Layer-by-layer assembled micro and nano carriers for drug delivery applications

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LAYER-BY-LAYER ASSEMBLED MICRO AND NANO CARRIERS FOR DRUG DELIVERY APPLICATIONS

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SYNOPSIS

In nano- or microparticulate drug delivery, both control of the drug release and interaction of the particles with the cellular environment are critical attributes. Polyelectrolyte layering of the particle surface offers an interesting and facile way to modulate both attributes. Layer-by-Layer (LbL) technology involves the consecutive assembly of oppositely charged polyelectrolytes based on electrostatic interactions onto charged template surfaces. The relative ease of preparation through the LbL self-assembly, accurate control over the wall thickness as well as the flexibility in the choice of constituents qualifies these carriers to constitute an influential domain in the field of particulate technology. The bioactive agents may either be encapsulated in the core/template or inside the multilayers, with the option to tailor the properties of the carrier to the specific needs of the active material and therapeutic application. Despite having great potential in the design of versatile LbL carriers, there are very few studies that report the design of carriers suitable for drug delivery applications. In particular, the characterization and applications of biodegradable, biocompatible carriers, coated with polyelectrolyte multilayers, have not been extensively studied to date

The objective is to fabricate, characterize and evaluate specific applications of polyelectrolyte multilayered carriers for drug delivery applications. Towards this objective, biodegradable and biocompatible micro and nano- LbL carriers were fabricated to study the drug release profiles, cellular interaction, intracellular transport and processing of polyelectrolyte multilayered carriers as delivery agents.

Chapter 3 explains the general methods for fabrication of protein loaded LbL coated CaCO_3 particles and their application to treat breast tumors. Up regulation of cysteine proteases in tumor cells constitute a major role in extra cellular matrix (ECM) degradation and metastatic invasion. Consequently inhibition of cysteine proteases (cathepsins) is envisaged as a promising strategy to restrict tumor growth. Towards this goal, this study reports the design of a biocompatible and biodegradable carrier for intracellular delivery of cathepsin B inhibitor (cystatin C) in breast tumor cells.

In an approach to expand the application of vascular drug carriers, LbL self-assembly was exploited to impart added functionalities in order to overcome the disadvantages associated with the conventional erythrocyte delivery system, as detailed in chapter 4. The concept of introducing LbL multilayer shells as a surface modification

strategy lead to unique means of controlling the release of bioactive molecules from erythrocyte carriers.

Chapter 5 describes the application of LbL technology to hydroxyapatite (HA) nanoparticles for gene delivery applications. This work investigates the integration of proteins as well as nucleic acids (pDNA) in the multilayer shells and the intracellular gene delivery. Following the carrier design, studies were performed to delineate the intracellular trafficking of system that encompasses the molecular pathways associated with the uptake and intracellular processing. In this work, we show that nanoparticles with PEM coats offer a promising strategy for the development of safe and efficient vectors for the delivery of genes/siRNA into cells.

Chapter 6 reports the design of LbL carriers endowed with biomimetic properties for passive targeting. In brief, membrane vesicles derived from human red blood cells were explored for surface modification of multilayer shells. Further, the influence of the membrane orientation on the phagocytic uptake by mononuclear phagocyte system (MPS) system was studied using monocytes as a model platform.

To summarize, this thesis illustrates the modifications of the LbL concept in different applications such as protein delivery, gene delivery, controlled release, and passive targeting. The high level of control over the architecture of LbL carriers, such as size, shape, multilayer shells material and their surface functionality makes them a versatile carrier for drug delivery applications.

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Chapter 1: Introduction

1.1 BACKGROUND

Drug delivery technology represents one of the most fascinating and challenging fields in the frontiers of current science; that encompasses multidisciplinary fields spanning chemistry, biology, materials science, pharmacy and medicine. Currently a \$50 billion industry, the field of drug delivery has grown tremendously over the past and is expected to contribute significantly to the overall healthcare portfolio over the next 25 years [1]. Despite the rapid advancements and promising future, a plethora of challenges are to be overcome for the successful application of carriers in clinical applications. These challenges can be addressed in part by precise engineering of the particulate technology that serves to overcome the disadvantages associated with the conventional delivery systems. Recently the layer-by-layer technology has emerged as a promising strategy to functionalise the particulate systems for tailored applications. This chapter reviews the background and development of layer-by-layer assembled carriers for drug delivery applications. More specifically, the different components in the design of system in terms of structural and functional properties of the template and multilayer shells are discussed.

