Studies on the release behavior of drugs in hyaluronic acid-nano/micro carrier composite hydrogels

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**STUDIES ON THE RELEASE BEHAVIOR OF
DRUGS IN HYALURONIC ACID-
NANO/MICRO CARRIER COMPOSITE
HYDROGELS**

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**SCHOOL OF MATERIALS SCIENCE AND
ENGINEERING**

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ENGINEERING**

A thesis submitted to Nanyang Technological University in
partial fulfilment of the requirement for the degree of Doctor of
Philosophy

2015

Dedicated to My Dear Lord Krishna

*Hare Krishna Hare Krishna, Krishna Krishna Hare Hare
Hare Rama Hare Rama, Rama Rama Hare Hare*

Abstract

Hyaluronic acid (HA) is a natural polymer that has gained significant attention as a potential biomaterial for a wide range of biomedical applications due to its unique physical and chemical properties. The mechanical and degradation properties of HA can be improved by physically or chemically crosslinking the chains into hydrogels. HA hydrogels have been extensively investigated as drug delivery carriers; however, the release of entrapped drugs is rapid and cannot be sustained significantly. Composite hydrogels of HA with drug loaded particulate carriers have been developed recently that combine advantages of physical/chemical properties of HA and sustained release property of entrapped drug carriers. These systems have not been studied in detail in terms of the transport behavior of drugs through the crosslinked networks. This transport is affected by several factors and understanding the mechanisms of drug transport would help to optimize the design of HA based composite hydrogels for specific therapeutic applications.

In this work, HA based composite hydrogels were developed with drug loaded egg phosphatidylcholine (EPC) liposomes, poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles (NPs), and PLGA microparticles (MPs) and investigated for *in vitro* drug release and physical stability of entrapped carriers. 5-Fluorouracil (5Fu) and latanoprost (Ltp) were selected as model hydrophilic and hydrophobic drug, respectively. HA was modified using methacrylic anhydride (MA) and adipic dihydrazide (ADH) moieties and then crosslinked to obtain hydrogels and characterized for morphology, swelling behavior,

rheology, and *in vitro* drug release. 5Fu was loaded into PLGA MPs and Ltp was loaded into EPC liposomes, PLGA NPs, and PLGA MPs. The delivery carriers were characterized for morphology, size distribution, % encapsulation efficiency (EE), and *in vitro* drug release. From drug release studies, it was found that 5Fu was rapidly released from HA hydrogels alone but encapsulating it inside PLGA MPs prolonged the release up to several days. Ltp was found to release slowly from HA hydrogels alone but the release was sustained from PLGA MPs and EPC liposomes for several days and weeks, respectively. In case of composite hydrogels, the release of 5Fu and Ltp was significantly retarded as compared to MPs and liposomes alone. Retardation from composite hydrogels was dependent upon hydrogel network structure surrounding the entrapped particulate carriers in case of 5Fu release but not for Ltp release. Degradation studies showed that there was no difference in the degradation rate of drug loaded PLGA MPs with or without hydrogel, thus indicating that the retardation of drugs from composite hydrogels was due to the additional diffusion barrier of gel network surrounding the MPs and liposomes.

Future work should involve further evaluation and optimization of various factors that affect drug transport in these HA based composite hydrogels to develop them into potential sustained release carriers for different types of drugs. Drug release from composite hydrogels in the presence of enzyme hyaluronidase would be useful to study for estimating the *in vivo* release behavior. In addition, quantitative estimation of diffusion coefficients and resistances would be important to understand the drug release kinetics of composite systems in detail and to develop a generalized model.

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List of Abbreviations

ACN	Acetonitrile
ADH	Adipic dihydrazide
CE	Cellulose ester
CS	Chitosan
DCM	Dichloromethane
DI	Deionised
DLS	Dynamic light scattering
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DPPC	Dipalmitoyl- phosphatidylcholine
EDC	1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EE	Encapsulation Efficiency
EPC	Egg phosphatidylcholine
FESEM microscope	Field emission scanning electron
5Fu	5-Fluorouracil
HPLC	High performance liquid chromatography
Hr/Hrs	Hour/Hours
HA	Hyaluronic acid
I2959	Irgacure 2959
IPA	Isopropyl alcohol
IV	Intrinsic viscosity
LBL	Layer-by-layer
Ltp	Latanoprost
LUVs	Large unilamellar vesicles
LVE	Linear viscoelastic range
MA	Methacrylic anhydride
MALS	Multi angle light scattering
Min/Mins	Minute/Minutes

MLVs	Multilamellar vesicles
MPs	Microparticles
MWCO	Molecular weight cut off
NPs	Nanoparticles
NMR	Nuclear magnetic resonance
o/w	Oil-in-water
PBS	Phosphate buffer saline
PC	Polycarbonate
PDI	Polydispersity index
PEGDE	Poly(ethylene glycol) diglycidyl ether
PGA	Poly(glycolic acid)
PLA	Poly(lactic acid)
PLGA	Poly(d,l-lactic- <i>co</i> -glycolic acid)
PS	Polystyrene
PVA	Poly(vinyl alcohol)
RT	Room temperature
Sec/Secs	Second/Seconds
SEC	Size exclusion chromatography
SEM	Scanning electron microscope
s/o/w	Solid-in-oil-in-water
TEM	Transmission electron microscope
TPP	Trisodium polyphosphate
UV/Vis	Ultraviolet/Visible
v/v	Volume by volume
w/o/w	Water-in-oil-in-water
w/v	Weight by volume

Chapter 1 Introduction

1.1 Background

There have always been extensive efforts directed towards the development of novel devices with potential pharmaceutical or clinical significance, for instance, novel systems for delivery of drugs and other biomolecules. This is to achieve the target or site specific delivery of therapeutic agents in a rate and/or time controlled manner for prolonged periods, enhancing patient compliance. Among the several types of polymeric delivery carriers that have been investigated for drug release, hydrogels have found considerable interest.

On account of their peculiar and remarkable advantages, such as, ability to swell in physiological environment, biocompatibility, ability to formulate them into variety of physical forms, tunable structural properties, and exhibiting wide range of chemical compositions and physical properties, they have been extensively investigated for several biomedical applications [1-3]. Among this wide range of applications, drug delivery systems based on hydrogel carriers have attracted significant research interest.

Hydrogels offer numerous advantages as delivery devices, for instance, protection of drugs from hostile environments (low pH in stomach, presence of enzymes), high porosity enabling loading of drugs in the gel matrix, and control of drug release from the matrix by changing its structure in response to environmental stimuli such as pH, temperature, and electric field [2]. Hydrogels can be synthesized using several synthetic and natural polymers. Among

various natural polymers used as hydrogel systems, HA has been widely explored for drugs, peptides, proteins, genes, and growth factors delivery applications. Biocompatibility, biodegradability, non-toxicity, non-immunogenicity, wound healing, and high molar mass associated viscoelasticity are the versatile properties of HA that make it a potential biomaterial.

Native, soluble HA has found several applications in drug delivery and ocular surgery. But its use is limited due to rapid rate of degradation and clearance *in vivo*, and lack of high mechanical strength [4]. These limitations could be improved by modifying HA chains with chemical derivatives and crosslinking them to obtain insoluble hydrogels. They exhibit favorable mechanical and degradation properties and still retain the biocompatibility, biodegradability, and viscoelastic properties of HA chains. Other useful properties include high aqueous swelling ratio, high porosity, susceptibility to hyaluronidase enzyme degradation, and resistance to cell adhesion *in vivo* [5].

1.2 Problem Statement

HA hydrogels have been widely investigated for oral, ocular, nasal, dermal, and other applications for sustained delivery of many therapeutic molecules [6, 7]. Various forms of hydrogel carriers such as films or membranes, NPs/MPs, injectable or implantable gels, etc have been utilized. Although the mechanical integrity of HA chains is greatly enhanced and the dissolution rate is reduced by crosslinking, it does not enable significant retardation in the release of entrapped drugs from gels alone. The rapid release of entrapped drugs from HA

hydrogels could be attributed to the macroscopic nature of their structure, with large mesh sizes and heterogeneous network crosslinked by randomly interconnected HA chains [8].

As a result, there is growing interest in composite hydrogel networks wherein the pharmacological limitations of hydrogels could be overcome by entrapping particulate systems inside the hydrogel matrix [3]. Drug loaded liposomes, NPs, MPs, and other types of particle-based drug delivery vehicles have been incorporated into synthetic as well as natural polymeric hydrogels to obtain composite systems. The combination is advantageous over the polymer or particles alone because it enables sustained release of drug for prolonged periods of time, preserves the bioactivity of drugs, improves the stability of particulate carriers, and enhances the bioavailability of drugs by restraining the carriers from being migrated away the target site [3, 9].

HA based composite hydrogels, obtained by dispersing drug loaded nano/micro carriers within HA hydrogel matrix, are the delivery systems that have not been widely explored for applications such as ocular drug delivery. These composite systems are particularly appropriate for ocular delivery applications because HA is found inside eye tissue as its natural component and possesses significant wound healing functions. The ocular residence time of drug loaded particulate carriers could be improved and prolonged sustained release kinetics can be obtained by entrapping them inside the crosslinked network of HA hydrogels exhibiting controlled mechanical and degradation properties and wound healing properties.

Composite hydrogel systems based studies have mainly focused on obtaining sustained release of drugs (reducing initial burst, increasing drug bioavailability) utilizing the drug-carrier and polymer matrix properties for a specific application. To date, there have been only a couple of studies to understand the factors affecting the transport of drugs through these crosslinked networks. While the transport behavior and release kinetics of solutes across HA ester films/membranes have been studied in detail [10-13], the same have not been evaluated for composite gels of HA with drug carriers such as liposomes or other polymeric NPs/MPs.

It would be valuable to understand the transport behavior of drugs in these composite systems which is dependent upon the properties of each component involved. This would improve designing of these delivery devices in terms of independent tunable properties of individual components. However, it is difficult to propose a unified model for release behavior of drugs from these composite systems. Some of the major challenges include complex geometries that combine hydrogel matrix network and drug carrier, morphologies and physiological properties of different phases, and complex interaction between them [14]. All these parameters have to be considered and optimized with respect to the overall device to evaluate the transport of drug from such a heterogeneous system.

In a composite system of drug loaded particles with HA hydrogel, release of drugs depend upon several factors that are hydrogel matrix, drug carrier, and drug solute specific. Among these factors, physiological properties of drug

(size, hydrophilicity, charge), composition and structure of drug carriers, interaction between drug/drug carrier with gel network, and the network structure of hydrogel matrix are most important. Understanding the effect of these factors on the release behavior of drugs would be very useful in devising means to control the release of drug from these composite hydrogels. This would help in the optimization of HA based delivery systems to target more novel therapeutic applications in ocular drug delivery.

Based on the information from literature and identification of the existing gaps thereof, the following problem statement has been proposed:

"There is inadequate understanding of drug transport mechanisms for HA based composite hydrogels systems with drug loaded nano/micro carriers"

1.3 Hypothesis

Composite systems, in general, are known to obtain sustained release of drugs for prolonged periods of time, and to improve stability of carriers and drugs due to combined benefits from individual components.

However, due to the complexity of these systems and the lack of detailed understanding of these systems in terms of drug transport and release kinetics, as stated in the problem statement above, it may be inappropriate to generalize the release behavior of drugs to HA based composite systems also. In view of this understanding, following hypothesis has been proposed for this work:

"HA based composite hydrogels, obtained by the incorporation of drug loaded nano/micro carriers in HA hydrogel matrix, affect the transport of hydrophilic and hydrophobic drugs differently and may improve the control of drug release from these delivery systems"

1.4 Objectives

Based on the problem statement and hypothesis mentioned above, the primary objectives of this work are to develop composite hydrogel systems of drug loaded nano/micro carriers with HA hydrogels and to elucidate the mechanisms of transport behavior of hydrophilic as well as hydrophobic drug through these systems. Further, the different systems would be compared and evaluated for some of the important factors affecting the release behavior of drugs through these HA based composite hydrogel systems.

1.5 Scope and Strategies

HA would be modified with chemical moieties and crosslinked to obtain hydrogels. 5Fu and Ltp are selected as model hydrophilic and hydrophobic drug, respectively for loading either into hydrogels directly or into different types of nano/micro carriers. The drug loaded nano/micro carriers would be mixed with modified HA macromer solution and then crosslinked to develop composite hydrogels. For nanocarriers, EPC liposomes and PLGA NPs would be prepared to entrap Ltp. For microcarriers, PLGA MPs would be prepared to entrap 5Fu and Ltp. These nano/micro carriers loaded HA composite hydrogels would be characterized for stability of entrapped drug carriers and *in vitro* release study to evaluate the transport behavior of 5Fu and Ltp.

Following strategies would be approached in order to achieve the objectives of this project:

1. Modification and synthesis of HA hydrogels – HA would be modified with MA and ADH derivatives and crosslinked via UV light and a chemical agent poly(ethylene glycol) diglycidyl ether (PEGDE), respectively to obtain hydrogels.
2. Characterization of HA hydrogels – Hydrogels would be characterized for their morphology, network structure, and mechanical properties.
3. Loading of drugs into hydrogels and *in vitro* release – 5-Fu and Ltp would be loaded into HA hydrogels and release profile for both the drugs would be obtained.
4. Preparation and characterization of drug loaded carriers – 5Fu would be loaded into PLGA MPs by solid-in-oil-in-water (s/o/w) emulsification method. Ltp would be passively loaded into EPC liposomes by thin film hydration technique and extrusion. Ltp would also be loaded into PLGA NPs and MPs via oil-in-water (o/w) emulsion method. These carriers would be characterized for size distribution, morphology, % EE, and *in vitro* release profile of 5Fu as well as Ltp.
5. Preparation of composite hydrogels – Modified HA (HA-MA and HA-ADH) macromers would be suspended in Ltp loaded EPC liposomes, mixed with 5Fu loaded PLGA MPs, and Ltp loaded PLGA NPs and MPs, and subsequently photocrosslinked or chemically crosslinked to develop composite hydrogels. These hydrogels would be characterized for *in vitro* release of Ltp and 5Fu and physical stability of liposomes, NPs, and MPs.
6. Evaluation of factors affecting the drug release behavior through composite hydrogels –

- Type of drug (hydrophilic vs hydrophobic drug)
- Type of drug loaded carriers (liposomes and PLGA NPs)
- Size of drug loaded carriers (liposomes, PLGA NPs, and PLGA MPs)
- Crosslinking density of hydrogel network (concentration of macromer, 2 % and 4 % HA-MA, and type of macromer, 4 % HA-MA and 4 % HA-ADH)

1.6 Outline of thesis

The thesis is broadly divided into five chapters. Chapter 1 presents a brief background to the field of hydrogels and drug delivery applications of HA hydrogels. This is followed by a detailed discussion of motivation and identification of the problem statement, hypothesis, objectives, and scope of the project and the strategies approached thereof. The chapter ends with a brief outline of the complete thesis. Chapter 2 deals with the review of past literature with respect to properties (structure, solution, degradation, and rheology), functions, and applications of HA, HA hydrogels, HA hydrogels based delivery systems, composite hydrogel drug delivery systems, release mechanisms of drug delivery systems, a brief discussion of model drugs used in this work, and the composite hydrogel systems that have been investigated for their delivery. The section of composite hydrogel drug delivery systems mainly focuses on systems comprising liposomes, PLGA NPs, and MPs as the drug carriers.

Chapter 3 describes materials and methods followed in the present work covering all the strategies mentioned above in detail. Results and discussion for

all the experiments conducted in this work are detailed in chapter 4. The chapter 4 has been further divided into different sections similar to chapter 3 for clarity of understanding of the main results of this work with respect to composite hydrogel systems. There are 3 main sections, first section describes results of 5Fu loaded PLGA MPs studies, followed by another sections describing results of Ltp loaded liposomes, PLGA NPs, and MPs. Finally, chapter 5 summarizes important conclusions for the current work, highlights the novelty of this project, and is followed by recommendations for the future work.

Chapter 2 Literature Review

2.1 Introduction to HA

Hydrogels can be defined as three-dimensional, crosslinked networks of hydrophilic polymers capable of absorbing large volumes of water, up to thousand times their actual dry weight. The hydrophilic polymers can be chemically or physically crosslinked to form the gels. Hydrogels have found a wide range of applications in drug delivery (controlled release systems), tissue engineering, prosthetic and biomedical devices, separation of cells or biomolecules, regenerative medicine, medical diagnostics, cellular immobilization, and coating or barrier materials for regulation of biological adhesions [1, 3]. Among the several polymers used for hydrogel formulations, HA represents one of the most attractive biomaterials for various cosmetic, pharmaceutical, and clinical applications owing to its remarkable properties such as biocompatibility, biodegradability, non-toxicity, non-immunogenicity, highly hydrated polyanionic structure, and high molar mass associated viscoelasticity [6, 15].

HA is a linear polysaccharide composed of a disaccharide unit of β -D-glucuronic acid (GlcUA) and N-acetyl- β -D-glucosamine (GlcNAc) as the monomer. The repeating units of this naturally occurring polymer are linked together by alternating β (1-3) and β (1-4) glycosidic bonds as shown in **Figure 1**. HA belongs to the group of polysaccharides known as glycosaminoglycans (GAGS) or mucopolysaccharides. It differs from other GAGS in being structurally simplest, non-sulphated, chain extension by monosaccharide

addition at reducing end of the chain, synthesis in plasma membrane, and not being covalently linked to a core protein to form proteoglycan [16].

Figure 1 Chemical structure of HA showing disaccharide unit linked by glycosidic bonds

2.1.1 Distribution

HA is found ubiquitously in the human body and other vertebrates. It represents the major constituent of extracellular matrix and has also been shown to occur intracellularly [17]. HA is found in high concentrations throughout the body in several tissues, highest being present in soft connective tissues, such as skin, synovial fluid, vitreous humor, and umbilical cord. It is also found in the cell coat or capsules of certain bacteria (e.g., group A *Streptococci*, *S. zooepidemicus*, *S. pyogenes*), but is absent in insects, plants, and fungi [18]. Among various animal sources, the largest content of HA is found in rooster combs. **Table 1** enlists the concentration of HA from different animal sources.

2.1.2 Synthesis and Turnover

Biosynthesis of HA, a highly controlled process, occurs in the plasma membrane of cells by the integral membrane protein called HA Synthase (HAS). HAS synthesizes linear HA molecules by alternate addition of activated

UDP derivatives of each disaccharide unit to the reducing end of the growing chain. HAS has three isoforms – HAS1, HAS2 and HAS3. All of them are capable of synthesizing HA but they differ in their reaction kinetics and the molecular weight of HA produced [6, 16, 17]. Turnover of HA is also highly organized and controlled as this would affect many physiological functions. The half-life of HA ranges from 1 day in epidermis to about 70 days in vitreous humor in humans. Within tissues, the turnover of HA occurs either through local degradation or secretion into the lymphatic/vascular system from the tissues. Local turnover occurs through enzymes, receptor-mediated endocytosis, and free-radicals within the tissues. In lymph nodes and liver, receptor-mediated endocytosis is responsible for degradation of HA whereas in kidney, clearance occurs by lysosomal degradation by specific enzymes and cellular uptake [6, 16].

Table 1 Occurrence of HA in different animal tissues and its content [18]

Tissue or body fluid	Concentration (µg/ml)
Rooster comb	7500
Human umbilical cord	4100
Human joint (synovial) fluid	1400–3600
Bovine nasal cartilage	1200
Human vitreous body	140–340
Human dermis	200–500
Human epidermis	100
Rabbit brain	65
Rabbit heart	27
Human thoracic lymph	0.2–50
Human urine	0.1–0.3
Human serum	0.01–0.1

2.1.3 Degradation

HA can be degraded by a number of physical, chemical, and biological factors. These factors include mechanical (shear) stress, acidic or alkaline hydrolysis,

irradiation, oxidation, thermal, and enzymatic degradation [6, 19]. One of the most common physical factors causing degradation of HA is ultrasonication. It causes a non-random, mechanical degradation of HA chains resulting into a bimodal distribution of molecular weights [6, 20]. Thermal degradation, on the other hand, cause random chain scission of HA at temperatures higher than 100 °C depending upon their exposure times [21].

Chemical factors include pH dependent hydrolysis and degradation by hydroxyl radicals during freeze drying. pH dependent hydrolysis, either at acidic or alkaline conditions, is due to random chain scission of glycosidic bonds. Under acidic conditions, nucleophilic attack on glucuronic acid residue leads to chain scission by ring opening and glycosidic bond cleavage in one step. Under basic conditions, electrophilic attack on N-acetylglucosamine residue leads to chain scission by glycosidic bond cleavage in two steps. HA solutions are most stable around neutral pH range ($5 < \text{pH} < 11$) and labile under strong acidic or basic pH [6, 19, 22].

Degradation of HA can also occur during freeze drying via the secondary reaction of hydroxyl radicals derived from the carboxyl residues. It has been found that free HA is more susceptible to hydrogen abstraction than sodium hyluronate and this degradation can be suppressed by using hydroxyl radical scavengers [6, 23].

Free-radical and enzymatic methods are those involved in the degradation of HA in biological environment. Both methods cause the HA solutions to degrade

randomly [6, 20]. The enzymes are known as hyaluronidases or hyases. They belong to a heterogeneous class of enzymes having broad range of HA hydrolysis specificities [24]. There are six associated genes in human genome and some of their important proteins involved in enzymatic activity include Hyal1, Hyal2, Hyal3, PH-20, and SPAM-1 [21]. The enzymes are hydrolases that cause hydrolytic cleavage of glycosidic bonds of HA.

Degradation by hyaluronidases proceeds by addition of water molecule across the cleavage site via double displacement mechanism while preserving the substrate conformation of HA [21]. In this mechanism, the carbonyl oxygen of N-acetyl group is positioned next to the catalytic site of glycosidic bond to form a covalent intermediate with the C-1 carbon of same sugar residue. This causes cleavage of glycosidic bond as well as inversion of anomeric configuration of C-1 atom. Next, the glucuronic acid group donates a hydrogen atom to the glycosidic oxygen (deprotonation) leaving the glycone part of HA unaltered. This is followed by reprotonation of GlcUA residue by hydrolytic cleavage of the intermediate bond between C-1 atom and carbonyl oxygen using water molecule and start of next catalytic cycle.

Degradation by reactive oxygen species (ROS) such as singlet oxygen, hydrogen peroxide (H_2O_2), superoxide anion, hydroxyl, and nitric oxide can occur in the presence of thiols or transition metals, UV irradiation, and freeze-drying. Although ROS have important roles like cell signaling in biological systems, the excessive production of such species during inflammatory processes, also leads to the degradation of HA [20, 21, 25].

2.1.4 Structure

The chemical structure of HA was first determined by Meyer and Weissman in 1954 by chemical and enzymatic methods [6, 20]. The linear primary structure was found to be composed of alternating disaccharide repeating units of GlcUA and GlcNAc linked together by β 1-3 and β 1-4 glycosidic bonds as shown in **Figure 2 A**.

Data from light scattering, nuclear magnetic resonance (NMR) spectroscopy, viscosity measurements, x-ray diffraction, (XRD) and molecular modeling experiments have shown the presence of ordered secondary structure in HA chains as shown in **Figure 2 B**. Each disaccharide unit in the linear chain is twisted through 180° with respect to the units lying ahead and behind of it and each tetrasaccharide unit of HA consists of up to five hydrogen bonds. This leads to the formation of a flat, tape-like 2-fold single helix secondary structure as two twists (totaling 360°) of the helix bring back the original conformation [20, 26, 27]. The two sides of this structure of HA are identical but antiparallel, that is, one side runs in opposite sense to the other. In addition, there exists an extensive hydrophobic patch formed by axial hydrogen atoms of CH units, about eight of them, stretching along three neighboring sugar units, on the alternating sides of the 2-fold helix of HA.

Bonding between these hydrophobic patches can cause aggregation of large number of HA molecules thus forming complex molecular networks or matrices. This molecular meshwork of HA, called β -sheet tertiary structure (**Figure 2 C**), is stabilized by intermolecular hydrogen bonding, countering

electrostatic repulsion and holding the meshwork together. The aggregates form and dissociate depending upon the surrounding conditions and this interconversion between different conformation states cause the overall structure to undergo varying degrees of flexibility [28]. The equilibrium between these states is a result of hydrophobic, steric, and hydrogen bonding interactions that occur specifically between HA chains and their surrounding environment [6].

Figure 2 Primary (A), secondary (B), and tertiary (C) structures of HA in solutions [6]

2.1.5 Solution properties

The solution properties of HA can be described in the following points:

1. At neutral pH and physiological ionic strength, HA molecule behaves as expanded and stiffened random coil in solutions. The expanded molecular conformation could be attributed to the combined effect of intramolecular hydrogen bonding, electrostatic repulsion between carboxyl groups, and solvent interactions [17, 27, 29].
2. The multiple hydrogen bonds in the molecular structure of HA restrict rotation and flexion at glycosidic bonds creating a stiff, twisted, but mobile polymer coil.
3. High concentrations strongly favor interchain hydrodynamic interactions and entanglement of chains influencing the flexibility and permeability of HA networks. However, the chains do not transform into gel-state and remain mobile. This suggests that the solutions possess weak and transient chain associations contributing to the non-ideal behavior [30].
4. HA molecules are highly hydrated and show excluded volume effect restricting the diffusion of other macromolecules through its network [30].
5. Properties of polyanionic HA are strongly dependent upon molecular weight, concentration, and degree of polymer ionization that in turn is determined by pH and ionic strength [29].
6. The HA chains show entanglement coupling behavior even at low concentrations contributing to the unique rheological properties. Newtonian to non-Newtonian transition in characteristics is exhibited by the aqueous solutions of HA with increase in concentration, molecular weight, and shear rate. Also, the viscoelasticity of solutions increases with increase in molecular weight and concentration [6, 31].

2.1.6 Rheology

HA solutions exhibit unique rheological behavior. The mechanical cushioning, lubrication and transport functions in cartilage, synovial fluid, and vitreous humor, respectively can be attributed to the viscoelastic nature of HA. Viscoelasticity is the property of solutions showing both viscous fluid and elastic body like behavior while undergoing deformation. That is, they show both Newtonian and Non-Newtonian fluid behavior depending upon the magnitude of shear rate [32]. Besides shear rate, the viscosity of HA solutions is also a function of concentration, molecular weight, temperature, pH, solvent, and frequency of the mechanical impact [6, 17-19, 32, 33].

Viscoelasticity can be observed by the changes in complex moduli under dynamic conditions. When storage (elastic) and loss (viscous) moduli are plotted over a range of frequencies two regions can be observed. The point at which the storage modulus crosses the loss modulus and increase in magnitude is known as cross-over point. The region below this cross-over point is called viscous flow region and above the point exists the elastic region. The cross-over point shifts towards the lower value of frequencies with increase in concentration and molecular weight [34].

Viscosity increases with increase in concentration and molecular weight as shown in **Figure 3 A** and **B**, respectively [33]. Differences in viscosity among samples are more pronounced towards the lower values of shear rate. It decreases with increase in shear rate up to a certain extent and then again stabilizes at higher shear values. This is the shear thinning behavior of HA

solutions, observed due to the disruption of weak network interactions, stretching, and alignment of the chains towards the flow direction. The constant viscosity at low shear rates can be represented as zero shear viscosity of the solution (that is, the viscosity of solution at resting state).

Figure 3 Shear rate dependence of viscosity on concentration (A) and molar mass (B) [33]

Viscosification (increase in viscosity with shear rate) has also been observed for dilute solutions of HA at very low shear rates. As the solution is sheared, the HA chains extend and this might cause them to interact and form hydrogen bonds with each other forming networks increasing the viscosity [35]. However, this occurs for a very short period of time as the viscosity becomes constant and the shear rate is too low to deform the formed network. High shear rates do not allow chains to interact and form network and the polymer chains just stretch and align in the flow field decreasing the viscosity.

Figure 4 shows the change in specific viscosity of solutions with increase in concentration. It could be observed that for dilute solutions, the viscosity is proportional to concentration. As concentration increases and reaches c^* (overlapping concentration), solution starts behaving as semidilute unentangled

where $\eta_{sp} \sim c^{5/4}$. Entanglement concentration (c_e) is reached with further increase in concentration making the solutions to behave as semidilute entangled [36].

Figure 4 Specific viscosity of Na-HA in phosphate buffer saline (PBS) at 25°C [36]

Another important factor affecting the viscosity of HA solutions is pH. Viscosity is independent of pH around the neutral pH range; below and above this range, the HA solutions undergo reversible or irreversible change in viscosity depending upon the exposure time. This occurs due to random chain scission (cleavage of glycosidic bonds) of HA chains under extremes of acidic and basic conditions [37, 38]. Another important observation was the sudden increase in viscosity at pH 2.5 unlike other pH values. This paste-like or gel-like behavior of HA solutions at pH 2.5 might be due to the balance of attractive and repulsive forces acting between the HA molecular chains [34].

