addition technique usually have the perfect consistency if prepared using the right concentration of CaCl2 and alginate. We found that one specific CaCl2 concentration can be used to produce all the way from easy flowing to very thick hydrogels, the final consistency depends on the amount of alginate added. In our experiments we tried three different CaCl2 concentrations and used three

different concentrations of LVG alginate for each. All these nine formulations could be extruded with perfect consistency and no phase separation.

We selected two formulations for the nanoparticle release study, and both of them showed very good release, about 25% in 24 hours. There are few reasons for such high release. First the amount of crosslinking agent added i.e. CaCl2 is very less compared to what we needed to use with CaSO4. Using CaSO4 the minimum concentration we could go was 0.19 %, to obtain a consistency score of at least 4. But with CaCl2 we could use a concentration of 0.01% and still manage to achieve the perfect consistency. Second reason for good release can be that the alginate-CaCl2 hydrogel has not reached the jelly state (lump) but it is in the form of a highly viscous thick paste of alginate. We could only manage to achieve this by the reverse addition technique. In this technique we don't depend on the diffusion of Ca++ ions into the alginate else we depend on the diffusion of alginate molecules into the CaCl2 solution. The CaCl2 solution is kept very dilute so that there are no excess Ca++ ions in the solution. Since the amount of Ca is so less, we don't really allow the formation of a hydrogel lump but instead we just make the alginate solution highly viscous. This is how we attain the perfect consistency along with fast release of encapsulated agents.

We performed the virus release study/ Hemagglutination assay on the same two formulations we used for the nanoparticle release study. The result of the hemagglutination assay was impressive. Both the hydrogels showed 100% hemagglutination compared to direct virus. This shows that the alginate-CaCl2 hydrogels are compatible with the virus and cause no effect on it's potency.

From the three in-vitro tests on the alginate-CaCl2 hydrogels, we found that they have very good extrusion consistency; fast release characteristics and they are compatible with biomolecules (influenza virus).

7. Conclusion

We have successfully found a type of alginate hydrogel composition that can be used for safe delivery of bioactive substances of diameter 200 nm or below to animals, that if delivered in an aerosol or free form, many have an harmful effect on that animal or other living organisms in the vicinity. The hydrogel composition has shown a fast release of encapsulated bioactive substances and is firm enough to be injected through a 26G needle without any phase separation or bleeding. This unique property allows this hydrogel to be used in parenteral drug delivery or vaccination. The hydrogel composition can be injected subcutaneously or intradermally with no aerosol formation while injection.

The most challenging part of the project was to imbibe both, good extrusion consistency and fast release characteristics in the same hydrogel. We studied three different types of hydrogels, namely alginate hydrogel prepared using internal setting method with CaSO4 as the crosslinking agent, supramolecular hydrogels and another type of alginate hydrogel prepared using CaCl2 as the crosslinking agent. The model biomolecule chosen was Influenza Virus. All the three types of hydrogels went through a three stage short listing process, which involved extrusion consistency test (to make sure the hydrogels don't bleed when extruded from a fine needle (26G)); nanoparticle release study (to study the % release of 200 nm particles) and finally the Virus release study or Hemagglutination

Test to study if the internal environment of the hydrogels is compatible for biomolecules and that it doesn't have any effect on the biomolecule integrity.

Out of the three, the two types of alginate hydrogels (approach I & III) seemed to maintain the integrity of the encapsulated biomolecule whereas the supramolecular hydrogels tend to react with biomolecule leading to its loss of potency. Thus we can conclude that alginate in itself doesn't react in any way with the biomolecules (at least not with Influenza Virus) and thus can serve as a good host to encapsulate drugs or vaccines.

The choice of the crosslinking agent in the preparation of alginate hydrogel plays a very important role since we have seen that, using calcium salts with different solubility's yield completely different type of hydrogel. We discovered that, when CaSO4 is used for gelation (solubility in water, 0.0025g/mL) [168] it continues to crosslink alginate for many days, as the crosslinking continues with the availability of free calcium ions (which become available at a slow rate as CaSO4 ionizes in water). On the other hand if we use CaCl2 (solubility in water, 0.745g/mL) [168], it ionizes instantly and all the free calcium ions binds to the alginate molecules. Also since we used the reverse addition method where add alginate dry powder into the CaCL2 solution, the rate-limiting step in gel formation is the rate at which the alginate molecules can pass into the solution. Therefore our third approach where we used CaCl2 as the crosslinking agent gave us completely crosslinked and consistent gels within a couple of hours.

Two formulations of the third approach (alginate-CaCl2 hydrogel) with specific concentration of alginate and the crosslinking agent were found to satisfy all the properties we desired in our hydrogel. Though we propose that these hydrogels can be used with any biomolecule, the studies performed in this project are only limited to Influenza Virus and individual tests must be performed for each biomolecule to test it's activity in these hydrogels.

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9. Appendix I: Materials

First Approach: - Alginate hydrogel prepared by internal setting method using $CaSO_4$ as crosslinker

LVG & LVM alginates, purchased from FMC, Biopolymers.

Trisodium metaphosphate, purchased from Sigma.

Calcium Sulfate dihydrate, purchased from Fluka.

Second approach: - Supramolecular Hydrogels made of PEO and cyclodextrins

Polyethylene Glycol (M.W. 10,000) (PEG or PEO) purchased from Alfa Aesar

α-Cyclodextrin purchased from TCI America

Third approach: - Alginate hydrogel paste using $CaCl_2$ as crosslinker

LVG Alginate, purchased from FMC, Biopolymers

Calcium chloride, dehydrated; purchased from Sigma Aldrich

General Materials, which were used in all the three approaches: -

200 nm Amine modified Fluorescence particles (FP), purchased from Invitrogen.

HBSS, 1X

Influenza Virus A/PR/8/34(H1N1)

DMEM medium

Trypsin (0.25% in 0.1% EDTA solution) (without Ca^{++}, Mg^{++} and sodium bicarbonate)

TPCK trypsin (Roche)

1X, Ethidium bromide-acridine orange (Cell Counting Solution)

Chicken Red Blood Cells (CRBC)

1.5mL eppendorf Tubes

24 well plate

U-bottom 96 well plate (transparent)