1.1.1 Layer-by-Layer technology

The fabrication and investigation of thin organic films are of great interest in nanotechnology as well as in biotechnology. Since the introduction of the layer-by-layer (LbL) assembly of oppositely charged polyelectrolytes onto flat and colloidal surfaces, the technique has received many potential applications. LbL technique entails the coating of nanometer-thick films on charged surfaces and colloids with multiple layers of various components utilizing electrostatic interactions for the layer build-up followed by the removal of the core/template [2]. The multilayer components range from polyelectrolytes to a variety of functional molecules including biological macromolecules (proteins, nucleic acids), surfactants, phospholipids, nano-particles, inorganic crystals and multivalent dyes [2]. Figure 1 illustrates the steps involved in the preparation of layer-by-layer coated particles followed by core dissolution to yield hollow microcapsules. The templates can be of different composition, size, and shape (micro particles, nanoparticles, cells, bacteria, hydrogel etc.). The first step (fig.1a) involves the deposition of a cationic polyelectrolyte layer (PAH) onto the negatively charged colloidal particle. The charged polymer that exhibits an opposite charge to the particle surface is added to the colloidal

suspension and allowed to adsorb on the surface by electrostatic interactions. (For representative purpose, the case of a negatively charged micron sized colloidal template and the most widely used polyelectrolyte pairs PAH/PSS are considered). Subsequently the unadsorbed polyelectrolytes are removed leaving behind the positively charged template. The next step (fig.1b) involves the introduction of anionic polyelectrolyte layer (PSS) to the colloidal templates. The positively charged particles now adsorb the negatively charged polyelectrolyte based on electrostatic self-assembly. Additional layers can be deposited by repeated deposition cycles, making use of the surface charge reversal that occurs upon adsorption of each layer (fig.1c). Following LbL coating, hollow capsules are produced by subsequent removal of the template (fig.1d) by chemical methods based on the nature of the template. (Core dissolving chemicals such as hydrochloric acid (HCl) in the case of silica particles, hydrofluoric acid (HF) in the case of melamine formaldehyde particles etc. can be used. Finally hollow microcapsules are obtained that are formed by a meshwork of interconnected polyelectrolyte thin films held together by electrostatic assembly (fig.1e).

Figure 1: Schematic illustration of the LbL technique for the preparation of hollow capsules. The initial steps (a, b) involve stepwise film formation by repeated exposure of charged templates to alternatively charged polyelectrolytes (cationic PAH and anionic PSS). The excess polyelectrolytes are removed by cycles of centrifugation and washing before the next layer is adsorbed. The coating steps are repeated until the desired number of multilayers is formed (c). The multilayer-coated particles are then exposed to core dissolving chemical to remove the template (d). Finally after the decomposition of the template, hollow microcapsules comprising thin polyelectrolyte films are obtained [2].

Various colloidal particles including biological as well as biodegradable materials like erythrocytes and PLGA/PLA or synthetic materials like silica or calcium carbonate have been employed. Depending on the template size, such polyelectrolyte multi-layered (PEM) particles can be produced in sizes ranging from 100nm-10 μ m. One of the interesting features is the formation of hollow microcapsules by subsequent core dissolution after multilayer formation [2-4].

1.1.2 Science of LbL

Decher *et al* reported the fabrication of multilayers by consecutive adsorption of polyanions and polycations onto planar substrates in which nano-scale arrangements of organic molecules can be controlled at least in one dimension [5]. The concept behind multilayer formations are, (i) repulsion of equally charged molecules and thus self-regulation of the adsorption and restriction to a single layer and (ii) the ability of an oppositely charged molecule to be adsorbed in a second step on the top of the first one [5]. LbL Technology was further extended to colloidal substrates from planar substrates [6]. It involves the consecutive assembly of oppositely charged polyelectrolytes on the template colloidal substrate followed by the dissolution of the core for the fabrication of hollow capsule. Although, LbL has also been extended to hydrophobic interactions [7, 8], hydrogen bonding [9, 10], DNA hybridization [11] etc., the electrostatic assembly remains the predominant mechanism.