Viscosity of HA is also affected by temperature conditions. Viscosity reduces with increase in temperature and this is attributed to the breaking of intermolecular hydrogen bonds and enhanced mobility of chains at elevated temperatures [35]. Cohesiveness of polymer can be defined as the degree to

which the molecular chains adhere to themselves. It depends on factors including concentration, molecular weight, and complex viscosity and can be measured in terms of cohesion-dispersion index (CDI). For HA, increase in molecular weight increases the CDI whereas an increase in concentration and complex moduli decreases it. The cohesive nature of HA can also be described by the viscosity and loss modulus values at high frequency [39].

Viscosity of aqueous solutions of HA can also be affected by presence of a small molecular agent, for example, phospholipids, guanidine, sodium chloride, and sugars [6]. While the former three lead to reduction in both elastic and viscous moduli, sugars appear to promote both the moduli. These effects on complex moduli are brought about by reduction of intermolecular interactions and entanglements between chains. Phospholipids present competition for binding to the hydrophobic patches present between HA chains whereas guanidine and sodium chloride tend to shield the electrostatic repulsion present between chains [6]. Sugars such as glucose and sucrose increase hydrodynamic radius and radius of gyration; firstly, by immobilizing water molecules by hydration of sugars and secondly by introducing structural changes through new hydrogen bond interactions [6, 40].

2.1.7 Functions

HA has several physiological and biological functions owing to its unique solution and rheological properties. Biological functions of HA, although wide in range, often regulate opposing effects inside body depending upon their molecular size. High molecular weight HA having more than 1000 to 5000

saccharides, exhibit space-filling, anti-inflammatory, anti-angiogenic, and immuno-suppressive properties. Low molecular weight HA having up to 500 disaccharide units have opposite functions; they are inflammatory, highly angiogenic, and immuno-stimulatory. Smaller oligosaccharides (4-6 size range) function as endogenous danger signals by inducing heat shock proteins and inhibiting apoptosis [18, 41].

The various functions of HA include [6, 16, 17, 42-44]:

Water homeostasis – HA networks exhibit a barrier against bulk flow of water, preventing rapid and excessive fluid fluxes through tissues. HA solutions also have osmotic buffering capacity useful in the regulation of tissue water content.

Exclusion and Transport – HA chains tend to exclude other macromolecules from the space of their network system. These properties regulate the transport of proteins through extracellular space and their distribution between the tissue compartments.

Matrix and cell surface interactions – HA plays structural role in cartilage and other tissues by virtue of its matrix interactions. In cartilage, HA binds to proteoglycan aggrecan through link proteins and form aggregates. HA also binds to certain cell surface receptor proteins known as hyaladherins, for instance, CD44 (cluster of differentiation 44), RHAMM (receptor for hyaluronan mediating motility), and LEC (liver endothelial cell clearance) receptor. By activation of signaling cascades through cell surface interactions, HA regulates many cellular activities such as mitosis, proliferation, migration,

differentiation, gene expression, angiogenesis, phagocytosis, and immune reactions.

Pericellular HA – Certain type of cells, like fibroblasts and mesothelial cells, form a thick pericellular layer of HA around themselves. This layer has functions of preventing direct contact between cells and giving protection against viruses, bacteria, and immune cells. Also, many cell-substrate interactions are regulated by this layer.

Other functions - HA is involved in inflammatory reactions, wound healing, growth factor action, tumorigenesis (cancer invasiveness and metastasis), morphogenesis, and embryonic development. It can also be used in the development of vascular grafts and stents due to its ability to inhibit platelet adhesion and aggregation and to stimulate angiogenesis [15]. Role of HA in wound healing has been attributed to phagocytosis, cell migration, proliferation, detachment, and mitosis [45].

HA in joints – The main functions of HA in joints include space filling, scavenging, lubrication, and cushioning. HA is being constantly secreted into the joint cavity and removed by synovium. This keeps the cavity open allowing extended joint movements. This synovial fluid shows viscous fluid and elastic body behavior under different conditions of mechanical impact (rate or frequency of energy input) [33, 46]. For example, under conditions of low shear rates or no load, the synovial fluid behaves as a viscous solution lubricating the joints. During conditions of high shear rates, the synovial fluid behaves as an

elastic body absorbing the mechanical energy (shock absorber) and preventing the surrounding cells and tissues from damage.

HA in vitreous humor –

The posterior cavity of the vertebrate eyes is filled with a transparent gel between the lens and retina. This gel, known as vitreous humor, is a delicate, complex, and highly hydrated double network of proteins and GAGS. It is composed of ~ 99 % water and ~0.9 % salts by weight [47]. The three main network components, collagen, HA, and proteoglycan (PG) chondroitin sulphate represent the rest of the small fraction of vitreous. Vitreous humor is avascular and nearly acellular apart from the presence of a few cells in the periphery called halocytes [47, 48]. The main functions of vitreous include developmental, to maintain the growth and shape of eye; optical, to maintain a clear path from lens to the retina by inhibiting migration and diffusion of cells and macromolecules and allowing circulation of nutrients and metabolites; and mechanical, to support and protect ocular tissues such as retina by absorbing shocks due to sudden compression, high oxygen tensions or other physical activities [29, 47-49].

The fine collagen protein fibrils (diameter~10 nm) found in vitreous humor are type II, V/XI and VI. The ordered network of water-insoluble protein fibrils immersed in the viscoelastic matrix of highly hydrated HA and PGs makes the vitreous humor a natural composite. The collagen fibrils run almost parallel to each other in small bundles except a few being orthogonal. The PGs can be linked to collagen fibrils making bridges between the bundles in the network.

They can also interact with HA through non-covalent bonding forming complexes that enable linking HA to the other network components.

The viscoelastic properties of vitreous body are mainly controlled by synergistic functions of collagen fibrils and HA chains [46]. Collagen fibrils behave as the load-bearing component maintaining the shape, strength, and flexibility of the network and providing resistance to tractional forces. Vitreous network is kept hydrated by the swollen chains of HA. They also provide chemical stability to collagen fibrils and prevent their aggregation by filling the space between them. Network hydration is to achieve the donnan equilibrium state which in turn keeps the collagen fibrils under internal tension. This tension would prevent any deformation in their structure thereby increasing the modulus. This swelling pressure keeps the collagen fibrils apart in the network and provides resistance to any compressive forces. Other factors involved might be the binding of HA with the fibrils and its excluded volume effect [47, 48].

The composition of vitreous can undergo changes due to ageing and diseases. This mainly involves change or reduction in HA concentration and molecular weight [46]. In healthy vitreous, HA concentration ranges from 65 to 400 $\mu\text{g/ml}$ and average molecular weight from 2 to 4 MDa [48]. Vitreous related complications including diabetic proliferative retinopathy, glaucoma, and tractional retinal detachment (retinal tear, choroidal detachment, macular holes or edema, vitreous hemorrhage) occur as a result of changes in macromolecular organization and viscoelastic properties [46, 47, 50].

2.1.8 Applications

HA has found several therapeutic and pharmaceutical applications owing to its versatile properties of biocompatibility, biodegradability, non-immunogenicity, and viscoelasticity. The various applications are as follows:

Ophthalmology – The two main applications of HA in eye include viscosurgery and viscoprotection. For viscosurgery, HA functions to protect delicate tissues as well as provide space for surgical manipulations. During cataract surgery and/or lens implantation, any loss in vitreous fluid is substituted by HA. It is also used to promote healing of tissue surfaces and protect them from dryness and harmful environmental agents [20, 33, 51].

Orthopedic applications – The main use of HA in joints include viscosupplementation. HA solution is injected intra-articularly to supplement or replace the synovial fluid and alleviate pain caused by arthritic disorders (osteoarthritis and rheumatoid arthritis) [18, 20, 51].

Anti-adhesion/Viscoseparation – HA pastes and hydrated membranes or films have been used to separate surgically traumatized connective tissue surfaces to prevent excessive scar formation during abdominal and pelvic surgery. These preparations prevent post-traumatic granulation that in turn prevents fibrous tissue formation and finally adhesion [33, 51]. HA can also increase blood compatibility of cardiovascular implants like vascular grafts and stents by reducing platelet adhesion and thrombus formation [17].

Otolaryngology – HA has been used to repair injured or scarred vocal cords, laryngeal or glottal insufficiency, and hearing disorders by viscoaugmentation [18].

Dermatology – HA has viscoaugmentation effect on skin. It can be used to fill in the facial wrinkles and depressed scars. Cosmetic preparations of HA are used to moisturize the skin and restoring elasticity to achieve anti-wrinkle or regenerative effect [18, 33].

Wound healing – Topical preparations of HA has been employed for treating skin wounds. HA also promotes healing of cornea, diabetic foot, nasal mucosa, and venous leg ulcers [6]. The anti-oxidant properties of HA also enable its use in wound dressing materials as an anti-inflammatory component [18].

Drug delivery – HA based carriers have been used for a number of sustained drug delivery systems. These include dermal, ocular, nasal, pulmonary, parenteral, liposomal, implantable, and gene delivery systems [6, 7].

Tissue engineering – HA has been explored to develop scaffolds or matrices to support growth of various types of cells for tissue engineering applications. It is either used alone or in combination with other polymers like collagen or chitosan (CS), to mimic *in vivo* matrix organization towards the reconstruction of many tissues including cartilage, bone, dermal, and adipose tissue [33].

2.2 HA Hydrogels

2.2.1 Introduction

A large number of drugs have been formulated with HA solutions for nonparenteral as well as parenteral delivery. For instance, pilocarpine and gentamicin dissolved in HA solutions have been used for ocular delivery with improved bioavailability or ocular residence time on account of solution's biocompatibility and mucoadhesive property. HA solutions with vasopressin enhanced the nasal absorption via mucosal tissues. Solutions of HA have been shown to maintain the stability of protein and peptide drugs intact and have been investigated for sustained release of proteins like human insulin like growth factor-I (hIGF-I) after parenteral delivery [6, 7].

Although unmodified and soluble HA has been utilized for many therapeutic applications, poor mechanical properties, rapid degradation, and clearance *in vivo* still exist as some of its limitations [4]. These limitations could be improved by modifying aqueous solutions of HA with chemical derivatives and then crosslinking them to obtain insoluble hydrogels. These hydrogels are not only mechanically and chemically robust but also retain the desirable properties of native HA chains including biocompatibility, biodegradability, and viscoelasticity. Other useful properties of these hydrogels are high aqueous swelling ratio, high porosity, susceptibility to hyaluronidase enzyme degradation, and resistance to cell adhesion *in vivo* [5].

Chemical modification of HA include methods of conjugation as well as crosslinking. Conjugation involves grafting of a molecule by a single bond onto

HA chain whereas in crosslinking, different HA chains are linked together by two or more bonds [52]. The carboxyl group of glucuronic acid unit, N-acetyl group of glucosamine unit, and hydroxyl groups (primary as well as secondary) are the main functional groups targeted for chemical modification of HA chains. Modifications of carboxylic acid groups include esterification, carbodiimide-mediated reactions, and amidation, and for hydroxyl groups it mainly includes etherification and esterification. Carboxyl groups can be crosslinked by dialdehydes, dihydrazides, or disulfide crosslinkers whereas hydroxyl groups can be crosslinked by divinyl sulfone or bis-epoxide [4, 6, 53].

The crosslinking methods of HA can be broadly divided into 3 types: direct crosslinking, crosslinking of modified derivatives of HA, and crosslinking between different HA derivatives to get hybrid hydrogels. Direct crosslinking can be achieved by addition and condensation reactions of which the important modifications include thiol or disulfide, haloacetate, dihydrazide, aldehyde, tyramine, and Huisgen cycloaddition (click chemistry). For example, direct crosslinking using chemical agents such as butanediol-diglycidyl ether (BDDE) and divinyl sulfone. HA hydrogels have also been obtained by double-crosslink strategy which involved exposing HA to alkaline and acidic conditions sequentially [52, 53].

The second type of crosslinking can be done by photopolymerization and electropolymerization of functionalized HA. These reactions include MA, glycidyl methacrylate, (crosslinking by UV light at 365 nm using a photoinitiator such as Irgacure 2959, I2959, by free-radical polymerization),

and electropolymerizable pyrrole-HA. HA-hydrazide or HA-amine functionalized derivatives can be crosslinked with homo or heterofunctional crosslinkers. Thiol modified HA, prepared by carbodiimide-activated hydrazide method, can be crosslinked by oxidation of thiols to disulfides in air.

Thiolated HA can be crosslinked using multifunctional electrophiles by Michael-type addition reaction. It involves reaction between a nucleophilic thiolate and an electrophile [54]. The reactive form of thiol reacts with an unsaturated ester macromer to form a thioether linkage. These multifunctional macromers include vinyl sulfones and acrylates such as PEG diacrylate. HA gels, capable of *in situ* crosslinking, have been developed by crosslinking tyramine (Tyr) grafted HA. Tyramine modification is done using 1-ethyl-3-[3-(dimethylamino)-propyl]-carbodiimide (EDC) and HOBt (1-hydroxybenzotriazole) and crosslinking of modified HA is achieved by mixing HA-Tyr with H₂O₂ and horseradish peroxidase (HRP) in two syringes [52, 53].

2.2.2 Characterization of hydrogels

Following are the major characterizations conducted for HA hydrogels [52]:

Structural characterization – NMR spectroscopy, a quantitative method, is used for the identification of modified derivatives and the peak integrals can be used to calculate the degree of substitution. Infrared spectroscopy (IR), especially, fourier transform IR (FTIR), is used to identify the type of bonds formed during the process of HA modification and complements the data obtained from NMR. Ninhydrin assay is a colorimetric test to detect and quantify free primary amine groups; for instance in amine-functionalized HA.

Morphology – Scanning electron microscopy (SEM), field emission SEM (FESEM), and transmission electron microscopy (TEM) are used to characterize the surface morphology of dried HA hydrogels and NPs. Particle size and shape can also be estimated. Atomic force microscopy (AFM) allows surface characterization (topography, roughness) of HA samples in aqueous conditions. Dynamic light scattering (DLS) is used to characterize the hydrodynamic behavior of NPs or MPs of HA in solution. It also enables quantification of diameter and size distribution of the particles.

Physical characterization – It includes swelling measurements, compression test, thermal analysis, molecular weight, and rheological measurements. Measurement of swelling is expressed in terms of swelling ratio that can be defined as a ratio between the swollen wet mass to the dried mass. Higher the density of the HA network, smaller is the mesh size, and lower is the swelling ratio or water uptake. Crosslinking density of HA gels can be estimated by uniaxial compression test. A defined force rate and compression rate is used to calculate the swollen modulus, G_e . Higher the modulus, higher is the crosslinking density, and lower is the swelling ratio. The thermal properties of HA hydrogels are analyzed by differential scanning calorimetry (DSC) technique. These thermograms also provide information regarding the hydration properties of gels. Dynamic mechanical thermal analysis (DMTA) can be used to study the effect of different types of crosslinkers and absorbed water content on complex moduli and loss factor of gels.

Size exclusion chromatography associated with multi angle light scattering (SEC-MALS) and asymmetric flow-field-flow fractionation (AF4) are used to measure molecular weights. In SEC-MALS, absolute molar mass is calculated using refractive index increment (dn/dc) defined for a polymer in a specific solvent and measurement parameters. Rheological measurements including steady shear and oscillatory mode can be used to study the HA gels. In steady shear experiment, viscosity is obtained as a function of shear rate. In oscillation mode, storage modulus (G' , reflecting elasticity) and loss modulus (G'' , reflecting viscous nature) can be determined as a function of frequency.

2.3 HA based drug delivery systems

2.3.1 Introduction

HA or HA derivatives have been extensively investigated as delivery vehicles for sustained release of drugs, peptides, proteins, antigens, growth factors, and genes in many applications such as ocular, nasal, pulmonary, and parenteral delivery. Several forms of carriers such as films/membranes, injectable/implantable hydrogels, nano/microspheres, coatings, hybrid or composite systems with NPs/MPs, other polymers, and liposomes have been investigated. Drugs can be loaded into the carriers by physical incorporation or by covalent linkage.

One of the first HA derivatives to be studied was the ester films and membranes prepared by esterification of the carboxyl groups with therapeutically active and/or inactive alcohols such as benzyl alcohol [10]. In these studies, transport

properties (diffusion and release kinetics) of different solutes (drugs such as hydrocortisone, 5Fu, and peptides/proteins) from HA ester films and membranes were evaluated in detail to develop controlled release systems [10-13].

HA-ADH-Poly (ethylene glycol) (PEG)-dialdehyde crosslinked HA films were evaluated for *in vitro* release of anti-bacterial and anti-inflammatory drugs [55]. HA was modified with ADH followed by crosslinking with the macromolecular reagent PEG–propiondialdehyde to obtain a crosslinked network. This fast-gelling and high swelling gel was characterized for morphology, thermal behavior, swelling kinetics, enzyme degradation, and *in vitro* release.

Besides films and membranes, another important HA hydrogel based carrier system includes injectable gels. A biocompatible *in situ* hydrogel system was synthesized using HA–Tyr conjugates by the catalytic enzyme HRP and evaluated for gelation kinetics and enzymatic degradation *in vitro* and *in vivo* [56]. The degradability of hydrogels was studied to provide valuable information towards developing an ideal implantable system for drug delivery.

Injectable HA microhydrogels were developed for controlled release of protein drug erythropoietin (EPO) [57, 58]. HA was first modified into HA-ADH and then crosslinked in presence of the drug. In one study, the HA-ADH was thiolated to obtain HA-S and EPO was loaded *in situ* during hydrogel synthesis by disulfide bond formation of HA-SH. In another study, EPO was loaded *in*

situ into the hydrogels through selective crosslinking reaction of the modified HA-ADH with bis(sulfosuccinimidyl) suberate. The microhydrogels were evaluated for morphology, thermal behavior, degradation, and drug release *in vitro* and *in vivo*. These microhydrogels could be further developed as controlled release systems of protein and peptide drugs.

Human growth hormone (hGH) is another protein for which injectable HA hydrogels were developed by crosslinking of thiolated HA (HA-SH) and aminoethyl methacrylated HA (HA-AEMA) through michael addition for prolonged controlled delivery [59]. The hydrogels were evaluated for enzymatic degradation, *in vitro*, and *in vivo* drug release.

Another form of modified and/or crosslinked HA drug carriers include NPs, MPs, and nanogels. HA microspheres were developed for delivery of plasmid DNA using combination of carbodiimide and ADH along with inverse emulsion method [60]. HA esters, prepared from alkyl halides, were used to form microspheres as carriers for delivery of hydrocortisone. Thiolated HA prepared using carbodiimide chemistry and containing siRNA, were crosslinked by disulfide bond formation to obtain nanogels [7].

Among the various types of conjugates for delivery systems, one of the first to be developed was HA-drug conjugates. It was shown that covalent conjugation of drugs to HA derivatives would enhance their therapeutic effect. Several drugs such as ibuprofen, hydrocortisone, paclitaxel, cisplatin, anthracycline antibiotics, peptides, proteins, and DNA have been conjugated to HA

derivatives for different applications [52]. The most commonly used derivative was HA-ADH and crosslinking method using EDC and NHS.

HA was conjugated to other polymers to form co-polymeric physical or chemical gels. HA gels were used to encapsulate pullulan nanogels to obtain a hybrid system for sustained delivery of therapeutic peptides and proteins [7]. HA was combined with CS to develop an insulin containing implant and drug release from this implant depended on quantity and composition of the formulation [6]. Another example includes co-polymeric HA-poloxamer hydrogels that were investigated for protein and drug delivery to enhance bioavailability and duration of activity.

In one study, graft copolymers were synthesized with mono amino-terminated poloxamer (MATP) and HA using EDC and NHS as coupling agents for sustained release of ciprofloxacin [61]. In another study, hydrogels were prepared by blending HA with two types of poloxamers (F68 and F127) to obtain an *in situ* forming thermosensitive hydrogel for controlled delivery of drug acyclovir [62]. These hydrogels were characterized for gelation temperature, viscoelastic properties, mucoadhesive force, and *in vitro* drug release.

Copolymers of HA with poly(*N*-isopropylacrylamide) (PNIPAAm) were developed to obtain injectable gels for controlled drug delivery applications. These temperature-sensitive gels were characterized for phase transition, viscosity measurements, and drug release *in vitro* and *in vivo* [63].

HA has also been conjugated to NPs and liposomes to form hybrid delivery carriers to enhance targeting and biocompatibility. HA was complexed with iron oxide (Fe_2O_3) NPs and the formulation was investigated for intracellular delivery of peptides [7]. In another study, HA was conjugated to PEG-PLGA to prepare 5Fu loaded NPs for Ehrlich ascites tumor inhibition [64]. siRNA delivery has also been investigated recently using HA-liposome conjugates [65].

Paclitaxel loaded phosphoethanolamine lipid NPs were covalently conjugated with HA to obtain tumor-targeted nanovectors [52]. Doxorubicin loaded PLGA NPs were conjugated to HA by covalent bonds via PEG spacer [52]. Amino groups of phosphatidylethanolamine of drug loaded liposomes were linked to carboxyl groups of HA using carbodiimide chemistry to obtain covalently conjugated HA-liposome carriers [52].

2.3.2 HA based composite systems

Crosslinking of HA into hydrogels greatly enhances the mechanical integrity and reduces the dissolution rate of its chains. However, the entrapped drugs are rapidly released from these hydrogels alone and sustained release cannot be obtained. This could be attributed to the macroscopic nature of these hydrogels having large mesh sizes and inhomogeneity in the crosslinking process (randomly crosslinked heterogeneous network of HA chains) [18, 66].

For this reason, there is growing interest in composite hydrogel networks wherein the pharmacological limitations of hydrogels could be overcome by

entrapping particulate carriers inside the hydrogel matrix [3]. HA-based composite hydrogel systems, wherein drug-loaded nano/micro carriers are homogeneously dispersed within the HA hydrogel matrix, could be potentially explored as the sustained release drug delivery systems.

Drug loaded liposomes, NPs, MPs, and other types of particle-based drug delivery vehicles have been incorporated into synthetic as well as natural polymeric hydrogels to obtain composite systems. The combination is advantageous over the polymer or particles alone because it improves the drug's half-life, preserves the bioactivity of drugs, improves the stability and biocompatibility of particulate carriers, and enhances the bioavailability of drugs by confining the carriers and preventing their migration away from the target site. Further, the composite system could enable sustained release of drug for prolonged or extended periods of time by reducing the burst release and also by providing an additional resistance or barrier to the diffusion of entrapped drugs (combination of carrier and surrounding gel network diffusion resistances) [3, 9].

Composite hydrogels of HA, that can be developed by dispersing drug loaded nano/micro carriers within the matrix of HA hydrogel, have not been widely explored as the carrier systems for drug delivery applications. HA based composite hydrogels have been mainly investigated for tissue engineering applications to utilize the properties of HA chains [67, 68].

Among the various drug delivery applications, HA based composite hydrogel systems are specifically appropriate for ocular delivery because HA is present inside eye as its natural component and also possesses significant wound healing functions. So if we can develop approaches to control the release of drugs from hydrogel matrix for prolonged periods then these HA based composite hydrogel systems can target many therapeutic applications.

Composite gels of HA and liposomes were developed by Lajavardi and coworkers for endotoxin-induced uveitis in order to achieve sustained release of a peptide and prolong ocular residence time while utilizing the anti-inflammatory properties of HA [69]. The liposomes loaded with vasoactive intestinal peptide (VIP) were conjugated with rhodamine and then combined with HA solution as the gel matrix. So the composite system did not use a crosslinked network of HA with peptide loaded liposomes. These composite gels were characterized for morphology, rheology, and *in vitro* drug release.

2.3.2.1 Composite Hydrogel Systems with Liposomes

Liposomes are spherical lamellar vesicles composed of an aqueous core surrounded by one or more phospholipid bilayer. **Figure 5** shows the structure of a liposome in which the aqueous core is surrounded by one bilayer. Liposomes are self-assembled amphiphilic systems ranging in size from few nanometers to microns. Upon exposure to an aqueous environment, these phospholipids first spontaneously arrange themselves into bilayer sheets due to hydration, and then fold themselves into closed vesicles known as multilamellar vesicles (MLVs) to minimize free energy as shown in **Figure 6** [70-72].

Figure 5 Structure of a liposome having one phospholipid bilayer

Figure 6 Schematic showing the self-assembly process of phospholipids into vesicles [72]

These MLVs can be further downsized to small unilamellar or large unilamellar vesicles (SUVs or LUVs) using various techniques such as extrusion and sonication. **Figure 7** shows the classification of these lamellar vesicles based on their size. Liposomes can be prepared by mechanical dispersion, solvent dispersion, or detergent removal methods [72, 73]. They have been widely used for therapeutic and diagnostic applications in medicine owing to their advantages such as ability to encapsulate hydrophilic and hydrophobic drugs, biocompatibility, biodegradability, non-toxicity, non-immunogenicity,

protection of entrapped moieties from external conditions or protection of healthy cells from exposure to toxic drugs, ease of tailoring their properties for specific applications, and targeted delivery of biomolecules. They have been used as sustained release carriers for delivery of several bioactive molecules including hydrophilic and hydrophobic drugs (such as doxorubicin, daunorubicin, amphotericin B, vincristine, cisplatin, acyclovir, cytarabine, topotecan, nystatin), genes, protein, peptides, and vaccines [9, 72-76].

Figure 7 Schematic showing the classification of lamellar vesicles based on size [72]

Despite the numerous advantages, liposomal delivery systems face certain drawbacks such as rapid clearance *in vivo*, inability to retain the entrapped drugs for prolonged periods of time, low solubility, short half-life, and instability [9, 76]. To improve upon these limitations, recently, a novel approach of composite systems of polymers and liposomes have been investigated to combine advantages of each component for sustained release of drugs for prolonged periods. Liposomal hydrogels, wherein drug loaded liposomes are entrapped inside hydrogel matrix (with or without drug), have

been investigated for several drug delivery applications using various synthetic and natural polymers.

Collagen was among the first natural polymer to be used with liposomes to develop composite hydrogels for the delivery of insulin [77]. Mulik and coworkers investigated the thermosensitive composite hydrogels of CS with liposomes for the delivery of cytarabine [78] and carboxyfluorescein [79]. These studies showed that multiple dosing could be avoided or reduced by obtaining sustained release of drugs for long periods from liposomal hydrogels. Microencapsulation of liposomes inside crosslinked microspheres, composed of alginate [80] and dextran [81] hydrogels, was investigated to evaluate the sustained release behavior of liposomes as well as the associated molecules.

Besides carboxyfluorescein, another low molecular weight molecule, Tirofiban, was loaded into liposomes which in turn were entrapped in CS-fibrin hydrogel and were shown to release the drug in a sustained manner maintaining the bioactivity of drug [82]. Liposomal hydrogels with gelatin-PEG were developed by DiTizio and coworkers as a coating for catheters and investigated for sustained release of the antibiotic ciprofloxacin and reduction in the bacterial adhesion to catheters [83].

Carbopols, polyvinyl alcohol (PVA), and poloxamers are some of the major synthetic polymers that have been widely explored for developing liposomal hydrogels. Release of liposome loaded ciprofloxacin was prolonged and its bioavailability was improved upon combining them with carbopol 940277 [84]

and PVA-polymethyl methacrylate (PMMA) [85] for ocular delivery applications. In another study, Mourtas and coworkers prepared griseofulvin and calcein loaded liposomes and complexed with carbopol 974 and hydroxyethyl-cellulose to investigate the effect of lipid and gel properties on drug release kinetics [86].

Liposomal hydrogels with poloxamer-407 were developed in order to obtain sustained release of biomolecules such as nucleic acids [87] and anti-cancer hydrophobic drug paclitaxel [88] for non-parenteral and parenteral delivery applications, respectively. Poly (N-isopropylacrylamide) (pNIPAAm) hydrogels embedded with calcein loaded liposomes were developed to study the interactions between drug carrier and hydrogel matrix, stability of the liposomes as well as the release kinetics of the encapsulated drug [89]. In another study, MacKinnon and coworkers immobilized liposomes in NIPAAm-acrylic acid copolymeric microgel beads and evaluated them for the stability of vesicles and their ability to sustain the release of encapsulated hydrophilic molecules [90].

2.3.2.2 Composite Hydrogel Systems with PLGA Nano/Microparticles

PLGA is a U.S. food and drug administration (FDA) approved biodegradable copolymer of poly lactic acid (PLA) and poly glycolic acid (PGA) (**Figure 8**) and belongs to the class of polyesters. PLGA and its various derivatives have been extensively investigated as controlled drug delivery carriers in the form of NPs as well as MPs for delivery of drugs, proteins, peptides, nucleic acids, vaccines, and other molecules [91-96]. This wide usage of PLGA as drug

delivery carriers could be attributed to its several advantages such as biocompatibility, biodegradability, tunable mechanical and erosion properties, sustained release, and ease of administration.

Figure 8 Chemical structure of PLGA (m and n are lactic and glycolic acid units resp.)

PLGA is most commonly synthesized by the ring-opening copolymerization of two different monomers which are cyclic dimers of lactic acid and glycolic acid. The lactide-to-glycolide (L:G) ratio can be varied leading to different compositions of PLGA with varying crystallinity and hence varying glass-transition (T_g) and melting temperatures (T_m) [93]. PLGA is degraded by the process of hydrolysis via breakage of its ester linkages in the presence of water. It undergoes bulk erosion and the acidic by-products of its degraded components are non-toxic and used up in the metabolic pathways of the body [97].

The most common method of formulation of PLGA NPs and MPs is emulsification-solvent evaporation technique (single or double emulsion). MPs can also be fabricated using coacervation and spray drying techniques and NPs can be fabricated by nanoprecipitation, layer-by-layer (LBL), and emulsion-diffusion techniques [92, 93, 98-100]. Recently, NPs and MPs have been combined with polymers to obtain composite hydrogel systems that would

prolong the sustained release of encapsulated drugs, improve the stability of particles, and their retention time at the target site.

Bovine serum albumin (BSA) loaded alginate MPs were combined with collagen hydrogels for ocular applications. The composite hydrogels were proposed to be useful as therapeutic lens for sustained release of drugs [101]. In another study, BSA loaded PLGA MPs were combined with alginate to develop composite hydrogels and evaluated as injectable, localized drug delivery systems [102]. BSA was found to be released for extended periods of time when compared with the microspheres or hydrogels alone suggesting that the combination can overcome the limitations of each carrier alone.

pNIPAAm-block-PMMA micelles loaded with prednisone acetate were entrapped in pNIPAAm to prepare composite hydrogels and characterized for morphology, swelling and thermal properties, and *in vitro* drug release. The controlled release property of composite hydrogels was better than hydrogels or micelles alone due to the presence of dual release barriers for diffusion of prednisone acetate [103].

Composite hydrogels of PVA incorporated with ibuprofen-loaded PLA microspheres were developed by Behnoodfar and coworkers and characterized for morphology, swelling and thermal properties, and *in vitro* drug release. The reduction in release rate as well as initial burst of ibuprofen from composite hydrogels indicated that they could be suitable as controlled release carriers [104].

Controlled delivery of basic fibroblast growth factor (bFGF) was obtained by entrapping the growth factor into heparin-conjugated PLGA nanospheres and dispersing them in fibrin hydrogel. The release rate was nearly zero-order and could be controlled by varying fibrinogen concentration. The biological activity of released growth factor was unaffected and localized, sustained release could be obtained [105].

Dexamethasone loaded PLGA microspheres were entrapped in polyacid containing PVA hydrogels to develop controlled delivery systems. These composite hydrogels were characterized for morphology, swelling, mechanical and degradation properties, and *in vitro* release. Dexamethasone was found to be released in a controlled manner for more than a month from the composite hydrogels as compared to the hydrogels alone. Further, the release rate was approximately zero-order. These hydrogels were proposed to be suitable as localized controlled delivery systems for coating applications on implantable devices such as glucose biosensors [106].

A multi-component system was developed by dispersing levonorgestrel loaded poly- ϵ -caprolactone (PCL) microspheres in poly (2-hydroxyethyl methacrylate) based hydrogel matrix for potential applications in implantable devices. The composite hydrogels were characterized for morphology, swelling and thermal properties, and *in vitro* release kinetics. The drug was found to be released in zero-order kinetics for extended periods and involved a complex combination of different release mechanisms (permeability, degradation of microspheres, and diffusion through hydrogel matrix). Hydrogel matrix not only provided

physical support to the drug loaded microspheres but could also act as another medium for additional drug loading [107].

PCL-PEG-PCL microspheres were loaded with camptothecine and entrapped in the thermosensitive hydrogels to develop injectable and biodegradable composite hydrogels for colorectal peritoneal carcinomatosis therapy. The composite gels were characterized for morphology, cytotoxicity, *in vitro* and *in vivo* release behavior of drug. The release of camptothecine from composite hydrogels was found to be much slower and sustained than the microspheres or drug alone [108].

Growth factor TGF- β 1 was encapsulated in PLGA microspheres which were then embedded into PEG based biodegradable hydrogels for cartilage repair. The composite hydrogels were characterized for morphology, degradation, bioactivity assay, and *in vitro* release. The burst release of TGF- β 1 was reduced due to incorporation of microspheres into the hydrogels and release was more controlled as compared to the microspheres alone [109].

In situ crosslinkable HA composite hydrogels were developed by entrapping PLGA NPs to obtain a hybrid system for intraperitoneal drug delivery and for preventing post-operative surgical adhesions. The hydrogel part would offer biocompatibility, ease of application, act as an additional barrier, prevent the migration of NPs whereas the NPs would allow controlled releases of encapsulated drugs. The hydrogels were characterized for morphology, *in vitro* cell viability assay, and biocompatibility tests. Incorporation of NPs did not

affect the crosslink-ability and the mechanical properties of the composite hydrogel [110].

PLGA MPs were entrapped in thermosensitive copolymeric hydrogels composed of PLGA and PEG copolymers to develop composite hydrogels for controlled release of proteins. Ribonuclease A was loaded into the MPs as well as in the hydrogel matrix and the composite hydrogels were characterized for morphology, rheology, and *in vitro* drug release. The release of proteins was controlled differently from MPs and hydrogel matrix and hence these composite hydrogels could be explored for delivery of multiple drugs with different release kinetics [111].

Composite systems of doxorubicin loaded poly(L-glutamic acid)-b-poly(propylene oxide)-b-poly (L-glutamic acid) micelles with aspirin loaded PVA-CS or PVA hydrogels were developed and characterized for *in vitro* release kinetics of the two different drugs. Aspirin was found to have a faster release profile as compared to the sustained release of doxorubicin. Analyzing the release profile using Peppas power law equation, aspirin showed anomalous transport and doxorubicin had Fickian type or anomalous transport depending upon the component of composite hydrogel systems [112].

2.4 Drug release mechanisms

“Drug release” could be defined as a process whereby drug solutes encapsulated or dispersed in a polymeric delivery carrier migrates from their initial position

towards the outer surface of the carrier and then into the release medium [113]. Drug release in a delivery system could occur by many different mechanisms depending on various factors related to the drug solute, delivery carrier, and release medium. The release might be either due to one mechanism, or a combination of different type of mechanisms and in the latter case, these mechanisms could either occur simultaneously at a given time or at different time points of the complete drug release process [113].

It is important to elucidate and understand the detailed release mechanisms involved in drug delivery systems. This helps to improve the designing and manufacturing of these systems and to evaluate any failure modes in the process all of which would be significant in developing controlled release formulations or systems [114]. Moreover, elaborate and clear description of these mechanisms is essential in the quality control and intellectual property procedures.

There are several different mechanisms proposed in literature for drug release process from any polymeric delivery system. These mechanisms are also interdependent in the complete release process. However, we should note that although different mechanisms might be responsible for drug release, the rate-controlling or rate-limiting mechanism is the one that occurs with slowest release rate. **Table 2** describes some of the major mechanisms involved and the factors affecting them depending upon the individual components of the process.

Table 2 List of drug release mechanisms (compiled from [113, 114])

Type of mechanism	Factors affecting release mechanism
Diffusion	Drug's physicochemical properties (solubility, size, molecular weight, charge, interaction with matrix, distribution within the matrix) Matrix properties (structure and composition, swelling, degradation)
Dissolution	Drug's physicochemical properties (solubility, size, charge) Matrix properties (structure and composition, swelling, degradation) Release/Sink medium (pH, temperature, ionic strength, enzymes)
Swelling	Matrix properties (structure and composition) Release/Sink medium (pH, temperature, ionic strength, enzymes)
Degradation and Erosion	Matrix properties (structure and composition) Release/Sink medium (pH, temperature, ionic strength, enzymes)
Partitioning	Drug's physicochemical properties (solubility, charge, interaction with matrix, distribution within the matrix) Release/Sink medium (pH, temperature, ionic strength, enzymes)
Osmosis	Drug's physicochemical properties (solubility, size, molecular weight, charge, interaction with matrix, distribution within the matrix) Matrix properties (structure and composition) Release/Sink medium (pH, temperature, ionic strength, enzymes)

2.4.1 Hydrogels

The transport and release mechanisms of drugs and other solutes from hydrogel networks involve a complex combination of diffusion/dissolution in the

polymer network, matrix swelling, hydrogel erosion, and mass transfer limitations occurring at the boundary of the polymeric carrier as well as its surroundings [2, 14, 115, 116]. For all the release systems, the diffusion coefficient of drugs or solutes from the polymeric networks depends on several factors including structure of pores, mesh size of network, water content, polymer composition, method of drug loading, and nature and size of the solute. Depending upon the mechanism of drug release from a particular system, hydrogel systems could be classified broadly into three categories. These include diffusion-controlled, swelling-controlled, and chemically-controlled release systems [2, 14, 115, 116].

Diffusion-controlled systems – Diffusion is one of the most common mechanisms determining the release kinetics of drugs from hydrogels. Drug diffuses through the network mesh or water filled pores into the surrounding. These systems are of two types – reservoir and matrix. Reservoir systems include geometries such as slabs, cylinders, capsules, or spheres. These systems comprise of a polymeric membrane surrounding a core drug depot and allows for time-independent, constant release rate that is limited by diffusion through the outer membrane. The constant release rate could be facilitated by using excess of drug in the core. This type of one dimensional release could be defined by Fick's first law of diffusion:

$$J_i = -D_{ip} \left(\frac{dC_i}{dX} \right)$$

where, J_i represents molar flux of drug (mol/cm^2), C_i , the concentration of drug, and D_{ip} is the diffusion coefficient of drug in the polymer. For steady-state diffusion, the release could be defined as:

$$J_i = K \left(\frac{Dip \Delta C_i}{\delta} \right)$$

where, δ represents thickness of hydrogel matrix and K is the partition coefficient roughly given by the ratio of drug concentration in the gel to that in the solution.

The matrix system is comprised of hydrogels in which the drug is distributed uniformly throughout the three-dimensional structure. The release rate of drug is time dependent and is proportional to one-half power of time. This non-steady diffusion of drugs in matrices could be defined using Fick's second law:

$$\frac{\partial C_i}{\partial t} = \frac{\partial}{\partial X} \left(D_{ip} \frac{\partial C_i}{\partial X} \right)$$

The above equation used for one dimensional transport can also be used for constant diffusion and concentration dependent diffusion coefficients.

Swelling controlled systems – In these systems, the drug is distributed in the glassy polymer chains. As the gel comes in contact with biological fluid, the fluid enters the network, lowers the glass transition of polymer, and relaxes the macromolecular chains. Drug diffuses through the swollen rubbery parts of the hydrogel and is released into the surrounding. The velocity and position of the moving front, that separates the glassy and rubbery phases of the polymer, controls the drug release rate and the time scale of polymer chain relaxation is the rate limiting step. The type of transport governing these systems is referred to as Case II transport and it leads to zero order kinetics. Several models have been proposed to explain the release kinetics from the swelling controlled systems. Drug release can also occur due to a combination of chain relaxation

and diffusion. The significance of macromolecular relaxation for drug release can be evaluated by fitting the experimental release data to the following equation of power law function:

$$\frac{Mt}{M_{\infty}} = k t^n$$

where, M_t and M_{∞} represent the amounts of drug released at time t and at equilibrium, respectively. k represents proportionality constant and n is the diffusional exponent.

Chemically controlled systems – These systems include two types, namely, erodible drug delivery systems and pendant chain delivery systems. The release of drugs in erodible systems is dependent on dissolution and/or degradation of the polymer. In pendant chain systems, the drug is linked to the polymer chains via degradable linkages. Drug release is mediated by the degradation of these linkages. In erodible matrices, drug is distributed within the three-dimensional structure and release is controlled by the diffusion rate of drug through the gel matrix as the polymer erodes. In pendant systems, the linkages can be enzymatically or hydrolytically broken down and release rate either follows first order kinetics or more complex kinetics depending upon the type of degradation.

Drug release mechanism from HA hydrogels

In case of HA hydrogels, drug diffusion and release were studied in detail only in the ester derivatives of HA. Partition coefficient of solute, membrane permeability, rate and extent of membrane hydration were the factors used as indicators of drug release profile in these studies [10-13] . The degree of

hydration of the membrane (H), the drug diffusivity in the hydrophobic (D_m) and hydrophilic (D_o) domains of the hydrated polymer, and the observed partition coefficient (K) were all related to the observed diffusivity (D_{tot}) in the following equation:

$$D_{tot} = [HD_o + (K - H) D_m]/K$$

It was observed that there exists a correlation between natural logarithm of diffusivity and inverse of membrane hydration for all the different types of drugs and membranes studied. For hydrophilic drugs, a linear relationship was found whereas for hydrophobic drugs, a non-linear relationship was observed. For proteins, the logarithm of diffusion coefficient was found to have a linear relationship with the logarithm of solute molecular weight. The coefficients were calculated from membrane permeability and partition coefficients. The latter was not correlated to the solute size. All these studies indicated that the main parameters governing the solute release from HA ester films or membranes included solute hydrophilicity/hydrophobicity, membrane permeability, polymer solubility (and/or ester hydrolysis), and type of drug loading.

2.4.2 Liposomes

Release of drug from liposomes occurs mainly by the mechanisms of diffusion and partitioning, each of which is affected by the properties of drug solute and liposomes [117-120]. Diffusion of drug solute is affected by its physiological properties such as solubility, charge, size, and molecular weight. Low molecular weight, smaller size drugs with higher aqueous solubility would be

released faster from liposomes and vice-versa. Charge of drugs might result in slower or faster release depending upon the charge of liposomes and ionic strength of the sink or release medium.

Size, lamellarity, and the bilayer permeability of liposomes are very important factors affecting the diffusion of drugs. Bigger the size of liposomes, or higher the order of lamellarity of liposomes, slower would be the release of drug due to increase in the diffusion path length. However, for similar size and type of lipids, the release of drugs from unilamellar vesicles might be faster than that from multilamellar liposomes because of the shape and lipid chain ordering effects. The unilamellar liposomes have greater curvature and subsequently weaker packing order of lipids resulting in faster release of drugs [118].

Drug loading and concentration of lipids also affect the diffusion rate of drugs from the liposomes. The bilayer permeability is related to the packing order of acyl chains and is a major determinant of drug diffusion. The bilayer chains are more fluidic and less rigid at temperatures above their transition temperature which varies with type of lipids. This would result in faster release of drugs. Inclusion of molecules like cholesterol reduces the fluidity, and increases the order and rigidity of chains in the bilayer decreasing the rate of drug release.

Partitioning of drugs is mainly affected by the interaction of drug with the lipid bilayer. The interaction is in turn depends upon the type of drug and lipid [117-119]. Permeability is one factor which is not only related to the diffusion coefficient across the lipid bilayer membrane but also to the partition coefficient

of drugs. As mentioned above, permeability of liposomes is mainly affected by their structure and composition [118, 120]. It is also affected by the physicochemical properties of drugs, size/lamellarity of liposomes, charge of lipids, pH and temperature of the release medium.

Lower the permeability of bilayer membrane, higher is the diffusion of drugs, slower is the partitioning, and hence faster is the release of drugs. Higher the interaction of drugs with lipid bilayer, higher is the partitioning, and slower is the release rate of drugs. This interaction is mainly determined by the charge of drug, charge of lipids, lipophilicity of drugs, and pH of the release medium.

2.4.3 PLGA NPs/MPs

The release of drugs from PLGA NPs and MPs is mainly governed by the mechanisms of diffusion, dissolution, and degradation/erosion [113, 121]. Osmotic pumping is another mechanism that also has been observed to be responsible for drug release from PLGA matrices. Each of these mechanism is affected by a number of factors which are either drug, drug carrier, and/or release environment dependent. Moreover, all the mechanisms are interdependent and the drug release process from PLGA particles is a combination of all three mechanisms.

Diffusion is affected by the solubility of drugs, loading of drugs, size and shape of PLGA particles, structure and composition of particles, pH and temperature of release medium, and presence of enzymes in the medium [114, 121]. Diffusion of drugs from PLGA particles occurs through the water-filled pores and/or the polymer matrix. This in turn depends upon the solubility and

crystallinity of drugs, molecular weight of PLGA, and degree of crystallinity of PLGA (L:G ratio, T_g , D-L ratio of lactide) [122, 123]. Higher the porosity of PLGA particles, faster is the release of drugs. Lower the crystallinity of drugs and PLGA particles, faster is the drug release.

Size and shape of particles also affect the diffusion of drug solutes through the PLGA polymer matrix. Dissolution of drugs depends upon their size and crystallinity and is governed by the rate of water uptake or diffusion into the polymer matrix [114]. Lower the size of drug solutes and their crystallinity, higher is the dissolution rate of the drugs.

Degradation of particles is one of the major determinants of drug release and it also affects the rate of diffusion and thus dissolution. It is mainly affected by the size, shape, structure and composition of PLGA particles, and temperature and presence of enzymes in the sink medium [123-125]. Higher the molecular weight of PLGA, higher the concentration of PLGA, higher the ratio of L:G, higher is the T_g and hydrophobicity and thus slower is the water absorption and degradation of particles. Smaller the size of particles, higher is the surface area:volume ratio, and faster is the release of drugs. The morphology and surface roughness of particles also affect the drug release of particles.

Finally, the end groups of PLGA chains also significantly affect the rate of degradation. The PLGA chains with acid end group degrade much faster than those with ester end groups thus facilitating higher release of drugs from the former [121]. This is because of the autocatalytic nature of the random scission

process of PLGA chains. Enzymes and reducing agents are known to enhance this process of polymer chains cleavage causing bulk erosion [114].

As the drug release from PLGA NPs and MPs can occur due to combination of any of the 3 mechanisms (diffusion, dissolution, and degradation), the release profile of drugs from PLGA is generally either bi-phasic (burst and diffusion controlled) or tri-phasic (burst, diffusion, and erosion controlled) [113, 121, 125, 126]. The phases of release profiles include burst and rapid release, burst and zero-order release, burst, short slow phase and then rapid release.

2.4.4 Composite hydrogel systems

Drug loaded nano/micro carriers in combination with many synthetic and natural polymers have been investigated as potential drug delivery systems. Studies based on these composite hydrogel systems have mainly focused on obtaining sustained release of drugs (by reducing initial burst, increasing drug bioavailability) utilizing the drug-carrier and polymer matrix properties for a specific application. It is difficult to understand the transport behavior and release kinetics of drugs in these composite systems due to the complexity of these systems in terms of the numerous parameters of the different phases involved. These parameters might act independently or in relation with each other to affect the drug transport and release.

To date, there have been very few studies to understand the factors affecting the transport of drugs through these crosslinked networks. Mourtas and coworkers, from their studies on liposomal hydrogels, have indicated that the major factors

determining the release of hydrophilic and hydrophobic drugs include rigidity of liposome membrane and drug loading levels inside liposomes, respectively [127]. Pjanovic and coworkers used effective diffusion coefficients, calculated from the mathematical model for drug diffusion from solids, to determine the diffusion resistances of hydrogels, liposomes, and liposomal hydrogels [128]. They found that the diffusion resistances of drugs was higher in liposomal composite hydrogels as compared to liposomes or gels alone thus concluding that presence of liposomes in the gel network leads to increase in overall diffusion resistance prolonging the release of drugs in complex systems. Phospholipid composition and method of liposome preparation were indicated as the main barriers to drug diffusion in the hydrogel systems.

2.5 5-Fluorouracil and Latanoprost for Ocular delivery

2.5.1 Introduction

5Fu is a hydrophilic drug (molecular weight-130.1 Da) mainly used for the treatment of several types of cancers. It is a fluorinated pyrimidine analogue as shown in **Figure 9** and belongs to the class of antimetabolites. 5Fu functions by interfering with nucleoside metabolism and inhibiting thymidylate synthase [129, 130].

Figure 9 Chemical structure of 5Fu

It can get incorporated into nucleic acids and cause base mismatch leading to chain termination and cell death. Besides its use as anticancer drug, the antifibrotic property (inhibition of proliferation of fibroblasts) of 5Fu has also enabled its use as an antimetabolite in clinical applications of ophthalmology. 5Fu is used as an antiscarring agent in glaucoma-filtration surgery. Glaucoma is the second leading cause for blindness and occurs due to increased fluid pressure inside eye (aqueous humor) [131]. If left untreated, this intraocular pressure (IOP), could lead to optic nerve damage with time and finally blindness.

The conventional surgical method is glaucoma filtration surgery that introduces an alternative path for drainage of excess fluid thus reducing IOP. However, as body's natural healing response, the subconjunctival fibroblasts start to proliferate and close the opening created in the surgery leading to failing and encysted blebs [132, 133]. To prevent this scarring of filtering blebs, subconjunctival serial injections of 5Fu is given either post-operatively or as single intraoperative injection. However, due to high toxicity of 5Fu (that might affect healthy cells), low retention of drug at target side, and poor patient compliance for serial injections, it is desirable to develop a delivery system for 5Fu that would sustain the release of 5Fu at target site for prolonged periods of time and reduce multiple dosing regimen.

Ltp is a lipophilic drug (molecular weight-432.6 Da) mainly used for treatment of primary open-angle glaucoma and ocular hypertension by reducing IOP. It is a prostaglandin $F_{2\alpha}$ analogue as shown in **Figure 10** and is an isopropyl ester of

its acid form [134]. Upon hydrolysis by esterases inside eye this ester prodrug yields isopropyl alcohol (IPA) and Ltp acid, latter of which is the main biologically active constituent in treatment [135, 136].

Figure 10 Chemical structure of Ltp [134]

Ltp is given in the form of eye drops either as oil/water emulsion, commercially known as Xalatan® (Pfizer, Catalent Pharma Solutions, Woodstock, IL, or as lipid/buffer emulsion [137]. Ltp functions by increasing the outflow of aqueous humor via trabecular or uveoscleral pathway by mediating extracellular remodeling via matrix metalloproteinases [136, 138].

2.5.2 Composite hydrogel systems for 5Fu and Ltp

Several polymeric (synthetic and natural) carriers have been used for delivery of 5Fu, mostly for therapeutic applications against different types of cancer [129]. 5Fu has also been investigated for ocular delivery applications [132, 139-141]. Among them, a couple of studies have focused on composite hydrogels using 5Fu loaded nanocarriers for ocular delivery. 5Fu loaded stable plurilamellar vesicles (SPLVs) were loaded into hydroxypropylmethyl cellulose (HPMC) to prepare liposomal hydrogels and characterized for *in vitro* release [142]. The release from liposomal hydrogels was found to be slower than the free drug solution as well as from the liposomes alone. Release from liposomes

alone varied depending upon the type and composition of lipids (addition of cholesterol reduced the initial release rate). The burst release, rate of release, and the extent of release of 5Fu over the study period were reduced by incorporation of liposomes into the hydrogels.

In another similar study, 5Fu loaded liposomes were entrapped in CS hydrogels and *in vitro* release was characterized [143]. The release rate of 5Fu from liposomal hydrogels was found to be significantly slower than 5Fu solution, hydrogels, or liposomes alone. The release from liposomal hydrogels was found to depend upon formulation variables such as lipid bilayer composition and the hydration conditions of dry lipid film. The steady-state release of 5Fu from composite hydrogels indicated the reservoir-system like function of liposomes inside the hydrogels for continuous delivery of 5Fu.

The nanocarriers investigated for Ltp delivery include liposomes and CS/alginate nanosheets. Natarajan and coworkers developed a liposomal delivery system for prolonged sustained release of Ltp that shows excellent efficacy *in vitro* as well as *in vivo* [135, 137, 144]. Ltp isopropyl ester was loaded into CS/alginate degradable nanosheets and these sheets were applied to rat cornea and assessed for local adverse effects, eye scratching movements, and IOP reduction to evaluate the potential of this delivery system for glaucoma management [145]. To date, HA based composite hydrogel systems with 5Fu or Ltp loaded nano/micro carriers have not been explored for ocular delivery applications.

Chapter 3 Materials and Methods

3.1 Materials

HA (molecular weight-1 MDa) was purchased from Samich (HK) Limited, China. EPC, Dipalmitoyl- phosphatidylcholine (DPPC), and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) were purchased from NOF Corporation, Japan. Cellulose ester (CE) dialysis membranes were purchased from Spectrum labs, USA. Polycarbonate (PC) filter membranes and drain discs were obtained from Northern Lipids Inc., Canada. Ltp (molecular weight-432.6 Da) was purchased from Chemical testing and Calibration Laboratories, China. 5Fu (molecular weight-130 Da) and CS (medium molecular weight) were obtained from Sigma Aldrich, USA. PLGA was purchased from Purac Asia Pacific Pte Ltd., Singapore. Chemicals sodium chloride (NaCl), sodium phosphate (Na_2HPO_4), potassium phosphate monobasic anhydrous (KH_2PO_4), potassium chloride (KCl), PVA, sodium azide, cholesterol, sodium hydroxide (NaOH), hydrochloric acid (HCl), mannitol, trisodium polyphosphate (TPP), MA ($[\text{H}_2\text{C}=\text{C}(\text{CH}_3)\text{CO}]_2\text{O}$), and deuterium oxide (D_2O) were obtained from Sigma Aldrich, USA and used directly without purification. PEGDE ($\text{C}_3\text{H}_5\text{O}_2-(\text{C}_2\text{H}_4\text{O})_n-\text{C}_3\text{H}_5\text{O}$), was obtained from Polysciences Inc, USA. ADH ($\text{NH}_2\text{NHCO}(\text{CH}_2)_4\text{CONHNH}_2$), and EDC ($\text{C}_8\text{H}_{17}\text{N}_3\cdot\text{HCl}$) were purchased from Alfa Aesar, USA. I2959 was purchased from Ciba Speciality Chemicals, Switzerland. Ethanol ($\text{C}_2\text{H}_5\text{OH}$), chloroform (CHCl_3), dichloromethane (DCM), methanol (CH_3OH), and IPA ($\text{C}_3\text{H}_7\text{OH}$) were obtained from Fisher Scientific, USA. Acetonitrile (ACN) (HPLC grade, 0.99 % purity) was purchased from Tedia, USA. Sieves (106 μm , 150 μm , and 212 μm) and sonication probes (3

mm and 6 mm) were obtained from Cole Parmer, USA. Syringe filters (0.22 μm and 0.45 μm) were purchased from Millipore, USA. Copper grids for TEM were purchased from Ted Pilla, Inc., USA.

3.2 Synthesis of HA Hydrogels Modification of HA

3.2.1 Modification of HA into HA-MA

Methacrylated derivative of HA, HA-MA, was synthesized as described previously [146]. **Figure 11** shows the chemical structure of MA. HA powder was dissolved in deionised (DI) water and allowed to stir at room temperature (RT) overnight to prepare 1.5 % (w/v) solution. Next day, pH of the HA solution was adjusted to 8 using 5 M NaOH while slowly adding 20 molar excess of MA to it. After addition of MA, HA was allowed to react with it for 2 hrs by maintaining the pH at 8. The HA-MA solution was stored at 4 $^{\circ}\text{C}$ for 24 hrs. It was then dialyzed against large volume of 0.1 M NaCl solution using a dialysis membrane (Spectra/Por 6 dialysis tubing, 10 kDa molecular weight cut off (MWCO), 29 mm diameter, flat width 45 mm) at RT for 48 hrs. Sink medium was completely replaced at intervals of 6 hrs on 1st day and then 12 hrs subsequently. After 48 hrs, HA-MA solution was dialyzed against alternating solutions of 1:4 $\text{C}_2\text{H}_5\text{OH}$ – H_2O (v/v) and ultrapure H_2O for 2 cycles. Finally, the HA-MA solution was frozen at -80°C for 2 hrs and then transferred to a freeze-drying jar and lyophilized for 72 hrs (Christ Alpha 1-4 LSC, Germany).

Figure 11 Chemical structure of MA derivative

3.2.2 UV Crosslinking of HA-MA

After freeze drying, the dried HA-MA fluffy powder was used to prepare 2 % and 4 % (w/v) solution in PBS, pH 7.4. HA-MA solutions were allowed to stir at RT overnight. Next day, 0.2 % or 0.4 % (w/w) of photoinitiator I2959, with respect to total mass of solution, prepared from 10 % (w/v) stock solution in 70 % ethanol, was added into the HA-MA solutions and manually mixed manually for few mins. 1 ml of this solution was then transferred into a stainless steel cylindrical mould (inner diameter 1.7 cm, height 1 cm) and exposed to UV lamp (VL-8.LC, Vilber Lourmat, France) at 365 nm for 10 mins. Crosslinked hydrogel was carefully taken out from the mould and used for further experiments.

3.2.3 Modification of HA into HA-ADH

Dihydrazide derivative of HA, HA-ADH, was synthesized as described previously [147]. **Figure 12** shows the chemical structure of ADH. HA powder was dissolved in DI water and allowed to stir at RT overnight to prepare 0.5 % (w/v) solution. Next day, 40 molar excess of ADH was added to this solution under continuous stirring. pH of the solution was adjusted to 4.75 using 5 M HCl. After all ADH is dissolved, 4 molar excess of EDC was added to the solution. After addition of ADH and EDC, HA was allowed to react with them for 2 hrs at RT and the pH of the reaction was maintained consistently at 4.75 using 5 M HCl. After continuing the reaction for 2 hrs, pH of HA-ADH solution was adjusted back to 7 to stop the reaction. HA-ADH solution was then dialyzed against large volume of 0.1 M NaCl solution using a dialysis membrane at RT for 48 hrs. Sink medium was completely replaced at intervals

of 6 hrs on 1st day and then 12 hrs subsequently. After 48 hrs, HA-ADH solution was dialyzed against alternating solutions of 1:4 C₂H₅OH– H₂O (v/v) and ultrapure H₂O for 2 cycles. Finally, the HA-ADH solution was frozen at – 80 °C for 2 hrs and then transferred to a freeze-drying jar and lyophilized for 72 hrs.