1.1.3 Drug delivery with LbL carriers

In recent years there has been great interest in studying such PEM particles and capsules as possible micro- or nano carriers for potential biomedical applications such as drug delivery systems, sensor particles for diagnosis etc. [2, 12]. The prime advantage of the polyelectrolyte layers is their large versatility and modularity with regards to the material properties and templates available. There are many advantages of the polyelectrolyte multilayer systems in drug delivery as compared to the existing carriers. Some of the main features of the system outlined are

- (i) Particles can carry biomolecules in different ways, encapsulated in the multilayer constituent, in the core template and in hollow capsules obtained following core dissolution [13, 14].
- (ii) Capsule wall protects the encapsulated agents from undesired extracellular degradation [15].
- (iii) Triggered and controlled release of the encapsulated agents can be facilitated by the integration of respective agents e.g. light sensitive [16], magnetic field sensitive [17] components in the multilayers of the PEM particles,
- (iv) Integration of reporters (e.g. labeled molecules) for diverse sensing applications [18], and

(v) Multilayer shells can be functionalized with biomimetic properties (lipid bilayer, virus components, peptides, antibodies to control specific particle/cell interaction and uptake) [19].

The capsules can be tuned by varying a number of parameters and can be functionalized with different types of macromolecules depending on the purpose. The electrostatic attraction is highly preferred as there is a least steric demand of all chemical bonds. Due to the versatility of the LbL technique, PEM particles have been tailored for a wide range of applications including reactor systems [20], sensor [21], immunoassay [22] and controlled carrier systems [23]. Recent research has shown that doxorubicin conjugated polyionic complexes (PIC) with protamine sulphate / poly(L-aspartic acid) were able to release doxorubicin into the cells, indicating the possibility of using LbL layers as carriers for dual application, drug and gene delivery [24].

1.1.4 Templates for LbL coated particles

A number of materials have been studied to serve as the template core. Some templates can be sacrificed after adsorption of the polyelectrolyte layers, resulting in hollow capsules with no core in the final product [2]. The selection of a suitable template is of prime importance as they play a main role in the carrier formation and they should satisfy the following requirements,

- (i) Stability of the template during the LbL process [2]
- (ii) Core dissolution conditions should not have any deteriorating effects on the multilayer shells and leave no residual components of the template after core dissolution [27].

Biocompatible cores such as cells [25, 26], calcium carbonate [14, 27, 28], poly (lactide-co-glycolide) (PLGA) [29, 30] etc. are more suitable for biomedical applications, and furthermore the dissolution of cells and calcium carbonate particles require milder chemicals [3, 14, 27, 28]. In addition, both micro-sized and sub-micron sized biocompatible particles have been used as templates. Synthesized colloidal particles give the advantageous possibility to encapsulate active substances within the core [14]. Silica [31, 32] and melamine formaldehyde (MF) particles [33, 34] have been utilized widely, but their dissolution requires harsh chemicals which may pose toxicity issues.

1.2 PROBLEM STATEMENT

Polyelectrolyte multilayered carriers (PEM) are promising micro- and nano-carriers due to the various possibilities to functionalize the core and shell [12]. However, even though quite a number of studies have been carried out regarding the fabrication and functionalization of LbL coated particles and capsules, there is still only little known about the mechanisms that control the uptake of PEM particle or capsules (PEMC) by cells as well as the degradation, subsequent release and processing of the multilayers [12].

Previous studies have mostly employed commercial, non-biodegradable templates such as Au, silica or melamine formaldehyde, which are also limited in that they only allow layer functionalization [15, 35-37]. Moreover most past reports focused on synthetic, non-biodegradable polyelectrolytes such as PAH/PSS [35]. On the other hand, synthesis of colloidal templates would allow the encapsulation of a wide range of agents and the identification of biocompatible and biodegradable cores and multilayers is a requirement to utilize these LbL capsules for biomedical applications[12]. There is an extensive gap and inadequate information about the application of these carriers in drug delivery applications. This limited information and understanding further led us to investigate the feasibility of LbL technology in functionalization of carriers for specialized applications.