Figure 12 Chemical structure of ADH derivative

3.2.4 Chemical Crosslinking of HA-ADH

After freeze drying, the dried HA-ADH fluffy powder was used to prepare 4 % (w/v) solution in PBS. HA-ADH solution was allowed to stir at RT overnight. Next day, crosslinker PEGDE (**Figure 13**) was added into the HA-ADH solution in 5 molar excess to the degree of modification and mixed thoroughly manually and allowed to stand at RT for 24 hrs for completion of crosslinking reaction. Next day, the crosslinked gel was carefully taken out from the glass vial and used for further experiments.

Figure 13 Chemical structure of PEGDE crosslinker

3.3 Characterization of HA hydrogels

3.3.1 Nuclear magnetic resonance

Proton (^1H) NMR spectra for unmodified HA and modified HA (HA-MA and HA-ADH) powders were obtained using Bruker NMR Spectrometer (Bruker DRX 400, Germany) at 400 MHz to determine the degree of modification. 20 mg of HA/HA-MA/HA-ADH powder was dissolved in 5 ml of DI water and stirred at RT overnight. Next day, the pH of the solutions was lowered to 4 using 1 M HCl and then incubated inside an oven at 100 °C for 2 hrs with intermittent stirring every 10 mins to degrade them completely. After 2 hrs, the pH of the degraded samples was adjusted back to neutral using 1 M NaOH and the samples were then lyophilized. The lyophilized degraded samples were dissolved in 1 ml of D_2O inside NMR tubes and analyzed for ^1H spectral data with 200 scans per sample.

3.3.2 Size exclusion chromatography-Multi angle light scattering

SEC-MALS was used to estimate the absolute molar mass of HA before and after modification. SEC system (Agilent 1100 series, Agilent Technologies Singapore Pte Ltd) was associated with light scattering detector (Wyatt Technology, USA) to estimate the molar mass of HA. Ultrapure water with 0.08 % (w/v) sodium azide and 5 mM sodium phosphate was used as the mobile phase. 1 mg/ml solution of HA, HA-MA, and HA-ADH were prepared in the same mobile phase and all samples were filtered through 0.2 μm filter before running in the system. The injection volume was 50 μl and d_n/d_c value of 0.165 was used for all the samples. The samples were run at a temperature of 35

°C for 21 mins with a post-run of 1 min. The light scattering data was collected and analyzed to calculate number average (M_n) molar mass, weight average (M_w) molar mass, and PDI of all the samples. **Table 3** shows raw data for the calculated values of these three parameters for unmodified and modified HA samples.

3.3.3 Swelling behavior of HA hydrogels

The HA-MA and HA-ADH hydrogels were characterized for their swelling behavior to evaluate their network structure. 2 % (w/v) HA-MA, 4 % (w/v) HA-MA, and 4 % (w/v) HA-ADH solutions were prepared in PBS and allowed to stir at RT overnight. Next day, they were crosslinked to obtain hydrogels, immersed in large volume of PBS, and incubated in an oven at 37 °C. The hydrogels were weighed everyday carefully removing the extra water and wet mass was recorded until day 6. PBS was then discarded and each sample was lyophilized for 72 hrs. After 72 hrs, dry mass of each sample was weighed and used to calculate swelling ratio. The swelling ratio values were then used for estimation of mesh size and crosslinking density of HA-MA and HA-ADH hydrogels.

3.3.4 Morphology of HA hydrogels

The morphology of HA-MA and HA-ADH hydrogels was observed under SEM (JSM 6360A Jeol, Japan). 2 % (w/v) HA-MA, 4 % (w/v) HA-MA, and 4 % (w/v) HA-ADH hydrogels, swollen in PBS buffer at 37 °C for 6 days, were cut longitudinally and transversely using a sharp cutter and rapidly quenched in

liquid nitrogen for few secs. The frozen samples were lyophilized for 72 hrs. Dried hydrogel samples were mounted on carbon tape over an aluminum stub and sputter coated with gold for 80 secs. The hydrogels were then imaged under different magnifications at an acceleration voltage of 5-10 kV.

Table 3 SEC-MALS raw data for estimated Mn, Mw, and PDI of HA samples

	HA Samples				
	Unmodified HA 1	Unmodified HA 2	Unmodified HA 3	Avg	Std dev
Number average molecular weight, M _n	1.03E+06	1.02E+06	1.02E+06	1.02E +06	4509. 25
Weight average molecular weight, M _w	1.07E+06	1.05E+06	1.05E+06	1.06E +06	11718 .93
Polydisper sity index, PDI	1.042	1.028	1.03	1.03E +00	0.007 572
	MA modified HA 1	MA modified HA 2	MA modified HA 3	Avg	Std dev
Number average molecular weight, M _n	9.40E+05	9.10E+05	9.46E+05	9.32E +05	19071 .71
Weight average molecular weight, M _w	9.92E+05	9.85E+05	1.01E+06	9.94E +05	10070 .92
Polydisper sity index, PDI	1.056	1.082	1.062	1.07E +00	0.013 614

	ADH modified HA 1	ADH modified HA 2	ADH modified HA 3	Avg	Std dev
Number average molecular weight, M_n	9.66E+05	1.61E+06	1.15E+06	1.24E +06	33194 8.5
Weight average molecular weight, M_w	1.87E+06	2.23E+06	1.98E+06	2.03E +06	18805 3.2
Polydisper sity index, PDI	1.932	1.387	1.72	1.68E +00	0.274 73
HA Samples		Average M_w		Standard deviation	
Unmodified HA		1055666.667		11718.93055	
MA modified HA, HA-MA		994033.3333		10070.91522	
ADH modified HA, HA-ADH		2025000		188053.184	

3.3.5 Rheology of HA hydrogels

HA-MA and HA-ADH hydrogels were also characterized for their rheological properties using a stress-controlled rheometer (Physica MCR 501, Anton Parr, USA). Parallel plate geometry (PP25/TGSN 6539, diameter 25 mm) was used as the measuring system for all the tests. All the experiments were performed at RT of ~23 °C and with zero normal force on samples to minimize the damage on to them. 2 % (w/v) HA-MA, 4 % (w/v) HA-MA, and 4% (w/v) HA-ADH hydrogels were swollen to equilibrium in PBS for 6 days at 37 °C and then used for amplitude and frequency sweep tests.

Amplitude sweep was conducted at an angular frequency of 10 rad/s to determine linear viscoelastic range (LVE). Dynamic moduli were obtained as a

function of % strain rate and the rates were varied from 0.01 % to 100 %. Oscillation shear tests were carried out at 0.1 % strain to determine storage moduli, loss moduli, and complex viscosity as a function of frequency, varying it from 0.1 to 10 Hz. **Table 4** shows the summary of all the characterization techniques used to evaluate the modified HA as well as the crosslinked hydrogels.

Table 4 Summary of characterization techniques for modified and crosslinked HA

Samples (n=3)	Characterization technique	Type of Characterization
Unmodified HA MA modified HA ADH modified HA	¹ H NMR - degree of modification	Structural
	SEC-MALS - molecular weight measurements	Physical
2 % HA-MA hydrogel 4 % HA-MA hydrogel 4 % HA-ADH hydrogel	SEM	Morphology
	Swelling measurements - swelling ratio (volumetric and mass), crosslinking density, mesh size	Physical
	Rheological measurements - amplitude and frequency sweep	Physical

3.4 Delivery Carriers for 5Fu

3.4.1 Preparation and Characterization of 5Fu loaded liposomes

Passive and Active loading of 5Fu into EPC liposomes

Liposomes were prepared using thin film hydration technique and 5Fu was encapsulated into EPC liposomes by active as well as passive loading.

For passive loading, 5Fu powder was dissolved in PBS buffer to make the solution for hydration of lipid. 10 mM EPC (with and without cholesterol 60:40) was dissolved in a co-solvent mixture of chloroform:methanol, keeping a ratio of 2:1 (v/v), in a round bottom flask (drug to lipid ratio was 0.23). The lipid was manually mixed in the solvents and then transferred to a rotary evaporator, maintained at 40 °C water bath, to remove the organic solvents for 2 hrs. After 2 hrs of solvent evaporation, a thin film of dried lipid was formed on the walls of round bottom flask which was then hydrated using the 5Fu-PBS solution. MLVs were formed upon complete hydration of the lipid film by manual shaking inside a water bath, maintained at 60 °C, for 10-15 mins. The MLVs suspension was then extruded 15 times through PC filters of sizes 0.2 µm and 0.08 µm that were fitted with drain disc onto a bench top extruder to obtain 5Fu loaded LUVs.

The 5Fu loaded LUVs suspension was transferred into a dialysis membrane (Spectra/Por CE dialysis tubing, 100 kDa MWCO, 10 mm diameter, flat width 16 mm) and dialyzed against 1 litre of PBS to remove the untrapped drug. At different time points (every 30 mins), 1 ml aliquot was withdrawn from PBS dialysis medium, filtered through 0.2 µm filter, and analyzed using high performance liquid chromatography (HPLC) (Agilent 1200 series, Agilent Technologies Singapore Pte Ltd). 1 ml sink medium was replaced with fresh

PBS buffer. After 4 hrs, the LUVs suspension was transferred from the dialysis membrane into an amber glass vial and stored at 4 °C overnight before characterization of % EE and *in vitro* release study.

For active loading, 5Fu powder was dissolved in 120 mM Na₂SO₄ + 1.2 mM Ca acetate solution. 10 mM EPC (with and without cholesterol 60:40) was dissolved in a co-solvent mixture of chloroform:methanol, keeping a ratio of 2:1 (v/v), in a round bottom flask. The lipid was manually mixed in the solvents and then transferred to a rotary evaporator, maintained at 40 °C water bath, to remove the organic solvents for 2 hrs. After 2 hrs of solvent evaporation, a thin film of dried lipid was formed on the walls of round bottom flask which was then hydrated using 120 mM Ca acetate solution (pH adjusted to 6). MLVs were formed upon complete hydration of the lipid film by manual shaking inside a water bath, maintained at 60 °C, for 10-15 mins. The MLVs suspension was then extruded 15 times through PC filters of size 0.2 µm and 0.08 µm that were fitted with drain disc on to a bench top extruder to obtain LUVs. After extrusion, the LUVs were characterized for size and pH.

The LUVs suspension was transferred into a dialysis membrane and dialyzed against 4 litres of 120 mM Na₂SO₄ + 1.2 mM Ca acetate solution (pH adjusted to 7) for 3 hrs to establish the pH gradient. After creating the gradient, the LUVs suspension was taken out from the dialysis membrane and characterized for size and pH. For loading, the LUVs suspension and 5Fu solution were heated for 2-3 mins inside a water bath (Polyscience, USA) at 60 °C. The LUVs suspension was then added into 5Fu solution and active loading was allowed to

continue for 1 hr at 60 °C with intermittent manual shaking of the solution every 10 mins. After 1 hr of loading, the 5Fu loaded LUVs was quenched in ice for 10 mins and then allowed to stand at RT for 5-10 mins. The LUVs were characterized for size and pH.

Finally, the 5Fu loaded LUVs suspension was transferred into a dialysis membrane and dialyzed against 1 litre of 120 mM Na₂SO₄ + 1.2 mM Ca acetate solution (pH adjusted to 7) to remove the untrapped drug. Every 30 mins, 1 ml aliquot was collected from the PBS dialysis medium, filtered through 0.2 µm filter, and analyzed using HPLC. 1 ml of sink medium was replaced with fresh buffer. After 4 hrs, the LUVs suspension was transferred from the dialysis membrane into an amber glass vial and stored at 4 °C overnight before characterization of % EE and release study.

Characterization of 5Fu loaded EPC liposomes

(a) Size

The average size as well as size distribution (polydispersity index, PDI) of 5Fu loaded EPC liposomes were estimated using the Malvern Zetasizer Nano ZS (Malvern Instruments Ltd, UK). A small aliquot of liposome suspension was diluted with a large volume of PBS (~1:100 v/v) and used for DLS measurement. The size was measured at RT with 3 runs (12 times in each run) for each sample.

(b) % EE

To determine the amount of entrapped 5Fu after extrusion, the LUVs (passively or actively loaded) were first broken down by adding IPA (1:4 v/v) and then the lipid mass was separated from the aqueous phase by centrifuging (Mikro 20, Hettich, Germany) the suspension at 13,000 rpm for 30 mins at RT. The supernatant was diluted 5X (total 25X) times using PBS buffer and estimated by HPLC. The concentration of 5Fu, calculated from clear supernatant, indicates the amount entrapped in the core of liposomes and the concentration from dialysis sink medium indicated the unentrapped amount present in continuous buffer phase.

HPLC system was used to estimate the amount of 5Fu entrapped in liposomes and also the unentrapped concentration in continuous phase. A reverse phase Eclipse-XDB C18 column (5 μ m, 4.6 mm IDX250 mm) was used with the mobile phase of H₂O:CH₃OH at 90:10 (v/v) ratio. The column was maintained at a temperature of 25 °C. Each sample was run at a flow rate of 1.0 ml/min for 5 mins including 1 min of post-run with the detector wavelength set at λ = 266 nm. The retention time of 5Fu was ~3.3 mins. Collected data was analyzed using Agilent's offline Chemstation software. 5Fu solutions with known concentrations (0.025 to 25 μ g/ml) were prepared by dissolving the 5Fu powder in PBS buffer. The solutions were filtered through 0.2 μ m filter and run in HPLC to obtain a standard curve (R^2 =0.999). Concentrations of 5Fu for the test samples were calculated using this calibration of HPLC system. This data from HPLC was used to calculate the % EE of EPC liposomes.

3.4.2 Preparation of 5Fu loaded Chitosan NPs

5Fu loaded CS NPs were prepared by ionic gelation using a polyanion TPP as shown in **Figure 14**. Briefly, CS solution was prepared in 1 % acetic acid and allowed to stir at RT overnight. 1 mg/ml TPP solution was also prepared and allowed to stir at RT overnight. The commercial 5Fu powder was crushed using a mortar and pestle to reduce its grain size. 5Fu powder was dissolved in CS solution and filtered through 0.45 μm filter. The pH of 5Fu-CS was adjusted to 4.7 using 5M NaOH. The solution was fixed to an overhead stirrer (IKA RW 20 digital, Germany) and speed was adjusted to 700 rpm.

TPP solution was filtered through 0.22 μm filter and then added to 5Fu-CS solution dropwise over 5-10 mins. As TPP was added, the clear solution started to turn opalescent. The ionic gelation reaction was allowed to continue for 1 hr. After 1 hr, a small aliquot was withdrawn from the solution for size measurement. The solution was then ultracentrifuged (Sorvall WX 100 Ultra Series, Thermo Electron Corporation, USA) at 25,000 rpm for 30 mins. Supernatant was used to detect untrapped 5Fu amount and pellet was resuspended thoroughly with 2 ml of ultrapure water, frozen at $-80\text{ }^{\circ}\text{C}$ for 1 hr, and lyophilized for 24 hrs.

Figure 14 Fabrication of 5Fu loaded chitosan NPs via o/w emulsification

Characterization of 5Fu loaded CS NPs

(a) Morphology and Size

The shape and surface morphology of 5Fu loaded CS NPs were observed under FESEM (JSM 6340F Jeol, Japan) and TEM (Carl Zeiss Libra 120 Plus, Germany). Dry NPs samples were suspended in ultrapure water in large dilution and sonicated for few secs in an ultrasonic cleaner (S60H Elma, Germany). For FESEM, 10-20 μl of NPs suspension was spread over clean silicon wafer substrate and allowed to dry overnight at RT. Next day, these wafers were mounted on double-sided adhesive carbon tape over an aluminum stub and sputter coated with platinum for 80 secs. The NPs were then imaged under different magnifications. For TEM, 8-10 μl of NPs suspension was spread over a lacey formvar/carbon coated copper grid (300 mesh) and allowed to dry

overnight at RT. Next day, the copper grids were fixed onto TEM holder and directly observed under microscope.

The average size as well as size distribution of 5Fu loaded CS NPs was estimated using the Malvern Zetasizer Nano ZS. 1 ml of NPs suspension was used for measurement. The size was measured at RT with 3 runs (12 times in each run) for each sample.

(b) % EE

After freeze drying, 5 mg of NPs was taken and dissolved in 20 ml of PBS (pH 5). The solution was sonicated for 30 mins and allowed to stir at RT for 24 hrs. Next day, the solution was probe sonicated (6 mm Probe, SONICS Vibra cell, USA) for 6 mins (with a pulse of 2 secs) at an amplitude of 40 % to extract the entrapped 5Fu completely. The solution was then ultracentrifuged at 25,000 rpm for 30 mins. The supernatant was used to calculate the entrapped amount of 5Fu in CS NPs. 5Fu is physically entrapped inside CS NPs and is also absorbed on the surface of NPs. The HPLC system was used to estimate the amount of 5Fu entrapped in NPs and also the unentrapped concentration in continuous phase. The concentration of 5Fu for all the test samples were calculated using the HPLC settings and calibration as described in section 3.4.1.

3.4.3 Preparation of 5Fu loaded Layer-by-layer NPs

20 mg of commercial hydroxyapatite NPs were washed in 10 ml ultrapure water by centrifugation at 10,000 rpm for 6 mins. The NPs were suspended in 0.5 ml of 0.5 M NaCl, vortexed (Labnet International VX100, USA), and sonicated

vigorously to obtain a homogeneous suspension. An aliquot was withdrawn to measure the zeta potential of uncoated NPs. The NPs suspension was added into 5 ml of 1 mg/ml CS solution, vortexed, and sonicated immediately for 20 mins at RT. The suspension was centrifuged (Thermo Electron Corporation B4i Multifunctional, USA) at 10,000 rpm for 6 mins. The supernatant was discarded and pellet was resuspended thoroughly using 0.5 ml of 0.5 M NaCl. An aliquot was withdrawn to measure the zeta potential of CS coated (1st layer) NPs. The NPs suspension was added into 5 ml of 5 mg/ml 5Fu solution, vortexed, and sonicated immediately for 20 mins at RT. The suspension was centrifuged at 10,000 rpm for 6 mins. An aliquot of supernatant was collected to estimate the amount of untrapped 5Fu and rest of the supernatant was discarded. The pellet was resuspended thoroughly using 0.5 ml of 0.5 M NaCl. An aliquot was withdrawn to measure the zeta potential of CS-5Fu (2nd layer) coated NPs.

The NPs suspension was added into 5 ml of 1 mg/ml CS solution, vortexed, and sonicated immediately for 20 mins at RT. The suspension was centrifuged at 10,000 rpm for 6 mins. An aliquot of supernatant was collected to estimate the amount of untrapped 5Fu and rest of the supernatant was discarded. The pellet was resuspended thoroughly using 0.5 ml of 0.5 M NaCl. An aliquot was withdrawn to measure the zeta potential of CS-5Fu-CS (3rd layer) coated NPs. The NPs suspension was added into 5 ml of 5 mg/ml 5Fu solution, vortexed, and sonicated immediately for 20 mins at RT. The suspension was centrifuged at 10,000 rpm for 6 mins. An aliquot of supernatant was collected to estimate the amount of untrapped 5Fu and rest of the supernatant was discarded. The pellet was resuspended thoroughly using 0.5 ml of 0.5 M NaCl. An aliquot was

withdrawn to measure the zeta potential of CS-5Fu-CS-5Fu (4th layer) coated NPs.

The NPs suspension was added into 5 ml of 1 mg/ml CS solution, vortexed, and sonicated immediately for 20 mins at RT. The suspension was centrifuged at 10,000 rpm for 6 mins. An aliquot of supernatant was collected to estimate the amount of unentrapped 5Fu and rest of the supernatant was discarded. The pellet was resuspended thoroughly using 2 ml of 0.5 M NaCl. An aliquot was withdrawn to measure the zeta potential of CS-5Fu-CS-5Fu-CS (5th layer) coated NPs. The suspension was centrifuged at 10,000 rpm for 6 mins. The supernatant was discarded, pellet was freeze dried, and characterized for size and release study.

Characterization of 5Fu loaded LBL NPs

(a) Size

The average size, size distribution, as well as zeta potential of LBL NPs was estimated using the Malvern Zetasizer Nano ZS. A small amount of dried NPs was diluted with a large volume of ultrapure water and sonicated for few secs in an ultrasonic cleaner. 1 ml of this suspension was used for measurement. The size and zeta potential were measured at RT with 3 runs (12 times in each run) for each sample.

3.4.4 Preparation of 5Fu loaded PLGA NPs

Water-in-oil-in-water (w/o/w) emulsion technique was used to prepare PLGA (0.4A intrinsic viscosity, IV) NPs encapsulated with 5Fu. 10-12 mg of this

crushed 5Fu powder was dissolved in 500 μ l of ultrapure water. The solution was vortexed and sonicated vigorously to dissolve the 5Fu completely. 100 mg of PLGA was added into 5ml DCM and vortexed vigorously until all the PLGA was completely dissolved. 1 % (w/v) PVA solution (100 ml) was prepared in DI water and filtered through 0.2 μ m filter (organic to aqueous phase ratio of 1:20). 40 ml of filtered PVA solution was kept under overhead stirrer at a speed of 1,000 rpm. 10 ml of PVA solution was kept under sonication (6 mm Probe). The 5Fu-H₂O solution was poured into PLGA-DCM solution and immediately vortexed vigorously to prevent precipitation. This 5Fu-H₂O-PLGA-DCM single emulsion was then poured into PVA solution under sonication. The solution turned milky as double emulsion is formed and it was sonicated for 3 mins at 30 % amplitude while shaking the glass vial intermittently to prevent the formation of precipitates. After 3 mins, the emulsion was immediately added into the 40 ml continuous phase of PVA and allowed to stir at 1,000 rpm at RT to evaporate the solvent for 1.5 hrs.

After 1.5 hrs, rest of the 50 ml PVA was added into the emulsion and solvent evaporation was continued for 3.5 hrs more. After 5 hrs, the emulsion was removed from the stirrer and centrifuged at 9,500 rpm for 16 mins at RT. An aliquot of supernatant was taken to estimate the amount of untrapped 5Fu and rest of the supernatant was discarded. The pellet was resuspended thoroughly using 10 ml of ultrapure water and washed twice. After each washing, an aliquot was collected and after second wash, the pellet was suspended in 1 ml of 1 % (w/v) mannitol (prepared in ultrapure water), frozen at -80 °C for 1 hr, and then freeze dried for 24 hrs.

Characterization of 5Fu loaded PLGA NPs

(a) Morphology and Size

The shape and surface morphology of 5Fu loaded PLGA NPs were observed under FESEM. For FESEM, 10-20 μ l of NPs suspension was spread over clean silicon wafer substrate and allowed to dry overnight at RT. Next day, these wafers were mounted on double-sided adhesive carbon tape over an aluminium stub and sputter coated with platinum for 80 secs. The NPs were then imaged under different magnifications.

The average size, size distribution, as well as zeta potential of PLGA NPs was estimated using the Malvern Zetasizer Nano ZS. A small amount of dried NPs was diluted with a large volume of ultrapure water and sonicated for few secs in an ultrasonic cleaner. 1 ml of this suspension was used for measurement. The size and zeta potential were measured at RT with 3 runs (12 times in each run) for each sample.

3.5 PLGA MPs for 5Fu Delivery

3.5.1 Preparation of 5Fu loaded PLGA MPs

s/o/w emulsion technique was used to prepare PLGA MPs encapsulated with 5Fu. 1 ml of DCM was first added into 25 mg crushed 5Fu powder and the mixture undergoes 2 mins of probe sonication (3mm Probe) at 30 % amplitude to further reduce the particles of 5Fu and disperse them homogeneously in DCM. 1 % (w/v) PVA solution (100 ml) was prepared in DI water, filtered

through 0.2 μ m filter, and the pH was adjusted to 1.7 using 5M HCl to reduce the solubility of 5Fu (organic to aqueous phase ratio of 1:100). 40 ml of this filtered PVA solution was kept under overhead stirrer at a speed of 1,000 rpm. 20 % concentration PLGA (0.4A IV or 0.4E IV), 300 mg, was added into the 5Fu-DCM suspension and vortexed vigorously until the PLGA was completely dissolved. The resulting mixture undergoes probe sonication for 2 mins and immediately added into the continuous phase of PVA using a glass pasteur pipette to form the emulsion.

The emulsion was stirred at 1,000 rpm, RT to evaporate the solvents for 1.5 hrs. After 1.5 hrs, rest of the 60 ml PVA was added into the emulsion and solvent evaporation was continued for 3.5 hrs more. After 5 hrs, the emulsion was removed from the stirrer and centrifuged at 8,000 rpm for 6.3 mins at RT. An aliquot of supernatant was taken to estimate the amount of untrapped 5Fu and rest of the supernatant was discarded. The pellet was resuspended thoroughly using 10 ml of ultrapure water and washed twice. After each washing, an aliquot was collected and after second wash, the pellet was suspended in 1 ml of 1 % (w/v) mannitol, frozen at -80°C for 1 hr, and then freeze dried for 24 hrs. Blank PLGA MPs were also prepared using the same procedure excluding the sonication steps and addition of 5Fu.

3.5.2 Characterization of 5Fu loaded PLGA MPs

3.5.2.1 Yield

After freeze drying, the 5Fu loaded MPs were sieved using 106 μm and 150 μm sieves and the dry mass before and after sieve were recorded. The sieved MPs were stored inside a desiccator until further use.

3.5.2.2 Morphology and Size distribution

The shape and surface morphology of 5Fu loaded PLGA MPs were observed under SEM. Dry MPs samples were spread over double-sided adhesive carbon tape which was then mounted on an aluminum stub and sputter coated with gold for 80 secs. The MPs were then imaged under different magnifications at an acceleration voltage of 5-10 kV. After imaging the MPs for shape and morphology characterization, the SEM images were used for size estimation of particles. ImageJ software was used with these images and they were scanned for a minimum of 1000 particles from different images for size analysis using area-equivalent radius/diameter.

3.5.2.3 % Loading and % EE

5Fu loaded PLGA MPs were extracted using DCM and PBS solvents to estimate the amount of 5Fu entrapped and calculate % loading and % EE. 20 mg of sieved MPs were taken and dissolved in 5 ml of DCM. The solution was vortexed until all the PLGA was dissolved. It was transferred into a 250 ml conical separating funnel and 10 ml of PBS was added to it. The funnel was capped and all the content was vigorously shaken for few mins to mix the PBS and DCM solution. The solutions are allowed to settle down or stand at RT for 5-10 mins. The immiscible phases of DCM and PBS separate. The solution was

again shaken and allowed for phase separation for 2 more cycles. After the 3rd cycle, the bottom phase of DCM was collected in a small glass beaker. The PBS was collected in a 50 ml centrifuge tube. The DCM was poured back into the funnel and fresh 10 ml of PBS was added.

The above steps were repeated for 4 cycles and PBS from first 4 cycles (40 ml) were collected together in the same 50 ml tube whereas the PBS solution from 5th cycle was collected in a separate 15 ml tube. The second tube would ensure if the entire amount of drug has been extracted from the particles or not. 1 ml each of the collected 5Fu-PBS solutions from both the tubes were filtered through 0.2 µm filter and analyzed using HPLC. The HPLC system was used to estimate the amount of 5Fu entrapped in PLGA MPs and also the untrapped concentration in continuous phase. The concentration of 5Fu for all the test samples were calculated using the HPLC settings and calibration as described in section 3.4.1.

3.5.2.4 Differential scanning calorimetry (DSC)

The thermal properties of 5Fu was evaluated before and after encapsulation inside PLGA MPs using DSC (Q10 V9.9 Build 303, TA Research Instruments, UK). Blank PLGA MPs and 5Fu crushed powder alone samples were also run for comparison. ~7 mg of each sample was taken in an aluminium pan and crimped with the lid. An empty pan was used as the reference. The following procedure was followed for temperature cycles: samples were cooled at 10 °C/min from RT to 0 °C, equilibrated at this temperature for 2 mins, heated at 2 °C/min to 330 °C, kept at this temperature for 2 mins, cooled back to 0 °C at 10 °C/min, and 1 more cycle of ramp to 330 °C at 2 °C/min was run. TA's

Universal analysis software was used to analyze the melting point of 5Fu and glass transition temperature of PLGA from the thermograms.

3.5.3 *In vitro* release of 5Fu from solutions

3.5.3.1 Release of 5Fu from HA solutions

5Fu crushed powder was weighed and dissolved in PBS buffer at a concentration of 0.3 mg/ml. After 5Fu is completely dissolved, HA powder was added to prepare 2 % and 4 % (w/v) solutions. The solutions were allowed to mix overnight on magnetic stirrer at RT.

For release study, 1 ml of each of the 5Fu-HA solution sample was taken into a CE dialysis membrane (10 kDa MWCO, 2.9 cm diameter, flat width 4.5 cm) and clipped at both ends using dialysis clips. The dimensions of dialysis membrane were maintained same for all the samples. The dialysis membrane was then placed into the release medium of PBS buffer in a screw capped bottle to avoid evaporation loss. The samples were then kept inside an orbital shaker incubator (LM-570RD, Yihder Technology, Taiwan) at 50 rpm maintained at 37 °C.

1 ml aliquots were withdrawn at various time intervals from the release medium, filtered through 0.2 µm syringe filter, and collected in HPLC sample vials (2 ml, Agilent). These samples were stored at 4 °C until analyzed by HPLC using the calibration as discussed in section 3.4.1. At every time point,

the release medium was completely replaced with fresh PBS buffer to avoid drug saturation and obtain a closer approximation of dynamic sink condition.

3.5.3.2 Release of 5Fu from PLGA MPs

For release study, 20 mg of 5Fu loaded PLGA MPs (equivalent of 0.3 mg/ml 5Fu) were weighed and placed directly into a dialysis membrane, immersed in PBS, and incubated at 37 °C inside shaker incubator. Rest of the procedure was same as described in section 3.5.3.1.

3.5.3.3 Release of 5Fu from PLGA MPs with HA solutions

HA powder was dissolved in PBS buffer to prepare 2 % and 4 % (w/v) solutions. The solutions were allowed to mix overnight on magnetic stirrer at RT. Next day, 5Fu loaded PLGA MPs (equivalent of 0.3 mg/ml 5Fu) were weighed and mixed in HA solutions manually using a spatula. After thorough mixing, the solutions were transferred into a dialysis membrane, immersed in PBS, and incubated at 37 °C inside shaker incubator. Rest of the procedure was same as described in section 3.5.3.1.

3.6 Composite Hydrogels of HA and 5Fu loaded PLGA MPs

3.6.1 Preparation of hydrogels

3.6.1.1 5Fu loaded HA-MA and HA-ADH hydrogels

5Fu crushed powder was weighed and dissolved in PBS buffer at a concentration of 0.3 mg/ml. After 5Fu is completely dissolved, HA-MA powder was added to prepare 2 % and 4 % (w/v) solutions and HA-ADH powder was added to prepare 4 % (w/v) solution. All the solutions were allowed to mix

overnight on magnetic stirrer at RT. Next day, HA-MA+5Fu solution (1 ml for each sample) was crosslinked using UV light as described in section 3.2.2. HA-ADH+5Fu samples (1 ml each) were chemically crosslinked using PEGDE as described in section 3.2.4.

3.6.1.2 5Fu-PLGA MPs loaded HA-MA and HA-ADH hydrogels

HA-MA powder was dissolved in PBS buffer to prepare 2 % and 4 % (w/v) solutions and HA-ADH powder was dissolved in PBS to prepare 4 % (w/v) solution. All the solutions were allowed to mix overnight on magnetic stirrer at RT. Next day, 20 mg of 5Fu loaded PLGA MPs (equivalent of 0.3 mg/ml 5Fu) were weighed and mixed in HA-MA and HA-ADH solutions, manually using a spatula (polymer to particles mass ratio of 1:1 for 2 % hydrogels and 2:1 for 4 % hydrogels). After thorough mixing, HA-MA+5Fu-PLGA MPs solutions were crosslinked using UV light as described in section 3.2.2. HA-ADH+5Fu-PLGA MPs sample was chemically crosslinked using PEGDE as described in section 3.2.4. **Figure 15** shows the schematic of photocrosslinking of HA-MA solution mixed with 5Fu loaded PLGA MPs to develop composite hydrogels.

3.6.2 Characterization of composite hydrogels

3.6.2.1 Physical stability of entrapped and released MPs

The physical stability of 5Fu loaded PLGA MPs entrapped inside HA hydrogels was observed under SEM. Composite hydrogels of Ltp loaded PLGA MPs and HA-MA/HA-ADH were swollen in PBS without dialysis membrane at 37 °C for 3 days. The swollen gels were taken out and cut longitudinally and transversally using a sharp cutter and immediately frozen at -80 °C and

lyophilized after 2 hrs. The sink medium was also collected and used to examine released MPs, if any. 10-20 μ l of this suspension was spread over clean silicon wafer substrate and allowed to dry overnight at RT.

Figure 15 Schematic showing the fabrication of HA-MA composite hydrogels incorporated with 5Fu loaded PLGA MPs

Next day, the dried hydrogel samples as well as the sink medium wafer samples were mounted on double-sided adhesive carbon tape over an aluminum stub and sputter coated with platinum for 80 secs. The hydrogel entrapped MPs were then imaged under different magnifications at an acceleration voltage of 5-10 kV.

3.6.3 *In vitro* release of 5Fu from hydrogels

3.6.3.1 Release of 5Fu from HA-MA and HA-ADH hydrogels

0.3 mg/ml 5Fu loaded 2 % HA-MA, 4 % HA-MA, and 4 % HA-ADH hydrogels were prepared as described in section 3.6.1.1. The crosslinked gels were carefully removed from the moulds or vials, transferred into a dialysis membrane, immersed in PBS, and incubated at 37 °C inside shaker incubator. Rest of the procedure was same as described in section 3.5.3.1.

3.6.3.2 Release of 5Fu from PLGA MPs and HA-MA/HA-ADH composite hydrogels

20 mg of 5Fu-PLGA MPs (equivalent of 0.3 mg/ml 5Fu) loaded 2 % HA-MA, 4 % HA-MA, and 4 % HA-ADH composite hydrogels were prepared as described in section 3.6.1.2. The crosslinked gels were carefully removed from moulds or vials, transferred into a dialysis membrane, immersed in PBS, and incubated at 37 °C inside shaker incubator. Rest of the procedure was same as described in section 3.5.3.1.

3.6.4 Degradation of 5Fu loaded MPs

3.6.4.1 Degradation of 5Fu loaded MPs

For degradation study, 5Fu loaded PLGA MPs were weighed and placed directly into a CE dialysis membrane and clipped at both ends using dialysis clips. The dimensions of dialysis membrane were maintained same as release study for all the samples. The dialysis membrane was then placed in PBS buffer medium in a screw capped bottle and incubated inside an orbital shaker incubator at 50 rpm maintained at 37 °C. For mass loss experiments, the MPs

were weighed and directly soaked in PBS buffer in a centrifuge tube to prevent losses during transfer steps before drying.

The release medium was completely replaced with fresh PBS buffer everyday similar to release study. Every 3rd day, the buffer medium was discarded, the sample was removed and washed 3 times with ultrapure water to remove salts. After washing, the samples were frozen at –80 °C for 1 hr, and freeze dried for 72 hrs. After 3 days, the dried samples were removed, dissolved in chloroform, and allowed to stir at RT overnight.

Next day, the samples were filtered through 0.2 µm syringe filter and analyzed by SEC system. The samples were run in a mobile phase of pure chloroform at a flow rate of 1 ml/min, an injection volume of 50 µl and at a temperature of 35 °C for 21 mins with a post-run of 1 min. The system was calibrated with polystyrene (PS) standards. The weight average molecular weights of all samples were calculated in relative to the standards. For mass loss experiment, the weight of dried MPs was recorded and knowing the initial weight, the % mass loss was calculated at different time points.

3.6.4.2 Degradation of 5Fu loaded MPs and HA-MA composite hydrogels

5Fu-PLGA MPs loaded 4 % HA-MA composite hydrogels were prepared as described in section 3.6.2.1. The crosslinked gels were carefully removed from moulds, transferred into a dialysis membrane, immersed in PBS, and incubated at 37 °C inside shaker incubator. Rest of the procedure was same as described in section 3.6.4.1 except the SEC sample preparation.

At every time point, after washing the samples to remove salts, and freeze drying, the gels were quickly transferred into fresh vials and broken into fine pieces manually using a spatula. Chloroform was added to these gel samples and allowed to stir at RT overnight to extract out the embedded MPs completely and dissolve them (HA-MA does not dissolve in chloroform). Next day, the samples were filtered through 0.2 μm syringe filter (only dissolved particles in chloroform passes through the filter retaining the gel pieces in the filter mesh) and analyzed by SEC system.

3.7 EPC liposomes for Ltp Delivery

3.7.1 Preparation of Ltp loaded EPC liposomes

EPC liposomes were prepared using thin film hydration technique and Ltp was loaded passively into them. 0.7 mM concentration of Ltp (stock in ACN) and 10 mM EPC were dissolved in a co-solvent mixture of 2:1 (v/v) ratio of chloroform:methanol in a round bottom flask (drug to lipid ratio was 0.07). The drug-lipid solution was manually mixed with the solvents in the flask and then transferred to a rotary evaporator, maintained at 40 °C water bath, to remove the organic solvents for 2 hrs. After 2 hrs of solvent evaporation, a thin, dried film of drug-lipid was formed on the walls of round bottom flask which was then hydrated using PBS buffer. The film was completely hydrated by manual shaking for 10-15 mins inside a water bath, maintained at 60 °C, to form MLVs. The MLVs suspension was then extruded 15 times through PC filters of size 0.2 μm and 0.08 μm that were fitted with drain disc on to a bench top

extruder to obtain Ltp loaded LUVs. Blank EPC liposomes were also prepared using the same procedure excluding the addition of Ltp.

3.7.2 Characterization of Ltp loaded EPC liposomes

3.7.2.1 Morphology and Size distribution

The morphology of Ltp loaded EPC liposomes were observed under TEM. 8-10 μ l of liposomes suspension was spread over a lacey formvar/carbon coated copper grid (300 mesh) and allowed to dry overnight at RT. Next day, the copper grids were fixed onto TEM holder directly and observed under microscope at different magnifications at an acceleration voltage of 120 kV.

The average size as well as size distribution of Ltp loaded EPC liposomes was estimated using the Malvern Zetasizer Nano ZS. A small aliquot of liposome suspension was diluted with a large volume of PBS and used for DLS measurement. The size was measured at RT with 3 runs (12 times in each run) for each sample.

3.7.2.2 % EE

Ltp is a lipophilic drug that distributes itself between the aqueous continuous phase and the lipid bilayer. The HPLC system was used to estimate the total amount of Ltp in liposomes and its concentration in continuous buffer phase and partitioned into the lipid bilayer. A reverse phase Eclipse-XDB C18 column (5 μ m, 4.6 mm IDX250 mm) was used with the mobile phase of ACN:Water at 70:30 (v/v) ratio. The column was maintained at a temperature of 25 °C. Each sample was run at a flow rate of 1.0 ml/min for 6 mins including 1 min of post-

run with the detector wavelength set at $\lambda = 210$ nm. The retention time of Ltp was ~4.4 mins. Collected data was analyzed using Agilent's offline Chemstation software.

Ltp solutions with known concentrations (1 to 25 $\mu\text{g/ml}$) were prepared in PBS buffer by diluting its stock dissolved in ACN. The solutions were filtered through 0.2 μm filter and run in HPLC to obtain a standard curve ($R^2 = 0.997$). Concentrations of Ltp for the test samples were calculated using this calibration of HPLC system.

To determine the total amount of Ltp after extrusion, the LUVs were first broken down by adding IPA (1:4 v/v) and then the lipid mass was separated from the aqueous phase by centrifuging the suspension at 13,000 rpm for 30 mins at RT. The supernatant was diluted 5X (total 25X) times using PBS buffer and estimated by HPLC. Ltp samples from MLVs suspension were also analyzed to estimate the concentration in aqueous phase and lipid bilayer and total amount of drug before extrusion.

For this, known volumes were collected in 2 microfuge tubes I and II. Suspension in tube I was diluted at 1:4 v/v ratio with PBS buffer and for tube II, IPA was added at same ratio to break down MLVs. The suspensions were centrifuged at 13,000 rpm for 30 mins at RT. Supernatants were diluted 5X (total 25X) times using PBS buffer and estimated by HPLC. The concentration of Ltp, calculated from clear supernatant of tube I, indicates the amount present

in continuous buffer phase. Therefore, subtracting this value from the total drug amount would give a measure of drug partitioned into the EPC bilayer.

3.7.3 *In vitro* release of Ltp

3.7.3.1 Release of Ltp from HA solutions

Ltp was dissolved in PBS buffer at a concentration of 0.3 mg/ml and to this solution, HA powder was added to prepare 2 % and 4 % (w/v) solutions. The solutions were allowed to stir overnight at RT.

For release study, 1 ml of each of the Ltp-HA solution samples was taken into a CE dialysis membrane (10 kDa MWCO, 2.9 cm diameter, flat width 4.5 cm) and clipped at both ends using dialysis clips. The dimensions of dialysis membrane were maintained same for all the samples. The dialysis membrane was then placed into the release medium of PBS buffer in a screw capped bottle. The samples were incubated inside an orbital shaker at 50 rpm maintained at 37 °C.

1 ml aliquots were withdrawn at various time intervals from the release medium, filtered through 0.2 µm syringe filter, and collected in HPLC sample vials. These samples were stored at 4 °C until analyzed by HPLC using the calibration as discussed in section 3.7.2.2. At every time point, the release medium was completely replaced with fresh PBS buffer.

3.7.3.2 Release of Ltp from EPC liposomes

For release study, 1 ml of Ltp loaded EPC liposomes (equivalent of 0.3 mg/ml Ltp) was taken and transferred directly into a dialysis membrane, immersed in PBS, and incubated at 37 °C inside shaker incubator. Rest of the procedure was same as described in section 3.7.3.1.

3.7.3.3 Release of Ltp from EPC liposomes with HA solutions

HA powder was dissolved directly in 1 ml of Ltp loaded EPC liposomes suspension (equivalent of 0.3 mg/ml Ltp) to prepare 2 % and 4 % (w/v) solutions. The solutions were allowed to mix overnight on magnetic stirrer at RT. Next day, the solutions were transferred into a dialysis membrane, immersed in PBS, and incubated at 37 °C inside shaker incubator. Rest of the procedure was same as described in section 3.7.3.1.

3.8 Composite Hydrogels of HA and Ltp loaded EPC liposomes

3.8.1 Preparation of hydrogels

3.8.1.1 Ltp loaded HA-MA and HA-ADH hydrogels

Ltp was dissolved in PBS buffer at a concentration of 0.3 mg/ml and to this solution, HA-MA powder was added to prepare 2 % and 4 % (w/v) solutions and HA-ADH powder was added to prepare 4 % (w/v) solutions. All the solutions were allowed to mix overnight on magnetic stirrer at RT. Next day, HA-MA+Ltp solution (1 ml for each sample) was crosslinked using UV light as

described in section 3.2.2. HA-ADH+Ltp samples (1 ml each) were chemically crosslinked using PEGDE as described in section 3.2.4.

3.8.1.2 Ltp-EPC liposomes loaded HA-MA and HA-ADH hydrogels

HA-MA powder was dissolved directly in 1 ml of Ltp loaded EPC liposomes suspension (equivalent of 0.3 mg/ml Ltp) to prepare 2 % and 4 % (w/v) solutions and HA-ADH powder was also dissolved directly in 1 ml of Ltp loaded EPC liposomes suspension to prepare 4 % (w/v) solution. All the solutions were allowed to mix overnight on magnetic stirrer at RT. Next day, HA-MA+Ltp-EPC liposomes solutions were crosslinked using UV light as described in section 3.2.2. HA-ADH+Ltp-EPC liposomes sample was chemically crosslinked using PEGDE as described in section 3.2.4. **Figure 16** shows the schematic of photocrosslinking of HA-MA solution mixed with Ltp loaded EPC liposomes to develop composite hydrogels.

Figure 16 Schematic showing the fabrication of HA-MA composite hydrogels incorporated with Ltp loaded EPC liposomes

3.8.2 Characterization of composite hydrogels

3.8.2.2 Physical stability of released liposomes

Composite hydrogels of Ltp loaded EPC liposomes with HA-MA and HA-ADH were directly immersed in PBS buffer without dialysis membrane to evaluate the release of liposomes and their stability thereof. The aliquots from sink medium were examined under TEM to image the released liposomes if any from the composite hydrogels and to evaluate the bilayer stability of released liposomes. 8-10 μ l of the medium was spread over a lacey formvar/carbon coated copper grid (300 mesh) and allowed to dry overnight at RT. Next day, the copper grids were fixed onto TEM holder and observed under microscope at different magnifications at an acceleration voltage of 120 kV.

Also, the aliquots from the sink medium of Ltp-EPC liposomes+HA-MA/HA-ADH composite hydrogels were used to check the size distribution of released liposomes using NanoSight LM10-HS and analyzed using Nanoparticle Tracking Analysis (NTA) software (Version2.3 Build 0008 BETA4, NanoSight, Amesbury, UK). A sample volume of 0.5 mL was measured for 30 secs with manual shutter and gain adjustments at RT. Mean and standard deviation for each sample were obtained using the NTA software.

3.8.3 *In vitro* release of Ltp from hydrogels

3.8.3.1 Release of Ltp from HA-MA and HA-ADH hydrogels

0.3 mg/ml Ltp loaded 2 %, 4 % HA-MA, and 4 % HA-ADH hydrogels were prepared as described in section 3.8.1.1. The crosslinked gels were carefully

removed from the moulds or vials, transferred into a dialysis membrane, immersed in PBS, and incubated at 37 °C inside shaker incubator. Rest of the procedure was same as described in section 3.7.3.1.

3.8.3.2 Release of Ltp from EPC liposomes and HA-MA/HA-ADH composite hydrogels

1 ml Ltp-EPC liposomes (equivalent of 0.3 mg/ml Ltp) loaded 2 %, 4 % HA-MA, and 4 % HA-ADH composite hydrogels were prepared as described in section 3.8.1.2. The crosslinked gels were carefully removed from moulds or vials, transferred into a dialysis membrane, immersed in PBS, and incubated at 37 °C inside shaker incubator. Rest of the procedure was same as described in section 3.7.3.1.

3.9 PLGA NPs/MPs for Ltp Delivery

3.9.1 Preparation of Ltp loaded PLGA NPs

o/w emulsion technique was used to prepare PLGA NPs encapsulated with Ltp. 4.67 mg of Ltp was dissolved in 2 ml of DCM and the solution was vortexed for few secs. 10 % concentration of PLGA (0.4A IV or 0.4E IV), 200 mg, was added into the Ltp-DCM solution and vortexed vigorously until all the PLGA was completely dissolved. 1 % (w/v) PVA solution (100 ml) was prepared in DI water and filtered through 0.2 µm filter (organic to aqueous phase ratio of 1:50). 40 ml of this filtered PVA solution was kept under overhead stirrer at a speed of 1,000 rpm. 10 ml of PVA solution was kept under sonication using 6 mm probe. The Ltp-PLGA-DCM solution was then poured into this PVA

solution under sonication. The PVA solution turns milky as the emulsion is formed and it was sonicated for 3 mins at 30 amplitude while shaking the glass vial intermittently to prevent the formation of precipitates. After 3 mins of sonication, the emulsion was immediately added into the 40 ml continuous phase of PVA and allowed to stir at 1,000 rpm, RT to evaporate the solvent for 1.5 hrs.

After 1.5 hrs, rest of the 50 ml PVA was added into the emulsion and solvent evaporation was continued for 3.5 hrs more. After 5 hrs, the emulsion was removed from the stirrer and centrifuged at 9,500 rpm for 16 mins at RT. An aliquot of supernatant was taken to estimate the amount of unentrapped Ltp and rest of the supernatant was discarded. The pellet was resuspended thoroughly using 10 ml of ultrapure water and washed twice. After each washing, an aliquot was collected and after second wash, the pellet was suspended in 1 ml of 1 % (w/v) mannitol, frozen at -80°C for 1 hr, and then freeze dried for 24 hrs. Blank PLGA NPs were also prepared using the same procedure excluding the addition of Ltp.

3.9.2 Preparation of Ltp loaded PLGA MPs

o/w emulsion technique was used to prepare PLGA MPs encapsulated with Ltp. 3.34 mg of Ltp was dissolved in 2 ml of DCM, 6 mg of Ltp was dissolved in 1 ml of DCM and the solutions were vortexed for few secs. 10 % (200 mg) and 20 % (300 mg) concentration of PLGA (0.4A IV or 0.4E IV) was added into the Ltp-DCM solutions and vortexed vigorously until all the PLGA was completely dissolved. 1 % (w/v) PVA solution (100 ml) was prepared in DI water and

filtered through 0.2 μm filter (organic to aqueous phase ratio of 1:50 or 1:100). 40 ml of this filtered PVA solution was kept under overhead stirrer at a speed of 1,000 rpm. After all the PLGA was dissolved, the Ltp-PLGA-DCM solution was immediately added into the 40ml continuous phase of PVA using a glass pasteur pipette to form the emulsion. The emulsion was stirred at 1,000 rpm, RT to evaporate the solvents for 1.5 hrs.

After 1.5 hrs, rest of the 60 ml PVA was added into the emulsion and solvent evaporation was continued for 3.5 hrs more. After 5 hrs, the emulsion was removed from the stirrer and centrifuged at 8,000 rpm for 6.3 mins at RT. An aliquot of supernatant was taken to estimate the amount of unentrapped Ltp and rest of the supernatant was discarded. The pellet was resuspended thoroughly using 10 ml of ultrapure water and washed twice. After each washing, an aliquot was collected and after second wash, the pellet was suspended in 1 ml of 1 % (w/v) mannitol, frozen at $-80\text{ }^{\circ}\text{C}$ for 1 hr, and then freeze dried for 24 hrs. Blank PLGA MPs were also prepared using the same procedure excluding the addition of Ltp.

3.9.3 Characterization of Ltp loaded PLGA NPs/MPs

3.9.3.1 Yield

After freeze drying, the Ltp loaded NPs/MPs were sieved using 106 μm , 150 μm , and 212 μm sieves and the dry mass before and after sieve were recorded. The sieved NPs/MPs were stored inside a desiccator until further use.

3.9.3.2 Morphology and Size distribution

The shape and surface morphology of Ltp loaded PLGA MPs were observed under SEM. Dry MPs samples were spread over double-sided adhesive carbon tape which was then mounted on an aluminum stub and sputter coated with gold for 80 secs. The MPs were then imaged under different magnifications at an acceleration voltage of 5-10 kV.

After imaging the MPs for shape and morphology characterization, the SEM images were used for size estimation of particles. ImageJ software was used with these images and they were scanned for a minimum of 1000 particles from different images for size analysis using area-equivalent radius/diameter.

The shape and surface morphology of Ltp loaded PLGA NPs were observed under FESEM. Dry NPs samples were suspended in ultrapure water in large dilution and sonicated for few secs in an ultrasonic cleaner. 10-20 μ l of this suspension was spread over clean silicon wafer substrate and allowed to dry overnight at RT. Next day, these wafers were mounted on double-sided adhesive carbon tape over an aluminum stub and sputter coated with platinum for 80 secs. The NPs were then imaged under different magnifications.

The average size as well as size distribution of Ltp loaded NPs was estimated using the Malvern Zetasizer Nano ZS. A small amount of dried NPs was diluted with a large volume of ultrapure water and sonicated for few secs in an ultrasonic cleaner. 1ml of this suspension was then used for DLS measurement of size at RT with 3 runs (12 times in each run) for each sample.

3.9.3.3 % Loading and % EE

Ltp loaded PLGA NPs/MPs were extracted using ACN and PBS solvents to estimate the amount of Ltp entrapped and to calculate % loading and % EE. 20 mg of sieved NPs/MPs were taken in a 15 ml centrifuge tube and dissolved in 2 ml of ACN. The solution was vortexed vigorously until all the PLGA was dissolved. 10 ml of PBS was added to the tube and immediately vortexed for few mins to prevent precipitation. The solution was then centrifuged at 7,500 rpm for 12 mins. The supernatant was collected in a 50 ml tube and to the pellet fresh 2 ml of ACN was added. These extraction steps were repeated for 4 more cycles and the supernatant from first 4 cycles (40 ml) were collected together in the same 50 ml tube whereas the supernatant from 5th cycle was collected in a separate 15 ml tube.

The second tube would ensure if the entire amount of drug has been extracted from the NPs/MPs. 1 ml each of the collected Ltp-ACN-PBS solutions from both the tubes was filtered through 0.2 μ m filter and analyzed using HPLC. HPLC system was used to estimate the amount of Ltp entrapped in PLGA NPs/MPs. The concentration of Ltp for all the test samples were calculated using the settings and calibration of HPLC system as described in section 3.7.2.2.

3.9.4 *In vitro* release of Ltp

3.9.4.1 Release of Ltp from PLGA NPs/MPs

For release study, 20 mg of Ltp loaded PLGA NPs/MPs (equivalent of 0.3 mg/ml Ltp) were weighed, placed directly into a CE dialysis membrane, immersed in PBS buffer and incubated at 37 °C inside shaker incubator. Rest of the procedure was same as described in section 3.7.3.1.

3.9.4.2 Release of Ltp from PLGA NPs/MPs and HA solutions

HA powder was dissolved in PBS buffer to prepare 2 % and 4 % (w/v) solutions. The solutions were allowed to mix overnight on magnetic stirrer at RT. Next day, 20 mg of Ltp loaded PLGA NPs/MPs (equivalent of 0.3 mg/ml Ltp) were weighed and mixed in HA solutions manually using a spatula. After thorough mixing, the solution was transferred into a dialysis membrane, immersed in PBS, and incubated at 37 °C inside shaker incubator. Rest of the procedure was same as described in section 3.7.3.1.

3.10 Composite Hydrogels of HA and Ltp loaded PLGA NPs/MPs

3.10.1 Preparation of hydrogels

3.10.1.1 Ltp-PLGA NPs/MPs loaded HA-MA and HA-ADH hydrogels

HA-MA powder was dissolved in PBS buffer to prepare 2 % and 4 % (w/v) solutions and HA-ADH powder was dissolved in PBS to prepare 4 % (w/v) solution. All the solutions were allowed to mix overnight on magnetic stirrer at

RT. Next day, 20 mg of Ltp loaded PLGA NPs/MPs (equivalent of 0.3 mg/ml Ltp) were weighed and mixed in HA-MA and HA-ADH solutions, manually using a spatula (polymer to particles mass ratio of 1:1 for 2 % hydrogels and 2:1 for 4 % hydrogels). After thorough mixing, HA-MA+Ltp-PLGA NPs/MPs solutions were crosslinked using UV light as described in section 3.2.2. HA-ADH+5Fu-PLGA MPs sample was chemically crosslinked using PEGDE as described in section 3.2.4.

3.10.2 Characterization of composite hydrogels

3.10.2.1 Physical stability of entrapped and released NPs/MPs

The physical stability of Ltp loaded PLGA MPs entrapped inside HA hydrogels was observed under SEM. Composite hydrogels of Ltp loaded PLGA MPs and HA-MA/HA-ADH were swollen in PBS without dialysis membrane at 37 °C for 3 days. The swollen gels were taken out and cut longitudinally and transversally using a sharp cutter and immediately frozen at -80 °C and lyophilized after 2 hrs. The sink medium was also collected and used to examine released MPs, if any. 10-20 µl of this suspension was spread over clean silicon wafer substrate and allowed to dry overnight at RT. Next day, the dried hydrogel samples as well as the sink medium wafer samples were mounted on double-sided adhesive carbon tape over an aluminum stub and sputter coated with platinum for 80 secs. The hydrogel entrapped MPs were then imaged under different magnifications.

The physical stability of Ltp loaded PLGA NPs entrapped inside as well as released from HA hydrogels was observed under FESEM. Composite

hydrogels of Ltp loaded PLGA NPs and HA-MA/HA-ADH were swollen in PBS without dialysis membrane at 37 °C for 3 days. The swollen gels were taken out and cut longitudinally and transversally using a sharp cutter and immediately frozen at -80 °C and lyophilized after 2 hrs. The sink medium was also collected and used to examine released NPs, if any. 10-20 µl of this suspension was spread over clean silicon wafer substrate and allowed to dry overnight at RT. Next day, the dried hydrogel samples as well as the sink medium wafer samples were mounted on double-sided adhesive carbon tape over an aluminum stub and sputter coated with platinum for 80 secs. The NPs were then imaged under different magnifications.

3.10.3 *In vitro* release of Ltp from composite hydrogels

3.10.3.1 Release of Ltp from PLGA NPs/MPs and HA-MA/HA-ADH composite hydrogels

20 mg of Ltp-PLGA NPs and Ltp-PLGA MPs (equivalent of 0.3 mg/ml Ltp) loaded 2 %, 4 % HA-MA, and 4 % HA-ADH composite hydrogels were prepared as described in section 3.10.1.1. The crosslinked gels were carefully removed from moulds or vials, transferred into a dialysis membrane, immersed in PBS, and incubated at 37 °C inside shaker incubator. Rest of the procedure was same as described in section 3.7.3.1.

3.10.4 Degradation of Ltp loaded MPs

3.10.4.1 Degradation of Ltp loaded MPs

For degradation study, Ltp loaded PLGA MPs were weighed, transferred directly into a CE dialysis membrane, immersed in PBS, and incubated at 37 °C inside shaker incubator. Rest of the procedure was same as described in section 3.6.4.1.