Problem statement: “The characterization and applications of biodegradable, biocompatible carriers, coated with polyelectrolyte multilayers, have not been extensively studied to date.”

Hypothesis: “Layer-by-layer assembly is a versatile technique for surface modification of particulate carriers for enhancing efficacy in drug delivery applications.”

1.3 OBJECTIVES

The main objective of this project is to fabricate, characterize and evaluate specific applications of PEM carriers, using a biodegradable/biocompatible core

Towards this objective, the thesis seeks to develop biodegradable and biocompatible micro and nano- LbL carriers, and study the drug release profiles, cellular interaction, intracellular transport and processing of PEM carriers. To achieve this goal the work has been divided into four specific aims

- 1) To develop carriers that allow core and layer functionalization with active biomolecules,
- 2) To identify strategies that allow controlled release of active agents from multilayered carriers, and to understand the mechanisms behind the control of release,
- 3) To develop strategies that allows passive targeting and thus control the *in vivo* lifetime of the PEM carriers,
- 4) To investigate the molecular mechanisms those underlie the interaction of the multilayered carriers with the cell; their uptake, processing and intracellular transport.

To complete the specific aims, we have identified bio-relevant polyelectrolyte multilayer particles in micro and nano sizes, as well as functionalization of the carriers with bio functional molecules aimed towards drug delivery applications. Thus we will focus on three different biocompatible and biodegradable templates, Calcium Carbonate (CaCO_3), red blood cells (RBC) and hydroxyapatite (HA), which are functionalized using the LbL technology for varied drug delivery applications. A comprehensive literature review is given at the introduction of every chapter to present an overview of previous research in the chosen field of application.

The novelty of the highly customizable and multifunctional PEM particle system lies in the LbL fabrication technology, which enables them to be specifically designed to fit the required purposes and yet have a simple production process. Novelty of these ideas is shown based on a thorough literature survey of work done in the field of LbL coated carriers toward drug delivery applications.

1.4 NOVELTY OF PROPOSED WORK

To achieve the following objectives, the research work followed the steps below.

Chapter 3: Functionalization of CaCO_3 particles with LbL technology

The current work is the first effort to introduce the pre-loaded core with biocompatible and biodegradable layers for identification of novel therapeutic target in tumor therapy. The versatility of the CaCO_3 particles are explored in different dimensions, (i) core encapsulation for formation of functionalized polyelectrolyte capsules, (ii) protein encapsulation in multilayer shells and (iii) study of bioactivity of encapsulated proteins. The first part focuses on the identification of protein carrier based on the encapsulation of model proteins BSA and lysozyme; and studies the bioactivity of the protein using these carriers.

The second part focuses on the application of the preformed carriers, polyelectrolyte microcapsules functionalized with a therapeutic protein cystatin for tumor therapy. The aim of the study is to design a drug carrier for intracellular delivery of cystatin C to inhibit cathepsin B, thereby inhibit tumor growth.

Chapter 4: Functionalization of LbL coated erythrocyte ghost carriers for controlled release of bioactive molecules

Human erythrocyte ghost carriers, which have been studied for over two decades is used in combination with the LbL system. Although several macromolecules have been successfully encapsulated in the erythrocyte carriers for various therapeutic applications, very little research has been done on controlled release from carriers. The LbL coating of the erythrocyte ghost carriers have been explored as a new dimension to modulate the release behavior of the encapsulated macromolecules. Besides controlled release the bioactivity of the molecules were studied to evaluate the proposed carrier system.

Chapter 5: Fabrication, functionalization and intracellular trafficking studies of LbL coated HA nanoparticles for intracellular drug delivery

For the first time hydroxyapatite (HA) nanoparticles (<200nm) were used as templates for LbL assembly. These multilayer shells have the possibility to functionalize the nanoparticles towards both protein and nucleic acid delivery. The cellular uptake and intracellular behaviour is still not well understood, though the fabrication of carriers has been studied extensively over the past decade. Here we demonstrate the design of nano-LbL carriers functionalised with pDNA in multilayer shells for gene delivery. In our study nucleic acid was chosen as a model compound to study the intracellular delivery. Furthermore studies to identify the intracellular pathway were accomplished using specific endocytic inhibitors. A detailed investigation of cellular interaction of nano carriers can be further extended towards various therapeutic applications requiring intracellular drug delivery.