3.10.4.2 Degradation of Ltp loaded MPs and HA-MA composite

hydrogels

Ltp-PLGA MPs loaded 4 % HA-MA composite hydrogels were prepared as described in section 3.10.1.1. The crosslinked gels were carefully removed from moulds, transferred into a dialysis membrane, immersed in PBS, and incubated at 37 °C inside shaker incubator. Rest of the procedure was same as described in section 3.6.4.2.

Chapter 4 Results and Discussion

4.1 HA hydrogels

4.1.1 Modification of HA and Synthesis of hydrogels

HA was modified to obtain MA and ADH derivatives that can be crosslinked using photopolymerization or PEGDE crosslinker, respectively to obtain hydrogels. Derivatization using MA and ADH groups and crosslinking reactions are very well established. Besides, the synthesis reactions could be performed at RT, under mild pH conditions, and the reaction was rapid and hence these two derivatives were chosen to develop HA hydrogels.

Modification into HA-MA and Photocrosslinking

Figure 17 shows the schematic of MA modification process and photocrosslinking of MA modified HA. The primary alcohol group of N-acetyl-D-glucosamine unit was replaced with methacrylate group of MA to modify HA. On account of addition of a double bond to the monomer unit, the modified moieties could now undergo a free radical polymerization upon exposure to UV light to crosslink the HA-MA derivative into hydrogel. Care was taken during the gelation or crosslinking of macromers to expose both sides of the gel holding mould to UV light to ensure uniform crosslinking across the 1 ml sample.

Modification into HA-ADH and Chemical crosslinking

Figure 18 shows the schematic of ADH modification process and chemical crosslinking of ADH modified HA. The carboxylic group of β -D-glucuronic acid unit was first activated with EDC and then hydrazide group of ADH was

added onto it directly to modify HA. The ADH moiety with amine groups on the modified HA can be crosslinked chemically by using a diepoxy agent, PEGDE. The epoxy-amine reaction is allowed to continue at RT crosslinking the HA-ADH derivatives to form the hydrogel.

Figure 17 Schematic showing the modification of HA using MA derivative and fabrication of HA-MA hydrogels (modified from [146])

4.1.2 Characterization of HA hydrogels

NMR

^1H NMR was used to determine the degree of modification or substitution for HA-MA and HA-ADH derivatives. Unmodified HA (**Figure 19**) was used for NMR characterization to confirm all the carbohydrate peaks used for calculations. As observed from HA-MA spectra in **Figure 20**, distinct methylene resonances from methacrylate were obtained at $\delta=5.63$ and 6.07

ppm. Relative integration of the N-acetyl methyl resonances at $\delta = 1.91$ ppm was used as the internal standard.

Figure 18 Schematic showing the modification of HA using ADH derivative and fabrication of HA-ADH hydrogels (modified from [55])

Figure 19 NMR spectrum of HA

The relative peak integrations of methylene signals to N-acetyl methyl protons were used to calculate the percent modification. It was observed that ~21 % of primary alcohols of HA were modified with methacrylate group. This is similar to that has been achieved earlier in previous work for HA-MA modification by the simple reaction of HA with MA without any catalyst [148].

Figure 20 NMR spectrum of HA-MA

From the HA-ADH spectra in **Figure 21**, we could observe that distinct methylene resonances from ADH were obtained at $\delta=1.51$, 2.13, and 2.27 ppm. Relative integration of the N-acetyl methyl resonances at $\delta = 1.91$ ppm was used as the internal standard. The relative peak integrations of methylene signals to N-acetyl methyl protons were used to calculate the percent modification. It was observed that ~65 % of carboxyl groups of HA were modified with dihydrazide moiety. Similar degrees of substitution have been

achieved earlier in previous work for modification of HA using EDC catalyzed reaction with ADH [55].

Figure 21 NMR spectrum of HA-ADH

SEC-MALS

The molar mass of HA before and after modification was estimated using SEC associated with MALS. This experiment was conducted to evaluate any changes in the molar mass of HA on account of modification using MA and ADH moieties under different reaction conditions. Wyatt Astra software version 5.1 was used to analyze the light scattering data to obtain the absolute molar mass without any calibration or reference to molar mass standards. Number average (M_n) molar mass, weight average (M_w) molar mass, and PDI were obtained for all the samples. M_w was plotted for all the samples and compared as shown in **Figure 22**.

Figure 22 Chromatogram showing average molar mass of unmodified and modified HA

The molar mass of each sample was calculated using the amount of light scattered at three different angles, concentration of samples using refractive index (RI) detector, and the specific refractive index increment, d_n/d_c , of sample in the solvent or mobile phase used. It was observed that the molar mass of HA was not altered significantly due to MA modification indicating that the mild reaction conditions did not degrade HA chains. However, the molar mass was almost doubled due to ADH modification. This might be because during modification, the ADH moiety got attached to two COOH groups of HA monomers present on two different chains (interchain bridge), thus linking them together and increasing the molecular weight significantly.

Swelling behavior of HA hydrogels

The network structure parameters of hydrogels were estimated from swelling study in PBS at 37 °C. For the estimation of mesh size and crosslinking density

of HA hydrogels, Flory-Rehner calculations were used as described previously [69]. The volumetric swelling ratio, Q_v , was calculated from Q_M , the degree of mass swelling, as:

$$Q_v = 1 + \frac{\rho_p}{\rho_s} (Q_m - 1) \quad (1)$$

where ρ_p is density of dry polymer (1.229 g/cm³) and ρ_s is density of solvent (1 g/cm³ for water in this case). Q_M was obtained from swelling ratio experiment. Wet mass of hydrogels (obtained after swelling equilibrium) was divided by dry mass (obtained after freeze drying of gels) to calculate the mass swelling ratio.

Now, Q_v could be used to determine $\overline{Mc}$, the average molecular weight between crosslinks using the following Flory-Rehner equation:

$$Q_v^{5/3} = \frac{\bar{v} \overline{Mc}}{V_1} \left(\frac{1}{2} - \chi \right) \quad (2)$$

where $\bar{v}$ is specific volume of dry polymer, V_1 is molar volume of solvent (18 mol/cm³ for water in this case), and χ is Flory polymer-solvent interaction parameter (0.473 for HA).

Once $\overline{Mc}$ is estimated, the number of crosslinked units per unit volume, effective crosslink density, v_e , was calculated as follows:

$$v_e = \frac{\rho_p}{\overline{Mc}} \quad (3)$$

The mesh size, ξ , of swollen hydrogels could be determined using the following equation:

$$\xi = Q_v^{1/3} \sqrt{\bar{r}_o^2} \quad (4)$$

where $\sqrt{\bar{r}_o^2}$ represents the root-mean square distance between crosslinks, which is given as the following equation for HA:

$$\sqrt{\left(\frac{\bar{r}_o^2}{2n}\right)} \cong 2.4 \text{ nm} \quad (5)$$

where n is the number of monomer (disaccharide) units for HA. For HA with a molecular weight (M_n) of 1×10^6 , n is ~2494 and thus, $\sqrt{\bar{r}_o^2}$ can be calculated as:

$$\sqrt{\bar{r}_o^2} = 0.1695 \sqrt{M_n} \text{ (nm)} \quad (6)$$

Combining equations (4) and (6) and substituting $\overline{M_c}$ for M_n , ξ , can be obtained as:

$$\xi = 0.1695 \sqrt{\overline{M_c}} Q_v^{1/3} \text{ (nm)} \quad (7)$$

The values obtained for Q_v , $\overline{M_c}$, v_e , and ξ could be considered as approximate values because the calculations took into account certain assumptions. However, they are still useful if we want to make comparisons between different hydrogels for network structure properties.

From the swelling ratios of HA-MA hydrogels, as shown in **Table 5**, it could be observed that increasing the concentration of macromer, HA-MA, led to increase in crosslinking density and decrease in swelling ratio and mesh size indicating higher degree of crosslinking. The network structure of HA-MA hydrogels was found to be different from HA-ADH hydrogels as observed from

Table 5. The swelling ratio of 4 % HA-MA was lower than 4 % HA-ADH hydrogels indicating that the gel network of former, obtained with photocrosslinking, was significantly denser with smaller mesh size for same concentration of macromer.

Although the degree of modification of HA using ADH moiety was significantly higher than MA derivative, the swelling ratio of HA-ADH hydrogels was higher than HA-MA gels. This could be due to the imperfect crosslinking of ADH derivatized HA solutions. As mentioned earlier, the molar mass of HA was almost doubled due to ADH modification that was possibly due to the interchain binding of COOH groups via ADH bridge. Besides this interchain binding, intrachain binding between COOH and ADH molecules might also occur. As the potential sites (free amine group in ADH moiety) for crosslinking got occupied by additional intra and inter chain binding with COOH group and as there was little control over the extent of these binding events, the efficiency of chemical crosslinking by PEGDE was greatly reduced. This, in turn, affected the crosslinking density of HA-ADH hydrogels leading to higher swelling ratio than HA-MA gels of same concentration.

Morphology of HA hydrogels

The SEM images in **Figure 23** show the cross-section of HA-MA and HA-ADH hydrogels. As the hydrogels were swollen and then rapidly quenched, the network structure was maintained. The surface of all hydrogels was smooth and pores could be observed on the surface as well as in the cross-section samples of hydrogels.

Table 5 Network properties of HA-MA and HA-ADH hydrogels

Samples	Q_M	Q_V	$\overline{Mc}$ (g/mol)	v_e (mol/cm ³)	ξ (nm)
2 % HA-MA	27	33	2.8×10^5	4.4×10^{-6}	287
4 % HA-MA	20	25	1.7×10^5	7.3×10^{-6}	203
4 % HA-ADH	29	36	3.2×10^5	3.8×10^{-6}	317

(a)

(b)

(c)

Figure 23 SEM images of HA hydrogels (a) 2 % HA-MA (b) 4 % HA-ADH (c) 4 % HA-MA

Cross-section of hydrogels shows the crosslinked network with pores. 2 % HA-MA and 4 % HA-ADH hydrogels looked more porous than 4 % HA-MA gels

as expected from differences in crosslinking density and swelling ratio. The overall network structure of different hydrogels however cannot be differentiated on the basis of pore size from SEM images alone.

Rheology of HA hydrogels

The mechanical properties of HA-MA and HA-ADH hydrogels were evaluated using dynamic oscillatory shear test wherein the hydrogels were subjected to specific sinusoidal strain displacement across a range of frequencies as the stress is measured. The constant % strain for this test was determined from the LVE region obtained from amplitude sweep experiment. The storage modulus, G' , was determined for all hydrogel samples at various frequencies and plotted as shown in **Figure 24** and **Figure 25**. G' of 4 % HA-MA gels was higher than the magnitude of G' of 2 % HA-MA gels indicating increase in stiffness of hydrogels with increasing concentration of macromer (**Figure 24**).

The G' of 4 % HA-ADH was found to be less than 4 % HA-MA as was expected according to the swelling study results as shown in **Figure 25**. The swelling ratio of HA-MA gels was smaller than HA-ADH gels indicating the storage modulus of former would be higher than the latter. As compared to 2 % HA-MA and 4 % HA-ADH hydrogels, the behavior of G' of 4 % HA-MA gels was slightly different, that is, the G' was not constant over the frequency range studied and it was gradually increasing with increase in frequency. This variation in the storage moduli of 4 % HA-MA gels (large error bars, high standard deviation) could be attributed to the inhomogeneity of crosslinked gel network. When the macromers were exposed to UV in the presence of initiator,

a homogeneous crosslinked network could not be formed and thus, there was low uniformity amongst 4 % HA-MA hydrogel samples for G'' .

Figure 24 Oscillatory shear test of 2 % and 4 % HA-MA hydrogels

Figure 25 Oscillatory shear test of 4 % HA-MA and HA-ADH hydrogels

4.2 Delivery Carriers for 5Fu

Liposomes

5Fu was loaded into liposomes using passive as well as active loading. The size of 5Fu loaded liposomes was around ~100 nm and distribution was narrow with a PDI of ~0.086. However, the % EE was found to be extremely low. 5Fu is a hydrophilic drug that distributes itself between the continuous aqueous phase and the aqueous core of liposomes. The % EE of liposomes was calculated using the following equation:

$$\% EE \text{ of } 5Fu = \left(\frac{\text{Amount of } 5Fu \text{ entrapped}}{\text{Initial amount of } 5Fu \text{ used}} \right) \times 100$$

Firstly, passive loading was tried but when the extruded 5Fu loaded liposomes were dialyzed to remove untrapped drug, it was observed that 5Fu was almost completely released from liposomes within 3 hrs. The EE of liposomes was very low for 5Fu and almost all of it was present outside the liposomes in the continuous aqueous phase. And even if a part of total drug was entrapped within the aqueous core of liposomes, the entire amount was released in a couple of hours in a burst release manner.

When passive loading of 5Fu could not be achieved, active loading was tried to increase the efficiency of 5Fu encapsulation. 5Fu is a weak acid drug with a pK_a of around 7.6-8 [130, 149]. It was actively loaded into liposomes using sodium sulphate-calcium acetate gradient method [150]. The results were same as passive loading. During the removal of untrapped drug, almost the entire amount of 5Fu was released within 1-2 hrs. Both the methods of loading were

tried with different types of lipids (EPC, DPPC, DMPC) and composition of lipids (inclusion of cholesterol) but none of them could enable the entrapment of 5Fu inside liposomes efficiently and release for longer periods. This inefficiency of encapsulation into liposomes or the rapid leakage from them might be because 5Fu is a very small molecular weight, hydrophilic, and highly membrane permeable drug [151]. Thus, it is very difficult to retain the drug inside liposomes for long periods of time.

Chitosan NPs

As the liposomes could not be used to entrap 5Fu for sustained release, CS was chosen to develop polymeric nanocarriers. CS is the deacetylated product of chitin, a naturally occurring polymer. **Figure 26** shows the chemical structure of chitosan. It is a linear, cationic polysaccharide and can physically crosslink with polyanions like TPP via ionic gelation.

Figure 26 Chemical structure of chitosan

It has been widely used as drug delivery carrier due to its biocompatibility, biodegradability, and mucoadhesive properties. In the present work, CS was crosslinked using TPP via ionic gelation technique to prepare 5Fu loaded NPs. The encapsulation of drugs inside CS NPs using this technique depends on factors such as molecular weight of CS, concentration of CS and drug, pH of CS and TPP solution (charge density), and degree of deacetylation of CS.

The pH of 5Fu-CS was adjusted to 4.7 to maximize the protonation of amino groups (pK_b for CS ~ 6.5) that complex with TPP as a result of electrostatic interaction (scheme I as shown in **Figure 27**). This results into reversible gelation of CS and it precipitates in the form of spherical particles. When the NPs form, 5Fu gets physically entrapped inside the particles and would also adsorb on the surface. The 5Fu loaded NPs ranged in size from ~ 250 - 280 nm but the % EE was found to be almost zero and the entire drug was detected in the supernatant during washing steps.

Figure 27 Synthesis of 5Fu loaded chitosan NPs - Scheme I

It was believed that the small molecular weight and affinity towards aqueous phase made it difficult to encapsulate 5Fu just by physical entrapment. As the physical entrapment of 5Fu inside CS NPs during complexation of amino groups with TPP was not found to be efficient, modifications were made to the original scheme I of NPs fabrication. The ionic gelation of CS-TPP to prepare 5Fu loaded NPs depends upon electrostatic interaction of CS with two different

anionic groups (5Fu and TPP). 5Fu is anionic around neutral pH (weak acid, $pK_a \sim 8$) and is capable of ionic interaction with CS [152]. The ionization of 5Fu occurs by deprotonation at either site N1 or N3, with N3 site being preferred under most reaction conditions [153]. The pH of TPP solution was ~ 9.5 and was not adjusted further.

Two schemes (II and III) were employed as modification to scheme I. In scheme II, as shown in **Figure 28**, the pH of 5Fu and CS solutions were adjusted to the value that would cause maximum ionization of the desired groups in both of them and NPs were prepared.

Figure 28 Synthesis of 5Fu loaded chitosan NPs - Scheme II

There would be competition between 5Fu and TPP ions to complex with amino groups but it was believed that ionic interaction would keep 5Fu with CS initially and crosslinking with TPP in the later step would ensure 5Fu was retained inside the NPs. However, no improvement in EE was observed. This might be because it was difficult to get maximum ionization for both 5Fu and

CS after they were mixed together. The pH shifted to favor ionization of only one of them because of the differences in their dissociation properties (5Fu is a weak acid and CS is a weak base).

In scheme III, as shown in **Figure 29**, the pH was adjusted so that the reacting groups were ionized to ~50-60 % for both 5Fu and CS. Mixing them together still maintained the ionization within this range and NPs were prepared. It was believed that although the interaction between 5Fu and CS had reduced as compared to scheme II due to lower degree of ionization, the affinity of 5Fu to CS was still higher than the scheme I.

Figure 29 Synthesis of 5Fu loaded chitosan NPs - Scheme III

However, it was observed that even with this scheme, 5Fu could not be encapsulated within the NPs. This might be because the ionic interaction between positively charged amino groups of CS and negatively charged anions of TPP was much stronger than the interaction with the anions of 5Fu [154].

Various other parameters involved in the fabrication of 5Fu loaded CS NPs were studied to optimize the formulation for maximum EE. This included varying concentration of CS, molecular weight of CS, % loading of 5Fu, CS:TPP ratio, reaction time, stirrer type and stirring speed, degree of deacetylation, and alginate coating of NPs. However, 5Fu could not be encapsulated inside CS NPs. Besides absence of encapsulation, another observation was aggregation of CS NPs after washing cycles. It was observed that NPs aggregated and the pellet was unable to be resuspended homogeneously in water. Reduction in centrifugation speed and time did not improve the problem of pellet aggregation and resuspension.

Layer-by-Layer NPs

As CS NPs alone could not encapsulate 5Fu efficiently, LBL NPs were prepared using 5Fu and CS solutions as the polyelectrolyte oppositely charged coating layers. The size and zeta potential values of NPs or sub-micron particles (as shown in **Table 6**) measured after each coating suggests the successful deposition of every layer. However 5Fu was not encapsulated in the NPs and the entire drug was detected in the supernatant during washing steps in between coatings.

Various parameters involved in the fabrication of LBL NPs were studied to optimize the formulation for maximum EE. This included varying number of coating layers, concentration of coating solutions, and type of coating solutions. However, no improvement in EE was observed. It was difficult to retain 5Fu inside the coating layers by electrostatic interaction that might be because the

charge density was not optimum for uniform coating and maximum retention between layers. Thus, being a small molecular weight drug and with strong affinity towards aqueous phase, 5Fu could not be loaded into LBL NPs.

Table 6 Size and zeta potential of 5Fu loaded LBL particles

Samples	Size (nm)	Zeta potential (mV)
Hydroxyapatite particles	1015.64	-2.64
CS (1 st coating)	958.37	9.27
5Fu (2 nd coating)	1947.67	-6.65
CS (3 rd coating)	941.17	14.14
5Fu (4 th coating)	2318	-6.41
CS (5 th coating)	794.57	13.37

PLGA NPs and MPs

After the failure of LBL NPs, PLGA was used to prepare 5Fu loaded NPs and MPs. 5Fu, as crushed powder, was loaded into PLGA NPs using w/o/w, a double emulsion technique and into PLGA MPs via s/o/w, a single emulsion technique. The solubility of 5Fu is ~10-12 mg/ml in water. For NPs, the ratio of organic to aqueous phase was maintained to be 10:1 so that the amount of soluble 5Fu could be increased for formulation.

The NPs were spherical in shape with smooth surface as shown in the FESEM images (obtained at different magnifications) in **Figure 30**. Size was ~280-300 nm with a PDI of ~0.1 and no aggregation was observed during or after freeze dry. However, upon extraction of these NPs, it was found that these NPs also

failed to encapsulate 5Fu similar to the other carriers investigated earlier (liposomes, CS NPs, and LBL NPs).

Although the PLGA used in this study, 50:50 L:G, was relatively less hydrophobic in nature than other types, it was difficult to load the hydrophilic drug into the NPs. The amount of 5Fu used for loading was possibly too less for entrapment inside NPs and everything was detected in the continuous phase PVA (and in water during washing steps). 5Fu preferred the aqueous continuous phase more than the hydrophobic phase of PLGA (and DCM) and thus the entire amount was phase separated into the PVA solution. Unless there is some covalent binding of 5Fu with the carrier system, it was found to be extremely difficult to load 5Fu into NPs by passive loading, ionic interaction, and emulsification.

Figure 30 FESEM images of 5Fu loaded PLGA NPs (a) X8, 000 (b) X20, 000

Although 5Fu could not be encapsulated into NPs, it could be entrapped inside PLGA MPs up to an EE of $\sim 35\%$. Some of the most important parameters controlling the amount of drug encapsulation inside PLGA MPs and *in vitro* release included size (dependent upon concentration and molecular weight of

PLGA, concentration of PVA), type of PLGA end group, % drug loading, and pH and volume of PVA solution. As PLGA MPs could be used as sustained release carriers for 5Fu, they were chosen to be entrapped inside HA hydrogels to develop composite hydrogels and evaluate the release behavior of 5Fu thereof.

4.3 Composite Hydrogels of HA for 5Fu delivery

4.3.1 Characterization of 5Fu loaded PLGA MPs

Yield

The % yield of 5Fu loaded MPs was calculated from the freeze dried and sieved MPs as the following:

$$\% \text{ Yield} = \left(\frac{\text{Dry mass of MPs after sieve}}{\text{Initial mass of PLGA used}} \right) \times 100$$

It was found to be 87 ± 2.5 % and consistent within batches of same formulation. Thus, the loss of MPs was very low during washing and freeze drying steps. This high recovery of MPs could be attributed to the optimization of the fabrication parameters to minimize formation of fibers and aggregation of particles during emulsification. The sieving was done to separate the debris or aggregated particles from the rest.

Morphology and Size distribution

The MPs were spherical in shape as can be observed from SEM images (obtained at different magnifications) in **Figure 31**. The surface of particles was found to be smooth.

(a) (b)
Figure 31 SEM images of 5Fu loaded PLGA MPs (a) X100 (b) X60

They might have micropores that should be confirmed with images having much higher magnification. The batches were found to have a broad size distribution and average size of particles was found to be $102 \pm 43 \mu\text{m}$. Similar distribution was observed among different batches with same formulation and lab conditions.

% Loading and % EE

5Fu was physically entrapped in its crystalline phase inside the PLGA MPs. 5Fu loaded PLGA MPs were extracted using the solvent phase separation method to estimate the % loading and % EE. The drug loaded MPs were first dissolved in DCM so that the drug was released completely into the organic solvent. Once this 5Fu+PLGA+DCM solution was exposed to aqueous phase (PBS, pH 7.4), 5Fu, being hydrophilic, separated itself into PBS and PLGA stayed with DCM. Repeating cycles of exposure to fresh PBS and vigorous shaking, extracted the entire amount of 5Fu from the MPs. 5 cycles of phase separation were optimized for complete extraction of 5Fu from PLGA MPs.

The concentration of entrapped and unentrapped 5Fu was calculated using HPLC system. A standard curve ($R^2 = 0.999$) was prepared using known

concentrations of 5Fu in PBS buffer and the calibration was used to estimate the concentration of drug samples from MPs. This data from HPLC was used to calculate % loading and % EE using the following equations:

$$\% \text{ Theoretical Loading} = \left(\frac{\text{Amount of 5Fu used}}{\text{Amount of PLGA used}} \right) \times 100$$

$$\% \text{ Practical Loading} = \left(\frac{\text{Amount of entrapped 5Fu}}{\text{Amount of PLGA MPs used for extraction}} \right) \times 100$$

$$\% \text{ EE} = \left(\frac{\text{Practical Loading}}{\text{Theoretical Loading}} \right) \times 100$$

For this study, 20 % of 0.4A or 0.4E IV PLGA was used to encapsulate 5Fu powder (theoretical loading ~8.33 %) to obtain a practical loading of 1.5 ± 0.15 % upon fabrication of 5Fu loaded MPs. Thus, the EE of PLGA MPs was 18 ± 2 %. This value, however, does not represent the highest % EE obtainable with 5Fu loaded PLGA MPs prepared by s/o/w. This formulation was chosen from a large set of batches that were prepared to study the parameters such as concentration of 5Fu/PLGA/PVA, type of PLGA, % 5Fu loading, and other MPs fabrication conditions and were optimized to obtain formulations with high % EE and sustained release of 5Fu.

4.3.2 *In vitro* release of 5Fu

5Fu was directly loaded into HA solutions and *in vitro* release was studied by a dialysis membrane technique to evaluate its transport properties. 5Fu solution in PBS alone was used to compare with other samples. At every time point, the release medium was completely replaced with fresh PBS buffer to avoid drug saturation and obtain a closer approximation of dynamic sink condition *in vivo*.

All release profiles were plotted as % cumulative release of drug against release time or period.

As we can observe from **Figure 32**, 5Fu solution and 2 % HA solution released ~85 % of 5Fu within 3 hrs while 4 % HA solution released ~70 % 5Fu. Almost 100 % of drug was released from all the solution samples within 48 hrs. There was no difference in the release trend by increasing the concentration of HA in solution (2 % and 4 % HA solution).

5Fu was released rapidly from both the samples similar to its solution in PBS alone. Being a very small molecular weight and hydrophilic drug, 5Fu diffused out rapidly through the swollen HA chains and was released into the sink medium. This indicates that 5Fu release cannot be sustained by HA solutions and the release was purely diffusion controlled.

Figure 32 Release profile of 5Fu from HA solutions

5Fu was loaded into PLGA MPs and they were characterized for *in vitro* release behavior. Also, the MPs were mixed with HA solutions and evaluated to study the effect of surrounding HA chains on the release behavior of 5Fu. The three main mechanisms of drug release from PLGA MPs include diffusion through water filled pores, diffusion through the polymer matrix, and degradation/erosion of the polymer matrix [121]. These mechanisms are interdependent; for instance, a factor affecting diffusion mechanism would affect the degradation rate and vice versa. Thus, in most of the cases, they always act in combination while affecting the drug release from PLGA MPs.

From **Figure 33**, we can observe that 5Fu was released until 10 days from PLGA MPs (0.4A IV) which is significant compared to the 48 hrs release from HA solutions alone. Release started with a short phase of slow release up to 3 days and then from day 4 onwards a second phase of fast release was observed that initially started as a steep increase in release rate and plateau thereafter. Burst release phase was absent and ~10 % of drug released within 24 hrs.

Figure 33 Release profile of 5Fu from 20 % PLGA MPs

In the first phase, 5Fu was found to be released slowly over a period of 3 days and then after day 4, there was a sudden increment in the amount of 5Fu released which continued for 5-6 days before plateau-off to 100 % release. As the crushed 5Fu powder was suspended in the PLGA-DCM solution during the formation of MPs via s/o/w emulsification, it was loaded in its crystalline form by physical entrapment and remained in the same form after encapsulation as shown by DSC. We could observe from the thermograms that the melting point of 5Fu remained same before (**Figure 34**) and after encapsulation inside MPs (**Figure 35**) thus indicating the presence of its crystalline form. Besides 5Fu's melting peak, the two small endothermic peaks at 45.15 °C and 162.83 °C in **Figure 35** correspond to the T_g of PLGA and T_m of PLA crystals, respectively.

Figure 34 DSC thermogram of crushed 5Fu powder

When the PLGA MPs were exposed to PBS buffer, 5Fu crystals had to be first dissolved into amorphous form before being released into the surrounding sink medium indicating that both drug dissolution and diffusion mechanisms were

involved as the mass transport processes. In the first phase of 5Fu release profile, as the buffer diffused into the PLGA polymer matrix, 5Fu started to dissolve and diffuse out into the medium. Thus, this short phase of slow release of 5Fu was governed by the mechanism of diffusion.

Figure 35 DSC thermogram of 5Fu loaded PLGA MPs

Once the polymer chains started to erode, the cleaved oligomers and monomers were released into the medium increasing the diffusion rate of 5Fu. In the second phase, the release of 5Fu was degradation controlled wherein, the sudden release of huge amounts of 5Fu could be attributed to the significant increase of porosity of MPs and diffusion of soluble by-products from the eroded polymer matrix. The bulk degradation of PLGA chains proceeds by hydrolytic cleavage of ester linkages [97]. Thus, the overall release behavior of 5Fu from PLGA MPs was biphasic and controlled by a combination of two mechanisms; diffusion and degradation; both of which were inter-dependent.

Similar to 0.4A MPs, a biphasic release profile of 5Fu was also obtained for 0.4E PLGA MPs with differences in the magnitude and length of phases. There was no burst release and ~100 % of drug was released in 26 days as compared to 10 days in 0.4A MPs. Also, the diffusion controlled first phase of slow release was extended by 12-13 days. These differences could be attributed to the slower degradation of ester-terminated chains of PLGA matrix than acid-terminated ones [121].

When the drug loaded MPs were mixed with HA solutions, release of 5Fu was slowed down extending the 100 % drug release period by 2 days as shown in **Figure 36**. 5Fu was released from HA+PLGA MPs solution in 12 days as compared to 10 days from MPs alone.

Figure 36 Release profile of 5Fu from 20 % PLGA MPs loaded HA solutions

Similar to the PLGA MPs alone, burst release was absent and ~9-10 % was observed within 24 hrs. The first short phase of slow release lasted up to 3 days

and then the second phase of fast release continued until day 9 when it started to plateau. Thus, the release of 5Fu from HA+PLGA MPs solution was also controlled by the combination of diffusion and degradation mechanisms. The release from solution entrapped MPs was significantly retarded in the second phase from day 4 onwards until day 9.

The presence of HA chains added a small barrier to the diffusion of 5Fu which first needed to be dissolved into its amorphous form before being released. The diffusion path length was increased as 5Fu was dissolved and released from MPs into the surrounding HA chains and then traveled through these chains before releasing into the sink medium extending the overall release period by 2 days. There was no further retardation in the release of 5Fu by increasing HA concentration (2 % and 4 % HA solution) and it was similar in both the solutions throughout the release time period indicating the diffusion barrier was independent of HA chain concentration. Once outside the MPs, the differences in the hydration levels between 2 % and 4 % HA solution did not affect the diffusion of 5Fu, a small molecular weight and hydrophilic drug, corroborating with its release results from HA solutions alone.

4.3.3 Characterization of HA+PLGA MPs composite hydrogels

5Fu loaded PLGA MPs were incorporated into HA-MA and HA-ADH hydrogels and characterized for morphology and *in vitro* release of 5Fu.

The network structure of composite hydrogels with entrapped MPs was examined using SEM. It was observed that the morphology of 5Fu loaded

PLGA MPs was not affected due to crosslinking reactions and they were homogeneously distributed across the hydrogels as shown in the SEM images in **Figure 37**. They were well dispersed on the surface as well as in the inner mesh network of composite hydrogels.

(a)

(b)

Figure 37 SEM images of HA hydrogels incorporated with 5Fu loaded PLGA MPs obtained at different magnifications (a) X50 (b) X60

The sink medium from composite hydrogels was also observed under SEM to check if any MPs had released from the gels. But no MPs were observed in the sink medium indicating they were well entrapped within the hydrogels and any surface bound particles had also not released into the medium.

Further, to evaluate the effect of crosslinking on the stability of 5Fu inside PLGA MPs, drug loaded MPs, directly or in presence of initiator, were exposed to UV (keeping other parameters same) and extracted for estimation of the amount of 5Fu using HPLC system. Similarly, the drug loaded MPs were also exposed to chemical crosslinker PEGDE and extracted to determine any loss of 5Fu. Upon comparison to unexposed 5Fu loaded MPs, it was observed that no drug loss occurred when the MPs were exposed to UV light or to chemical

crosslinker. Thus, the stability of MPs entrapped drug was ensured under the crosslinking reaction conditions.

4.3.4 *In vitro* release of 5Fu from hydrogels

5Fu was directly loaded into HA hydrogels (HA-MA and HA-ADH) and *in vitro* release was studied by a dialysis membrane technique to evaluate its transport properties. 5Fu solution in PBS alone was used to compare with other samples.

As we can observe from **Figure 38** and **Figure 39**, 5Fu solution released ~85 % of 5Fu within 3 hrs while 2 % HA-MA, 4 % HA-MA, and 4 % HA-ADH gels released ~68-74 % 5Fu within 3 hrs. Almost 100 % of drug was released from all the hydrogel samples within 48 hrs. Similar to HA solutions, the release was purely diffusion controlled.

Figure 38 Release profile of 5Fu from HA-MA hydrogels

Figure 39 Release profile of 5Fu from 4 % HA-MA and HA-ADH hydrogels

Crosslinking of HA chains into hydrogel network, either HA-MA or HA-ADH hydrogels, did not retain the drug for longer periods. Both type of gels showed similar release profiles.

There was no difference in the release trend either by increasing the concentration of macromer in hydrogels (2 % and 4 % HA-MA hydrogels) or changing the type of macromer (4 % HA-MA and 4 % HA-ADH hydrogels). Although the swelling ratios of hydrogels were different, it hardly affected the diffusion rate of a small molecular weight and hydrophilic drug like 5Fu. It diffused out rapidly through the crosslinked networks and released into the sink medium similar to its solution in PBS alone. This indicates that 5Fu release cannot be sustained by HA hydrogels and was diffusion controlled.

4.4 Release behavior of 5Fu from composite hydrogels

Figure 40 and **Figure 41** compares the release of 5Fu from composite hydrogels and MPs alone. The release trend of 5Fu from composite hydrogels was similar to the MPs alone (biphasic release), that is, the release was controlled by a combination of diffusion and degradation mechanisms. But the rate was significantly retarded in composite hydrogels and the release period was extended by 2-3 days. Also, the extent of retardation was dependent upon the hydrogel network surrounding the MPs.

Figure 40 Release profile of 5Fu from 20 % PLGA MPs loaded HA-MA hydrogels

In case of HA-MA hydrogels, the release rate was found to retard further with increase in macromer concentration (2 % and 4 % hydrogels). This increase in retardation was significant in the degradation-controlled second phase of release period and the overall extension period was same. Similarly, there was difference in retardation between 4 % HA-MA and 4 % HA-ADH hydrogels

(effect of type of macromer) with the release rate of 5Fu being slower in the former as shown in **Figure 41**.

A hydrogel network surrounding the MPs can retard the release rate of drugs from the particles either by providing an additional barrier to the diffusion of drugs, by reducing the exposure of aqueous medium to the entrapped MPs thus delaying their degradation and/or erosion and therefore the drug release, or by chemically interacting with the drug and/or drug carrier. The additional barrier of the gel network increases the diffusion path length or thickness of the diffusion layer thus reducing the rate of drug release from entrapped MPs. The drug is first released from MPs into the gel network and then it diffuses through the network before being released into the sink medium. So the drug is travelling a longer distance before its final release or detection.

Figure 41 Release profile of 5Fu from 20 % MPs loaded HA-MA and HA-ADH hydrogels

In this study, it was assumed that there was no chemical interaction between HA hydrogels and PLGA MPs. 5Fu release from HA solutions and hydrogels indicated that HA chains did not interact with the drug either. Thus, the retardation of 5Fu from PLGA MPs entrapped inside HA-MA and HA-ADH hydrogels could be either due to diffusion resistance by the gel network and/or delay in the degradation and/or erosion of MPs.

From the degradation study of MPs, it was observed that the 5Fu loaded MPs degraded in the same rate whether alone or entrapped inside hydrogels as shown by the average molar mass of particles estimated by SEC. 4 % HA-MA hydrogels showing maximum retardation of 5Fu release were chosen for degradation study. As we can observe in **Figure 42**, the rate of degradation was same indicating that diffusion rate of water into MPs was not interfered whether they were entrapped inside hydrogel network or not.

Figure 42 Degradation profile of 5Fu loaded PLGA MPs with or without HA-MA hydrogel

The impact of water might be slightly different on the MPs adsorbed on the surface of the gel network than from those entrapped inside. Here we assumed that the impact was similar to all the particles. Thus, the function of HA-MA gel network, in retarding the release of 5Fu in composite hydrogels, could be to act as an additional diffusion barrier. As the retardation was mainly observed in the second phase, another important factor possibly responsible for drug retardation could be differences in erosion profiles of PLGA MPs with and without hydrogels that needs to be further investigated.

From **Figure 39** we can observe that the drug alone, when dispersed in hydrogel network, was not retarded by any of the gel samples. In composite hydrogels, although 5Fu release from MPs was retarded by the diffusion resistance of gel network (additional barrier), the release period was not prolonged significantly. The retardation of release could be observed from day 4 onwards but it was not sustained for prolonged periods. Thus, the barrier of hydrogel was unable to offer high resistance to the diffusion of drugs once they were released into the network from the entrapped particles corroborating with the results of 5Fu release from HA hydrogels alone.

Besides the dialysis membrane release study, the composite hydrogels were also soaked directly in PBS buffer and *in vitro* release was conducted. This was to evaluate any affect on 5Fu release trend due to the release of drug loaded MPs from inside the hydrogels into the sink medium. Without a dialysis membrane, the MPs (empty or drug loaded), if released from hydrogels, do not have any barrier and they would get released into the release medium along

with free drug. As observed from **Figure 43**, the release trend of 5Fu was similar whether the hydrogels were placed inside dialysis membrane or immersed directly in the release medium.

Figure 43 Release of 5Fu from 20% MPs loaded hydrogels with or without dialysis bag

This confirmed the imaging results indicating MPs were not released from the hydrogels and they released the drug completely while being entrapped within the crosslinked network. As the mesh size was in nanometer range, the micron sized particles remained entrapped inside the hydrogels and released the drug there which then diffused through the hydrogel and released into the sink medium.

4.5 Composite Hydrogels of HA for Ltp delivery using liposomes

Ltp was loaded passively into EPC liposomes by thin film hydration technique and extrusion. These liposomes were characterized for morphology, size distribution, % EE, and *in vitro* release study of Ltp.

4.5.1 Characterization of Ltp loaded EPC liposomes

Morphology and Size distribution

Ltp loaded EPC liposomes were observed as circular vesicles with unilamellar (single bilayer) structure as shown in the cryo-TEM images in **Figure 44**.

Figure 44 cryo-TEM images of Ltp loaded EPC liposomes obtained at different sections

A few MLVs were also observed along with LUVs in the images of liposomes suspension. From size measurement, a unimodal, narrow size distribution was observed for the Ltp loaded EPC liposomes as shown in **Figure 45**. The intensity mean diameter or average size of these LUVs was 94.17 ± 0.94 nm with the average PDI of 0.047 ± 0.007 .

Figure 45 Size distribution plot of Ltp loaded EPC liposomes

High stability for Ltp loaded liposomes have been reported in literature for prolonged periods when stored at 4 °C as compared to blank EPC liposomes [137]. It was concluded that entrapping Ltp did not destabilize the bilayer and instead improved the stability of these EPC liposomes under the storage conditions behaving similar to the stabilizers like cholesterol [137].

% EE

Ltp is a lipophilic drug that distributes itself between the aqueous continuous phase and the lipid bilayer. For each liposome sample, the amounts of drug before extrusion, in aqueous phase as well as bilayer, and after extrusion were estimated. As Ltp is capable of distributing itself in both aqueous phase and lipid bilayer, the concentration present in the aqueous phase of MLVs could be used as a measure of untrapped drug. This amount when subtracted from total drug amount would give an estimate of the drug that retained inside liposomes and could be used to calculate % EE of EPC liposomes.

Drug loaded liposomes, before and after extrusion, can be broken down by IPA and after separation of lipid mass by centrifugation, the supernatant could be used to estimate the total amount of drug. As the untrapped drug was not separated from the extruded liposomes, the supernatant from broken liposomes gives an estimate of total amount of drug. Concentration of drug from broken liposomes, before and after extrusion, would give an approximate estimate of drug loss during the process of extrusion.

The concentration of untrapped Ltp and total amount of Ltp, before and after extrusion, were calculated using HPLC system. A standard curve ($R^2 = 0.997$) was prepared using known concentrations of Ltp in PBS buffer and the calibration was used to estimate the concentration of Ltp for all the test samples. This data from HPLC was used to calculate the drug loading efficiency of liposomes using the following equation:

$$\% EE \text{ of Ltp} = \left(\frac{\text{Amount of Ltp entrapped}}{\text{Initial amount of Ltp used}} \right) \times 100$$

In this work, the untrapped Ltp amount was found to be 7 ± 3 %. Thus, the entrapment efficiency of EPC liposomes was found to be 93 ± 3 %.

4.5.2 *In vitro* release of Ltp

Ltp was directly loaded into HA solution and *in vitro* release was studied by a dialysis membrane technique to evaluate its transport properties. Ltp solution in PBS alone was used to compare with other samples. At every time point, the release medium was completely replaced with fresh PBS buffer to avoid drug saturation and obtain a closer approximation of dynamic sink condition *in vivo*.

As we can observe from **Figure 46**, Ltp solution in PBS released ~58 % of drug within 3 hrs whereas 2 % and 4 % HA solution released ~33 and ~44 % of Ltp, respectively. Almost 100 % of drug was released from Ltp solution within 24hrs and from all solution samples in 5 days indicating Ltp release was retarded from both HA solutions unlike the drug solution in PBS alone. As Ltp was not molecularly dispersed in HA chains, it took time to first dissolve and then release from the chains when they were exposed to the aqueous medium.

Release of Ltp, a small molecular weight and hydrophobic drug, was thus delayed from HA chains into the surrounding aqueous environment. However, there was no difference in the release trend by increasing HA concentration in solution (2 % and 4 % HA solution) indicating no effect on the dissolution and diffusion of Ltp by increasing the viscosity of solution. Thus, Ltp release could be sustained from HA solutions and was diffusion controlled.

Figure 46 Release profile of Ltp from HA solutions

Ltp was passively loaded into EPC liposomes and they were characterized for *in vitro* release behavior. **Figure 47** shows the % cumulative release of Ltp from EPC liposomes over time and it could be observed that Ltp was released in a slow and sustained manner for over 3 weeks. ~25 % of drug was released within 24 hrs and subsequently it slowed down and almost 80 % was released at the end of 2 weeks. This release of Ltp was purely controlled by partitioning mechanism rather than diffusion. The partition coefficient of drug depends upon the drug:lipid ratio or the % drug loading.

Ltp, being hydrophobic, has higher affinity towards lipid bilayer and a major percent of the loaded drug resides there predominantly. Also, the favorable interactions between drug and lipid bilayer retained the drug longer in bilayer and also stabilized the vesicles sustaining the release for prolonged periods [137, 144].

Figure 47 Release profile of Ltp from EPC liposomes

When liposomes were exposed to release medium, diffusion of drug started quickly releasing the drug from the continuous aqueous phase of liposomes. The entire release period was controlled by partitioning of Ltp between bilayer and continuous phase indicating that the physico-chemical interactions of Ltp with bilayer controls its diffusion from the liposomes.

When the drug loaded liposomes were mixed with HA solutions, release of Ltp was slowed down, extending the 100 % drug release period by 4 days as shown in **Figure 48**. Ltp was released from HA+EPC liposomes solution in 27 days as compared to 23 days from liposomes alone. ~18 % of drug was released within 24 hrs and subsequently it slowed down and almost 70 % was released at the end of 2 weeks.

Figure 48 Release profile of Ltp from EPC liposomes loaded HA solutions

Incorporating liposomes in HA solutions retarded the release of Ltp through them indicating an additional resistance to the diffusion of Ltp as the path length increased due to the presence of HA chains around the liposomes. Ltp

would be released from liposomes into the HA chains first, where they might partition themselves in the HA chains before diffusing slowly through these chains to get released into the sink medium.

There was no difference in the release trend by increasing HA concentration in solution (2 % and 4 % HA) indicating no further effect on the diffusion resistance of Ltp on account of increasing solution viscosity. This might be because the release of Ltp was mainly partition controlled. Thus, Ltp release could be retarded from liposomes incorporated HA solutions.

4.5.3 Characterization of HA+EPC liposomes composite hydrogels

Ltp loaded EPC liposomes were incorporated into HA-MA and HA-ADH hydrogels and characterized for physical stability of entrapped liposomes and *in vitro* release of Ltp.

Aliquots from the sink medium of liposomes loaded composite hydrogels were analyzed using cryo-TEM and NanoSight NTA system to evaluate the bilayer stability of released liposomes and to estimate the size distribution of liposomes released at different time points, respectively. From the TEM images of sink medium of composite hydrogels in **Figure 49**, it was observed that the bilayer of released liposomes was intact indicating that the lamellar structure was unaffected during gelation and maintained their stability in crosslinked systems.

Figure 49 cryo-TEM images of EPC liposomes released from composite hydrogels of HA

For size measurement, the size distribution of liposomes released from both HA-MA and HA-ADH hydrogels was plotted and compared to the size distribution profile of original liposomes suspension before incorporation into hydrogels for each time point. As depicted in in **Figure 50**, it was observed that not only the liposomes were released from the composite hydrogels into the sink medium but also that there was no significant difference between the size distribution profile of original liposomes suspension and those released from both HA-ADH and HA-MA hydrogels at different time points.

The average particle size for original liposome suspension was ~88 nm, and the average size for released liposome varied in a range of 84-103 nm for HA-MA hydrogel and 81-93 nm for HA-ADH hydrogel indicating that the incorporated liposomes were stable during crosslinking of hydrogels and were released from the hydrogels without any significant change in their size and shape.

Figure 50 Size distribution of EPC liposomes released from HA-MA and HA-ADH hydrogels

Further, to evaluate the effect of crosslinking on the stability of Ltp inside EPC liposomes, drug loaded liposomes, directly or in presence of initiator, were exposed to UV (keeping other parameters same) and extracted for estimation of the amount of Ltp using HPLC system. Similarly, the drug loaded liposomes were exposed to chemical crosslinker PEGDE and extracted to determine any loss of Ltp. Upon comparison to unexposed Ltp loaded liposomes, it was observed that no drug loss occurred when the liposomes were exposed to UV light or to chemical crosslinker. Thus, the stability of liposomes entrapped drug was ensured under the crosslinking reaction conditions.

4.5.4 *In vitro* release of Ltp from hydrogels

Ltp was directly loaded into HA hydrogels (HA-MA and HA-ADH) and *in vitro* release was studied by a dialysis membrane technique to evaluate its transport properties. Ltp solution in PBS alone was used to compare with other samples.

As we can observe from **Figure 51** and **Figure 52**, Ltp solution alone released ~58 % of drug within 3 hrs. 2 % HA-MA, 4 % HA-MA, and 4 % HA-ADH gels released ~37 %, ~23 %, and ~28 % Ltp, respectively within 3 hrs. Almost 100 % of drug was released from Ltp solution within 24 hrs. As Ltp was not molecularly dispersed in HA hydrogel, it took time to first dissolve and then release from the network when they were exposed to the aqueous medium. Release of Ltp was thus delayed from the HA hydrogel network into the surrounding aqueous environment.

Figure 51 Release profile of Ltp from HA-MA hydrogels

Crosslinking of HA chains into hydrogels retained the drug approximately up to a week and it was possibly dependent on the crosslinking density of the network. 4 % HA-ADH and 4 % HA-MA hydrogels released the drug completely in 5 and 7 days, respectively. Ltp was released relatively faster from 2 % HA-MA and 4 % HA-ADH gels than 4 % HA-MA gels. This could be because of the more hydrophobic network of 4 % HA-MA hydrogels leading to

increased retardation of Ltp dissolution and slower release. Thus, Ltp release could be sustained from HA hydrogels and was diffusion controlled.

Figure 52 Release profile of Ltp from 4 % HA-MA and HA-ADH hydrogels

4.6 Composite Hydrogels of HA for Ltp delivery using PLGA NPs/MPs

Ltp was encapsulated into PLGA NPs and MPs by o/w emulsion method. These NPs and MPs were characterized for morphology, size, % EE, and *in vitro* release study of Ltp.

4.6.1 Characterization of Ltp loaded PLGA NPs/MPs

Yield

The % yield of Ltp loaded NPs and MPs was calculated from the freeze dried and sieved particles as the following:

$$\% \text{ Yield} = \left(\frac{\text{Dry mass of NPs/MPs after sieve}}{\text{Initial mass of PLGA used}} \right) \times 100$$

For NPs, the yield was found to be 62 ± 3 % and for MPs, it was 85 ± 5 %. The lower yield of NPs could be attributed to the loss of particles in the supernatant during centrifugation or washing steps. The centrifugation speed and time could not be increased beyond that was used in the procedure because it would lead to hard pellets that could not be resuspended homogeneously. For MPs, the sieving was done to separate the debris or aggregated particles from the rest.

Morphology and Size distribution

Figure 53 shows FESEM images of Ltp loaded PLGA NPs obtained at different magnifications. The NPs were spherical in shape with a smooth surface. No pores could be observed on their surface. The size distribution from DLS measurement was found to be unimodal and narrow as shown in **Figure 54**. The intensity mean diameter or average size of NPs was 283 ± 4.5 nm with a low PDI of 0.077 ± 0.026 .

Figure 53 FESEM images of Ltp loaded PLGA NPs (a) X10, 000 (b) X30, 000

Figure 55 and **Figure 56** shows the SEM images of Ltp loaded 10 % and 20 % PLGA MPs, respectively, obtained at different magnifications. MPs were spherical in shape and surface of particles was smooth.

Figure 54 Size distribution plot of Ltp loaded PLGA NPs

(a)

(b)

Figure 55 SEM images of Ltp loaded 10 % PLGA MPs (a) X60 (b) X100

(a)

(b)

Figure 56 SEM images of Ltp loaded 20 % PLGA MPs (a) X40 (b) X100

They might have micropores that should be confirmed with higher magnification images. MPs had a broad size distribution as measured from the

ImageJ software using SEM images. For 10 % PLGA MPs, average size of particles was found to be $53 \pm 19 \mu\text{m}$. For 20 % PLGA MPs, average size of particles was found to be $126 \pm 42 \mu\text{m}$.

% Loading and % EE

Ltp, being hydrophobic drug, was molecularly dispersed in the PLGA NPs and MPs matrix via o/w emulsification. Ltp loaded PLGA NPs and MPs were extracted using ACN and PBS as solvents to estimate the % loading and % EE. The drug loaded NPs and MPs were first dissolved in ACN so that the drug was completely released into the solvent. Once PBS (pH 7.4) was added to this Ltp-PLGA+ACN solution, the transparent solution turned milky. PLGA doesn't dissolve in PBS but Ltp dissolves in both ACN and PBS, the extent of solubility being different though. Upon centrifugation, PLGA precipitated into a pellet but the dissolved Ltp separated into the supernatant. Repeating cycles of exposure to fresh ACN and PBS, followed by centrifugation, extracted the entire amount of Ltp from the NPs and MPs.

The concentration of entrapped and unentrapped Ltp was calculated using HPLC system. A standard curve ($R^2 = 0.997$) was prepared using known concentrations of Ltp in PBS buffer and the calibration was used to estimate the concentration of drug samples from NPs and MPs. This data from HPLC was used to calculate % loading and % EE using the following equations:

$$\% \text{ Theoretical Loading} = \left(\frac{\text{Amount of Ltp used}}{\text{Amount of PLGA used}} \right) \times 100$$

% Practical Loading

$$= \left(\frac{\text{Amount of entrapped Ltp}}{\text{Amount of PLGA NPs/MPs used for extraction}} \right) \times 100$$

$$\% EE = \left(\frac{\text{Practical Loading}}{\text{Theoretical Loading}} \right) \times 100$$

For MPs study, 10 % of 0.4A IV PLGA was used to encapsulate Ltp solution (theoretical loading ~2 %) to obtain a practical loading of 1.5 ± 0.16 % upon fabrication of Ltp loaded MPs. Thus, the EE of PLGA MPs was 75 ± 7 %. Also, 20 % of 0.4A or 0.4E IV PLGA was used to encapsulate Ltp solution (theoretical loading ~1.67 %) to obtain a practical loading of 1.5 ± 0.1 % upon fabrication of Ltp loaded MPs. Thus, the EE of PLGA MPs was 90 ± 5 %. For NPs study, 10 % of 0.4A or 0.4E IV PLGA was used to encapsulate Ltp solution (theoretical loading ~2.33 %) to obtain a practical loading of 1.5 ± 0.2 % upon fabrication of Ltp loaded NPs. Thus, the EE of PLGA NPs was 64 ± 8 %.

4.6.2 *In vitro* release of Ltp from PLGA NPs and MPs

Figure 57 shows the % cumulative release of Ltp from PLGA NPs (0.4A IV). A high burst release of ~47-50 % was observed within 24 hrs from NPs. This high burst release indicated that a large amount of drug was adsorbed on the surface of NPs which came out as soon as they were exposed to aqueous medium within 24 hrs. ~100 % of drug was released in 6 days. After the burst release, Ltp was found to be released slowly in the second phase for the rest of the release period of 5 days. The profile did not show any change in release trend due to the degradation of NPs. Thus, the release of Ltp from NPs was biphasic starting with burst release followed by the diffusion controlled release in the second phase.

Figure 57 Release profile of Ltp from 10 % PLGA NPs

When the drug loaded NPs were mixed with HA solutions, release of Ltp followed exactly the same trend as NPs alone. ~100 % of drug was released in 7 days as shown in **Figure 58**. Burst release was high similar to NPs alone. No retardation in the release of Ltp was observed upon increasing the concentration of HA solution from 2 % to 4 % indicating no effect on the diffusion of Ltp by increasing solution viscosity. The release was diffusion controlled in the second phase similar to NPs alone. Thus, Ltp could not be sustained from NPs incorporated HA solutions.

Figure 59 shows the % cumulative release of Ltp from 10 % PLGA MPs (0.4A IV). In contrast to NPs, burst release of Ltp was absent from MPs indicating majority of the loaded drug was entrapped inside the MPs. ~27 % of drug was released within 24 hrs from MPs. However, drug release was not sustained for prolonged periods and ~100 % of drug was released in 8 days. The release

profile did not show any change in rate (change in shape of release curve) due to degradation or onset of erosion of MPs.

Figure 58 Release profile of Ltp from 10 % PLGA NPs loaded HA solutions

Thus, the overall release trend was monophasic and controlled by diffusion mechanism alone [155]. When the Ltp loaded 10 % PLGA MPs were mixed with HA solution, the release rate was similar to MPs alone as shown in **Figure 60**. The release rate was retarded marginally towards the later stages extending the 100 % drug release period by 2 days. However, no retardation in the release of Ltp was observed upon increasing the concentration of HA solution from 2 % to 4 % indicating no effect on the diffusion of Ltp by increasing solution viscosity. ~100 % of drug was released in 10 days and the release of Ltp was diffusion controlled similar to MPs alone.

Figure 59 Release profile of Ltp from 10 % PLGA MPs

Figure 60 Release profile of Ltp from 10 % PLGA MPs loaded HA solutions

Figure 61 shows the % cumulative release of Ltp from 20 % PLGA MPs (0.4A IV). Similar to 10 % MPs, burst release was absent indicating majority of the loaded drug was entrapped inside the MPs. ~20 % drug was released within 24

hrs from 20 % MPs. The release rate of Ltp from 20 % MPs was less than 10 % MPs due to larger size and slower degradation of the former. However, drug release was not sustained for prolonged periods and ~100 % of drug was released in 12 days. The overall release trend was monophasic and governed by the mechanism of diffusion alone similar to 10 % MPs.

Figure 61 Release profile of Ltp from 20 % PLGA MPs

When the drug loaded MPs were mixed with HA solution, release rate was similar to MPs alone as shown in **Figure 62**. The release rate was retarded marginally towards the later stages extending the 100 % drug release period by 2 days. However, no further retardation was observed in the release of Ltp upon increasing the concentration of HA solution from 2 % to 4 % indicating no effect on the diffusion of Ltp by increasing solution viscosity. ~100 % of drug was released in 14 days and the release was diffusion controlled similar to MPs alone.

Figure 62 Release profile of Ltp from 20 % PLGA MPs loaded HA solutions

Similar to 0.4A NPs and MPs, biphasic and monophasic release profiles of Ltp was also obtained for 0.4E PLGA NPs and MPs, respectively with differences in the magnitude and length of phases. In 0.4E NPs and MPs, ~100 % of drug was released in 9 days and 30 days, respectively as compared to 6 and 12 days in 0.4A NPs and MPs. Burst release of Ltp was almost similar for NPs. No burst release was observed in 0.4E MPs similar to 0.4A MPs. The phase of slow release was extended by 3 to 18 days as compared to 0.4A NPs and MPs, respectively. The longer duration in 0.4E NPs/MPs than 0.4A NPs/MPs is due to the slower degradation of ester-terminated chains of PLGA matrix than acid-terminated ones [121].

4.6.3 Characterization of HA+PLGA MPs composite hydrogels

Ltp loaded PLGA NPs and MPs were incorporated into HA-MA and HA-ADH hydrogels and characterized for morphology and *in vitro* release of Ltp.

The network structure of composite hydrogels with entrapped MPs, surface as well as cross-section samples, was examined using SEM as shown in **Figure 63**. It was observed that the morphology of Ltp loaded PLGA MPs was not affected due to crosslinking reactions and they were homogeneously distributed across the hydrogels. They were well dispersed in the surface as well as the inner mesh network of composite hydrogels.

(a)

(b)

Figure 63 SEM images of surface and cross-section of HA hydrogels incorporated with Ltp loaded PLGA MPs at X100 (a) surface (b) cross-section

The Ltp loaded PLGA NPs were found to aggregate within the hydrogels as observed from the FESEM images, obtained at different magnifications, in **Figure 64**, although during preparation they were manually dispersed homogeneously. However, it needs to be investigated further why the NPs aggregated inside hydrogel network and how this aggregation could affect the mechanical properties of hydrogels and/or the release behavior of Ltp.

The released medium, in which the composite hydrogels were soaked and incubated at 37 °C, was collected and spread over silicon wafers, dried, and observed under SEM and FESEM to check released MPs and NPs, respectively if any. No MPs were observed in the sink medium indicating that they were all

entrapped within the hydrogels and any surface bound particles had not released into the medium. NPs, however, were detected in the sink medium as aggregates indicating that they were released from the hydrogels.

Figure 64 FESEM images HA hydrogels entrapped with Ltp loaded PLGA NPs obtained at different magnifications (a) X10, 000 (b) X15, 000

Further, to evaluate the effect of crosslinking on the stability of Ltp inside PLGA NPs/MPs, drug loaded NPs/MPs, directly or in presence of initiator, were exposed to UV (keeping other parameters same) and extracted for estimation of the amount of Ltp using HPLC system. Similarly, the drug loaded NPs/MPs were exposed to chemical crosslinker PEGDE and extracted to determine any loss of Ltp. Upon comparison to unexposed Ltp loaded NPs/MPs, it was observed that no drug loss occurred when the NPs/MPs were exposed to UV light or to chemical crosslinker. Thus, the stability of NPs/MPs entrapped drug was ensured under the crosslinking reaction conditions.

4.7 Release behavior of Ltp from composite hydrogels

4.7.1 EPC liposomes loaded HA hydrogels

Figure 65 and **Figure 66** compares the release of Ltp from composite hydrogels and liposomes alone. Although the release trend of Ltp from composite hydrogels was similar to the liposomes alone, the rate was significantly retarded in HA-MA and HA-ADH hydrogels and release period was extended by 4 days. In this case also, the release of Ltp was controlled by partitioning mechanism alone.

Figure 65 Release profile of Ltp from EPC liposomes loaded HA-MA hydrogels

Similar to 5Fu studies, the retardation in the release of Ltp from composite hydrogels was due to the combination of liposomes and HA hydrogel where the gel network surrounding the liposomes acted as the additional barrier to the diffusion of drug. However, unlike 5Fu release, the release of Ltp from liposomes incorporated composite hydrogels was not dependent upon the gel

network environment. Although the hydrophobicity was different across the gel samples, drug retardation was similar in 2 %, 4 % HA-MA, and 4 % HA-ADH gels because the release was mainly governed by partitioning rather than diffusion. Thus, Ltp release could be sustained from liposomes incorporated HA hydrogels and was partition controlled.

Figure 66 Release profile of Ltp from liposomes loaded HA-MA and HA-ADH hydrogels

Besides the dialysis membrane release study, the composite hydrogels were also soaked directly in PBS buffer and *in vitro* release was conducted. This was to evaluate any affect on Ltp release trend due to the release of drug loaded liposomes from inside the hydrogels into the sink medium. Without a dialysis membrane, the liposomes, if released from hydrogels, do not have any barrier and they would get released into the release medium along with free drug. The released liposomes might be empty if they have released the drugs while being

entrapped inside hydrogel network or they could be partially loaded with Ltp if they were released outside before complete drug release.

As observed from **Figure 67**, the release trend of Ltp was almost similar whether the hydrogels were placed inside the dialysis membrane or immersed directly in the release medium. At every time point, the % cumulative release from composite hydrogels inside dialysis bag was ~5 % less than the amount released without dialysis bag extending 100 % drug only by 1 day. This indicated that liposomes released ~100 % of drug while being entrapped within the crosslinked network of hydrogels. Thus, empty liposomes were released into the sink medium after releasing all the entrapped drug from within the hydrogel network and they were the ones detected from TEM images and estimated from size measurement (section 4.5.3).

Figure 67 Release profile of Ltp from liposomal hydrogels with or without dialysis bag

4.7.2 PLGA NPs loaded HA hydrogels

Figure 68 and **Figure 69** compares the release of Ltp from composite hydrogels and PLGA NPs alone. The release of Ltp from NPs incorporated composite HA hydrogels followed similar pattern as NPs alone with ~100 % of drug being released in 7 days. The burst release was reduced to ~10-13 % in 2 % HA-MA and 4 % HA-ADH hydrogels, respectively. No reduction in burst release was observed in 4 % HA-MA hydrogels.

The overall release was retarded in 2 % HA-MA hydrogels as compared to 4 % HA-MA or 4 % HA-ADH hydrogels. There was no effect of different type of gels on release behavior and the rate was faster upon decreasing the concentration of HA macromer from 4 % to 2 % in HA-MA hydrogels.

Figure 68 Release profile of Ltp from 10 % PLGA NPs loaded HA-MA hydrogels

Figure 69 Release profile of Ltp from 10 % NPs loaded HA-MA and HA-ADH hydrogels

However, this effect was not consistent between different studies indicating that there was no significant effect on retardation of Ltp release by changing either the type of macromer in hydrogels or by increasing crosslinking density within same type of gel. This could be because of aggregation of NPs inside the hydrogels and large amount of surface adsorbed drug. Thus, Ltp release could not be sustained from NPs incorporated HA hydrogels and was biphasic, governed by diffusion mechanism after burst release similar to NPs alone.

Besides the dialysis membrane release study, the composite hydrogels were also soaked directly in PBS buffer and *in vitro* release was conducted. This was to evaluate any affect on Ltp release trend due to the release of drug loaded NPs from inside the hydrogels into the sink medium. Without a dialysis membrane, the NPs, if released from hydrogels, do not have any barrier and they would get released into the release medium along with free drug. The released NPs might

be empty if they have released the drugs while being entrapped inside hydrogel network or they could be partially loaded with Ltp if they were released outside before complete drug release.

As observed from **Figure 70**, the release trend of Ltp was almost similar whether the hydrogels were placed inside the dialysis membrane or immersed directly in the release medium. At every time point, the % cumulative release from composite hydrogels inside dialysis bag was ~5 % less than the amount released without dialysis bag extending 100 % drug only by 1 day similar to EPC liposomes. This indicated that NPs released ~100 % of drug while being entrapped within the crosslinked network of hydrogels. Thus, empty NPs were released into the sink medium after releasing all the entrapped drug from within the hydrogel network and they were the ones detected from FESEM images.

Figure 70 Release of Ltp from 10% NPs loaded hydrogels with or without dialysis bag

4.7.3 PLGA MPs loaded HA hydrogels

Figure 71 and **Figure 72** compares the release of Ltp from composite hydrogels and 10% PLGA MPs alone. The release trend of Ltp from composite hydrogels was similar to the MPs alone but the rate was significantly retarded in HA-MA hydrogels extending the release period by 3 days. No burst release was observed similar to MPs alone. ~18-22 % of drug was released within 24 hrs and a monophasic release was followed with a long slow release phase throughout the release period. ~100 % of drug was release in 11 days.

There was no further retardation in release by increasing the macromer concentration in HA-MA hydrogels from 2 % to 4 %. Also, HA-ADH gel was unable to retard the release as compared to HA-MA hydrogels. In this case also, the release of Ltp was controlled by diffusion mechanism throughout the release period.

Figure 71 Release profile of Ltp from 10 % PLGA MPs loaded HA-MA hydrogels

Figure 72 Release profile of Ltp from 10 % MPs loaded HA-MA and HA-ADH hydrogels

Figure 73 and **Figure 74** shows the release of Ltp from composite hydrogels and 20 % PLGA MPs alone. Similar to 10 % PLGA MPs, the release trend of Ltp from composite hydrogels was similar to the MPs alone but the rate was significantly retarded in HA-MA and HA-ADH hydrogels extending the release period by 3 days.

No burst release was observed similar to MPs alone. ~18-22 % of drug was released within 24 hrs and a monophasic release was followed with a long slow release phase throughout the release period. ~100 % of drug was release in 11 days. There was no further retardation in release by increasing macromer concentration in HA-MA hydrogels from 2 % to 4 % (no effect of concentration of macromer). Also, HA-ADH hydrogels showed similar retardation as HA-MA hydrogels (no effect of type of macromer). Release of Ltp was controlled by diffusion mechanism throughout the release period similar to MPs alone.

Figure 73 Release profile of Ltp from 20 % PLGA MPs loaded HA-MA hydrogels

Figure 74 Release profile of Ltp from 20 % MPs loaded HA-MA and HA-ADH hydrogels

Besides the dialysis membrane release study, the composite hydrogels were also soaked directly in PBS buffer and *in vitro* release was conducted. This was to evaluate any affect on Ltp release trend due to the release of drug loaded

MPs from inside the hydrogels into the sink medium. Without a dialysis membrane, the 10 % or 20 % PLGA MPs (empty or drug loaded), if released from hydrogels, do not have any barrier and they would get released into the release medium along with free drug. As observed from **Figure 75** and **Figure 76**, the release trend of Ltp was similar whether the hydrogels were placed inside dialysis membrane or immersed directly in the release medium.

Figure 75 Release of Ltp from 10 % MPs loaded hydrogels with or without dialysis bag

This confirmed the SEM imaging results indicating that MPs were not released from the hydrogels and they released 100 % of drug while being entrapped within the crosslinked network. As the mesh size was in nanometer range, the micron sized particles remained entrapped inside the hydrogels and released the drug there which then diffused through the hydrogel and released into the release medium.

Figure 76 Release of Ltp from 20 % MPs loaded hydrogels with or without dialysis bag

Similar to 5Fu study, it was observed from the degradation study of Ltp loaded MPs that they degraded in the same rate whether alone or entrapped inside hydrogels as shown by the average molar mass of particles estimated by SEC in **Figure 77**. 4 % HA-MA hydrogels were used for degradation study (retardation was similar in all hydrogel samples). Thus, the retardation observed in the release rate of Ltp in composite hydrogels of HA with 10 % or 20 % PLGA MPs was due to the additional diffusion resistance or barrier by the hydrogel network surrounding the MPs. Similar to 5Fu MPs, the erosion profiles of Ltp loaded PLGA MPs with and without hydrogels might be different. Also in case of Ltp, another important factor possibly responsible for drug retardation could be partitioning of drug between MPs and gel network. The effect of these two factors on Ltp release retardation needs to be further investigated.

Figure 77 Degradation profile of Ltp loaded PLGA MPs with and without HA-MA hydrogel

4.8 Comparison of drug release profiles from composite hydrogels

The release profiles of nano/micro carriers incorporated composite hydrogels were compared to evaluate the effect of three factors; namely, type of drug, type of carriers, and size of carriers on the release behavior of 5Fu and Ltp and their retardation thereof. 5Fu and Ltp loaded PLGA MPs with HA hydrogels were used to understand the effect of type of drug, Ltp loaded PLGA NPs and EPC liposomes with HA hydrogels were used for type of carriers, and Ltp loaded PLGA NPs and PLGA MPs with HA hydrogels were used to understand the effect of size of carriers.

Figure 78 compares the release profiles of 5Fu and Ltp loaded 0.4A IV PLGA MPs with and without 4 % HA-MA hydrogels. As we can observe, type of

drugs significantly affected their release patterns from PLGA MPs. Both 5Fu and Ltp were significantly retarded from PLGA MPs loaded HA hydrogels but the extent of retardation was much higher for 5Fu than Ltp that might be due to differences in their hydrophilicity. For both drugs, the retardation from composite hydrogels was due to the additional diffusion barrier of hydrogel network surrounding the MPs.

Differences in erosion profiles of PLGA MPs with and without hydrogel and partitioning of drug might be other possible factors responsible for retardation of drugs from composite hydrogels. In case of 5Fu, differences in the extent of retardation between composite hydrogel samples could also possibly be explained by differences in erosion profiles of entrapped MPs that can be affected by swelling of gel network.

Figure 78 Release profiles of 5Fu and Ltp from PLGA MPs loaded 4 % HA-MA hydrogels

Figure 79 compares the release profiles of Ltp loaded 0.4A PLGA NPs and EPC liposomes with and without 4 % HA-MA hydrogels. As we can observe, type of carriers significantly affected the release of Ltp due to differences in the properties of the carriers and release mechanisms involved (diffusion/partitioning/degradation).

Release of Ltp from the slowly degrading NPs (0.4E IV) also showed similar release profile and there was no retardation from composite hydrogels. Absence of reardation of Ltp from NPs loaded composite hydrogels could be due to aggregation of NPs inside the gel network and large amount of surface adsorbed drug. Retardation of Ltp from liposomes loaded HA hydrogels was due to the additional diffusion barrier of gel network surrounding the liposomes. Partitioning of drug might also be affected due to this gel network barrier retarding the release rate.

Figure 79 Release profiles of Ltp from NPs and liposomes loaded 4 % HA-MA hydrogels

Figure 80 compares the release profiles of Ltp loaded 0.4A PLGA NPs and MPs with and without 4 % HA-MA hydrogels. Ltp was significantly retarded from PLGA MPs loaded HA hydrogels but not from NPs loaded gels. MPs degraded slower than NPs, burst release was absent, diffusion path length was longer due to large size, and hence the higher retardation of Ltp from MPs than NPs. However, it was difficult to infer if the size of carriers played a role in retardation of Ltp release from composite hydrogels because all the particulate carriers were degradable. Retardation in the release of Ltp was mainly observed in the later stages of release when degradation mechanism were also involved as mass transport process although the complete release period was controlled by diffusion.

Figure 80 Release profiles of Ltp from PLGA NPs and MPs loaded 4 % HA-MA hydrogels

Chapter 5 Conclusion and Future work

HA hydrogels (both chemical and physical) have been extensively explored as drug delivery systems wherein the primary focus has been to reduce initial burst effect, enhance drug bioavailability, and to obtain sustained release of drugs by modifying parameters such as gelation kinetics and other crosslinking reaction variables (affecting mechanical and/or degradation properties) and type of copolymer. However, these hydrogels have been unable to retard the release of drugs for prolonged periods just by varying crosslinking conditions, thus motivating to explore composite hydrogels wherein a drug loaded sustained release delivery carrier is entrapped inside hydrogel network. HA based composite hydrogels with nano/micro carriers have been developed mainly for tissue engineering applications, but they have not been systematically investigated for the transport behavior of drugs to evaluate their potential as sustained release drug delivery systems. It is critical to understand the transport behavior of drugs through these crosslinked networks to improve the design and efficacy of these composite hydrogel delivery systems.

5.1 Conclusion

In this work, HA was first modified to attach two different functional moieties to its repeating unit to enable it for crosslinking. Well-established methods of modification were chosen based on the mild and rapid reaction conditions using MA and ADH as chemical moieties. MA derivatized HA, modified at primary OH group of monomer, ADH derivatized HA, modified at COOH functional

group, were successfully crosslinked using UV light and chemical crosslinker PEGDE, respectively to develop hydrogels.

These HA-MA and HA-ADH hydrogels were characterized for degree of substitution, molecular weight, network structure, morphology, mechanical properties, and *in vitro* drug release. HA-MA hydrogels were prepared in two different concentrations, 2 % (w/v) and 4 % (w/v) to understand the effect of macromer concentration within same type of gels. 4 % gels of HA-MA and HA-ADH were prepared to understand the effect of different types of macromer/hydrogels. Higher degree of modification of HA was achieved with ADH moiety as compared MA that might be due to higher efficiency of the reaction with the former. Upon estimation of molecular weight, it was found that modification with MA did not affect the M_w of unmodified HA but it was almost twice the magnitude after modification with ADH. HA chains were not degraded by any of the modification methods and the gain in M_w in HA-ADH chains could be attributed to the interchain binding of COOH groups via ADH bridge.

4 % HA-MA hydrogels were found to be crosslinked with higher density and thus having smaller network mesh size as compared 4 % HA-ADH hydrogels. Effect of macromer concentration on increasing the crosslinking density was observed from HA-MA hydrogels. Dynamic oscillatory shear tests also confirmed the results from morphology and swelling studies with 4 % HA-MA hydrogels exhibiting higher storage modulus than 2% HA-MA and 4% HA-ADH hydrogels.

2 %, 4 % HA-MA, and 4 % HA-ADH hydrogels were then used for *in vitro* release study of two model drugs, 5Fu and Ltp, either alone or with entrapped nano/micro carriers as composite hydrogels. The release behavior from hydrogels alone and composite hydrogels were compared to drug solution alone, drug loaded HA solution, and HA solution with entrapped drug loaded nano/micro carriers. 5Fu release could not be sustained from any of the solution or hydrogel samples and it was rapidly released within 2 days. Ltp, however, was released slower than 5Fu but release was not retarded beyond 5-7 days from HA solutions and hydrogels. The burst release was still very high for Ltp. It was concluded that the release of 5Fu as well as Ltp was diffusion controlled.

As HA solutions and hydrogels alone could not sustain the release of 5Fu and Ltp, several polymeric nano/micro carriers were investigated for the same. For 5Fu loading, liposomes, CS NPs, CS LBL particles, PLGA NPs, and PLGA MPs were developed. However, due to its small molecular weight and hydrophilicity, it could be encapsulated only in PLGA MPs with PLGA concentration 20 % and above and was found to release in sustained manner. For Ltp, liposomes, PLGA NPs, and PLGA MPs were developed and found to successfully entrap the drug and release it in sustained manner. The sustained release carriers for 5Fu and Ltp were characterized for morphology, size distribution, % loading and % EE, and *in vitro* release.

From 5Fu release studies, it was found that 5Fu was retarded significantly from all the MPs loaded composite hydrogel samples. Retardation from HA-MA composite hydrogels was higher than HA-ADH composite hydrogels. This

retardation was due to the combination of PLGA MPs and HA hydrogels because hydrogels alone cannot sustain the release of 5Fu. As the degradation rate of MPs alone or inside hydrogels was found to be similar, the retardation in release of 5Fu from composite hydrogels was due to the diffusion resistance of drugs by the additional barrier of gel network surrounding the MPs. Although the release was retarded in composite hydrogels, it was not sustained for prolonged periods. Release of 5Fu from PLGA MPs was governed by a combination of diffusion and degradation mechanisms in a biphasic pattern without any burst release phase and similar trend was observed from composite hydrogels.

For 5Fu release, 2 % HA-MA, 4 % HA-MA, and 4 % HA-ADH composite hydrogels showed significant difference in the rate of retardation indicating that the diffusion resistance of drug was dependent upon the type of network structure surrounding the drug carriers. Increasing HA-MA macromer concentration decreased the release rate further. Change in macromer types also affected the release rate in composite hydrogels.

From Ltp-PLGA MPs release studies, it was found that Ltp was retarded significantly from MPs loaded HA-MA and HA-ADH composite hydrogels. Although there was difference in retardation of drug alone from the hydrogel samples, the % retardation of Ltp was similar in all the composite hydrogel samples irrespective of macromer type and concentration. The release of Ltp, in spite of being retarded in composite hydrogels, was not sustained for prolonged periods similar to 5Fu. Unlike 5Fu release, however, the retardation

in release of Ltp was independent of the network structure of gels. In case of Ltp loaded NPs, the composite hydrogels did not show any retardation in release due to aggregation of particles inside hydrogels and presence of large amount of surface adsorbed drug.

Similar to 5Fu, as the degradation rate of Ltp loaded PLGA MPs alone or inside hydrogels was similar, the retardation in release of Ltp from composite hydrogels was due to the additional diffusion barrier of gel network surrounding the MPs.

Release of Ltp from PLGA MPs was governed by diffusion mechanism alone in a monophasic pattern without any burst release phase and similar trend was observed from composite hydrogels. Release of Ltp from PLGA NPs was biphasic, high burst release followed by second phase of slow release governed by the mechanism of diffusion. The release of Ltp from MPs alone was slowest in 20 % PLGA MPs, followed by 10 % MPs, and then 10 % NPs. Similar trend was followed in composite hydrogels also indicating that large sized particles, having smaller surface area:volume ratio and slower rate of degradation, retarded the release of Ltp by increasing the diffusion path length.

For Ltp loaded liposomes, the release of Ltp was controlled by partitioning mechanism throughout the release period and follow the same in composite hydrogels. Similar to Ltp loaded PLGA MPs, the release of Ltp was significantly retarded from HA-MA and HA-ADH composite hydrogels. Also, the % retardation was similar in all the composite hydrogel samples irrespective

of macromer type and concentration. The release of Ltp, inspite of being retarded in composite hydrogels, was not sustained for prolonged periods similar to 5Fu. Thus, the hydrogel network is acting as an additional diffusion barrier to drug surrounding the liposomes. Further, the partitioning effect of drug might also be different in composite hydrogels as compared to liposomes alone. After releasing from liposomes into the hydrogel, the drug might partition itself in the network and then slowly diffuse out into the surrounding aqueous medium.

From the release trends of Ltp, it could be concluded that these composite systems of Ltp loaded liposomes and PLGA MPs with HA hydrogels could be explored for designing for zero-order rate drug release carriers by optimizing their properties and the various parameters involved in their synthesis.

Besides the dialysis membrane enclosed samples, release study was also conducted for HA-MA composite hydrogels by immersing the samples directly in PBS buffer without the membrane. The release trend of the composite hydrogel samples was exactly similar with or without dialysis membrane for 5Fu and Ltp loaded MPs. Upon imaging the release medium from composite hydrogels, absence of any MPs confirmed the similarity of release trends of hydrogels with and without dialysis membrane and it was concluded that all the MPs remain entrapped inside the hydrogel network and released the loaded drug from there itself.

In case of Ltp loaded NPs and liposomes also, release rate of drug from composite hydrogels was similar when they were immersed in PBS buffer with or without dialysis membrane. Upon analyzing the release medium from NPs (imaging) and liposomes (imaging and size measurement) loaded hydrogel samples, it was found that NPs and liposomes were released into the sink medium. As the release trend was similar in the composite hydrogels with or without dialysis membrane and the nanocarriers (liposomes and PLGA NPs) were detected in the sink medium, it was concluded that all the drug was released from the carriers while they were still entrapped inside the gel network and only empty carriers were released.

The imaging of composite hydrogels ensured that the morphology of MPs was not affected due to the crosslinking procedure. However, the NPs were found to be aggregated inside the hydrogels as compared to the homogeneously dispersed MPs. In case of liposomes entrapped composite hydrogels, the size distribution and imaging of samples from released medium ensured the stability of vesicular structure of liposomes during crosslinking. It was found that there was no significant difference in the size of released liposomes as compared to the original suspension of liposomes before crosslinking.

Thus, with reference to the proposed hypothesis for the current work, it could be concluded that HA based composite hydrogels could retard the release of hydrophilic and hydrophobic drugs differently but the release could not be sustained for prolonged periods. For a specific drug, the retardation would depend upon the type of delivery carrier entrapped inside hydrogels. Also, the

drug diffusion barrier/resistance of hydrogels is dependent upon the network structure properties.

The drug release profiles of 5Fu loaded PLGA MPs with and without HA hydrogels, and of Ltp loaded PLGA MPs and EPC liposomes with and without HA hydrogels attained the therapeutic dosage required per day for either most or complete part of the release period. However, it is important to highlight that further optimization of design parameters is believed to improve these composite hydrogels formulations and hence they still remain potential drug delivery carriers for sustained release. We can improve the % drug loading in these MPs and liposomes, tailor the physico-chemical properties of PLGA MPs, and hydrogels to improve the sustained release above therapeutic dosage for prolonged periods of time.

In this work, the release studies aimed to improve the patient compliance by reducing multiple dosing regimen by prolonging the sustained release of drug above the therapeutic dosage required per day. Also, in case of 5Fu, the harmful effects to surrounding healthy cells can be minimized by releasing the drug at target site and restraining the entrapped microcarriers in the target site. Ocular residence time also improves by increasing the bioavailability of drug. Moreover, the release period was targeted for at least 2 weeks. So the overall duration of release obtained, achieved the targeted timeframe set for this study. They can still be improved to further reduce the dosing regimen and sustain the release for longer periods of time.

Thus, these composite hydrogels can be used as either injectable formulations, in case of HA-MA gels, or implantable formulations, in case of HA-ADH gels for ocular drug delivery. Besides the sustained release at target site and enhanced ocular residence time, these formulations would have additional advantage of possessing the wound healing properties of HA chains that can be confirmed after *in vivo* studies. Also, the mechanical and degradation properties of these composite hydrogels can be controlled. The reagents used in the modification and crosslinking reactions are biocompatible at lower concentrations and thus, these delivery systems can be potentially utilized for entrapment of sustained release carriers such as MPs or liposomes inside HA hydrogels for several ocular therapeutic applications.

The important contributions of this work includes the following:

- (a) development of composite hydrogel systems of HA with Ltp loaded liposomes that were shown to retard the release of Ltp significantly while maintaining the physical stability of Ltp as well as liposomes.
- (b) investigation of composite hydrogels of HA with PLGA NPs/MPs for the *in vitro* release behavior of hydrophilic as well as hydrophobic drugs using 5Fu and Ltp, respectively.
- (c) evaluation of the effect of hydrogel network structure on retardation of the release of hydrophilic and hydrophobic drugs from HA based composite hydrogels. The additional barrier of drug diffusion from the hydrogel network structure surrounding the PLGA MPs and liposomes was found to be dependent upon the type of drug and type of carrier for a specific drug.

5.2 Future work

The HA based composite hydrogels studied in this work needs to be further optimized to develop them into sustained release carriers for different types of drugs. For a specific drug and drug carrier, a systematic evaluation of various factors that affect the transport behavior of drugs would be significant. These could include physiological properties of drugs, structure and surface charge of drug carriers, crosslinking reaction variables, and different molecular weights of HA.

Crosslinking methodology for gels includes parameters such as concentration of crosslinking agent and reaction conditions that could affect the crosslinking density and gelation kinetics of hydrogels. Changes in these factors would in turn modify the swelling behavior, network morphology (structure and size of mesh), mechanical and degradation properties of HA thereby influencing the transport behavior of drugs through the composite hydrogels.

The interactions of HA hydrogel with the drug and/or drug carrier (affected by varying the physiological properties of drugs and type of drug carriers) and diffusion of carriers are also important to understand to obtain favorable transport behavior and release kinetics. Nature of drug-HA interactions would significantly influence the transport of hydrophobic and charged solutes through HA hydrogels and is thus an important factor to be considered. This significance could be attributed to the amphiphilic nature of HA networks that results from a combination of its polyanionic nature of carboxyl groups and the hydrophobic patches along its molecular chains.

The network morphology, crosslinking density of gels, and type of interactions between carriers and HA network would determine if the carriers (drug-loaded or empty) are released into the surrounding environment or if they remain entrapped and the drug diffuses out from them and gel network. To study this, fluorescently tagged carriers could be used for *in vitro* drug release experiments and fluorescence can be followed over time to estimate the diffusion behavior of carriers qualitatively as well as quantitatively.

Biodegradability is one of the desirable properties of HA and should not get affected as a result of crosslinking reaction conditions. In addition, rate of degradation of polymeric gel network would significantly affect the release of drugs by controlling the diffusion of drugs and/or drug carriers through the network. The bioavailability of drugs would be affected by the diffusion of drug carriers depending upon the degradation rate of surrounding hydrogel network. Thus, it is very important to evaluate the effect of enzymatic degradation of composite hydrogels on the release of entrapped drugs or drug carriers to get insights for the release behavior *in vivo*. Enzymatic degradation studies of HA composite hydrogels with a specific crosslinking density could be conducted using different concentrations of hyaluronidase in PBS buffer at 37 °C and vice-versa.

The present work has demonstrated that MPs and liposomes loaded HA composite hydrogels retard the release of drugs. However, we need to further investigate the details of the mechanism of resistance offered by the twin barriers of nano/micro carriers and HA gel network to the transport of drugs.

This could be achieved by quantitatively estimating the diffusion behavior in terms of effective diffusion coefficients and diffusion resistances of drugs and/or nano/micro carriers in the composite HA hydrogels. Also, investigation of other factors that might be responsible for retardation of drug release and differences in the extent of retardation among composite hydrogel samples should be considered. These factors include erosion profiles of PLGA MPs alone as well as inside HA-MA and HA-ADH hydrogels and partition coefficient of drugs (partitioning between carriers and gel network).

Understanding the transport behavior and release kinetics of drugs through HA composite hydrogel systems with nano/micro carriers would help to obtain a set of controllable parameters that could be optimized to improve the design and efficacy of these systems for a specific application. For instance, the HA-liposome or HA-PLGA NPs composite systems could be useful for delivery of hydrophobic drugs in applications such as ocular delivery, wherein the mucoadhesive nature and wound healing effect of HA could be exploited while delivering the hydrophobic drug in a sustained manner for prolonged periods. Entrapping the drug carriers in HA network would enhance their ocular residence time improving the bioavailability of drug. Thus more novel ocular delivery applications could be found for HA-based systems by controlling the efflux of drugs from the composite hydrogels.

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1. **Bora, M.**, Widjaja, L.K., Chan, P.N.P.H., Wong, T.T.L., Venkataraman, S.S. (2013, September). Nanocomposite Hyaluronic acid Hydrogels for Ocular drug delivery. Paper presented at The 25th European Conference of Biomaterials, Madrid, Spain.
2. Kottapalli, A.G.P., **Bora, M.**, Asadnia, M., Miao, J.M., Venkataraman, S.S., Triantafyllou, M. (2013, September). Hydrogel microstructures with encapsulated nanofibers mimic the cupulae of the blind cave fish. Paper presented at The 25th European Conference of Biomaterials, Madrid, Spain.

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