Chapter 6: Functionalization of LbL multilayer shells with RBC membranes towards passive targeting

The classical concept of targeted drug delivery via receptor-ligand interaction can be applied to the LbL carriers by functionalization of the multilayer shells with respective ligands. Very few successful studies have been reported on the surface modification of the carriers for passive targeting and still active research is carried out towards immune

camouflage of these drug delivery agents. The concept of erythrocyte derived vesicles towards surface modification for passive targeting is reported for the first time and studies are underway for the feasibility of these biomimetic (RBC like) carriers.

Chapter 2: Review of Literature

2.1 Protein delivery

A major challenge in the field of drug delivery is how to overcome the unfavorable physicochemical nature of molecules such as proteins and oligonucleotides, which are unable to penetrate the cell membrane in order to reach their intracellular targets [38, 39]. Proteins, peptides and oligonucleotides are useful therapeutic tools; however, their applications are limited because delivery to the desired location is not easily achievable [38, 39]. Penetration into intracellular space can be achieved by adjusting hydrophilicity, by which small-molecule based pharmacological agents have been successfully developed [40]. However it is difficult to modify hydrophilicity of proteins and peptides without influencing biological functions [40]. Drug delivery carriers have been developed as a solution to this challenge, which serve as transporters for cellular entry of therapeutic molecules [41]. Despite the advent of numerous types of carriers in the rapidly evolving field of drug delivery, the search for an ideal carrier is under active research. Moreover, there has been significant progress towards the use of particulate-based protein delivery systems for therapeutic applications [41].

This chapter deals with the fabrication and functionalization of CaCO_3 particles with the LbL technology for protein delivery. It also discusses a novel method for the fabrication and functionalization of CaCO_3 particles with LbL technology for protein delivery. CaCO_3 microspheres in the micrometer range ($\sim 5 \mu\text{m}$) are of great interest due to their potential for encapsulation of large quantities of guest molecules by providing compartmentalization on the nanometer scale, which can be used as functionalized templates[14]. Since a detailed explanation on the design of LbL carriers has been given in the introduction, we proceed to outline the application of the carriers in a therapeutic condition. A detailed review of the pathology of the disease, the mechanism of action and the potential therapeutic target for application of LbL carriers is given below.

2.1.1 What is tumor?

Tumor refers to a mass of cells characterized by uncoordinated or irregular growth of cells. They are characterized by a set of histological changes displayed by the tumor cells and the microenvironment in which they develop and function. Cancer develops through various successive stages characterized by changes in gene expression and protein function at multiple levels [42]. Cancer is caused fundamentally due to damaged

DNA leading to abnormal gene expression patterns leading to uncontrolled growth of cells [42]. In order for a primary tumor to expand growth beyond its site of origin, the cells must first detach and migrate to establish growth at a distant site [43].

The focus of our investigation in this study is on breast tumor. Breast cancer is the most common malignant disease prevalent among western women. The mortality of the patients suffering from breast cancer is mainly attributed to the metastases at distant sites rather than to the primary tumor [44].

2.1.2 Metastasis and Tumour progression

Metastasis is the process in which cells from a primary tumor disseminate to form tumor masses at distant sites, which represents a stage of malignant progression [45]. Figure 2 illustrates a general overview of the steps involved in the process of tumor invasion. The first stage in tumor pathology involves the formation of neoplasm; group of cells characterized by progressive or uncontrolled proliferation of cells (fig. 2a). The second step involves local proteolysis resulting from the release of specific hydrolytic enzymes synthesized by cancer cells (fig. 2b). Detachment itself depends upon the extent of cell adherence brought about by the interaction of the plasma membrane with the surrounding extracellular matrix (ECM). Invasion through tissues requires the expression of proteolytic enzymes such as matrix metalloproteinases, cysteine proteases (matrix degrading enzymes) etc. which degrades collagen and allows passage through the cell matrix. As the disease progresses, the tumor cells detach from the site of attachment and invade surrounding tissues and metastasize (fig. 2c). Tumor invasion and metastasis are aggressive stages at which the tumor cells invade, migrate and proliferate.

Figure 2: Schematic illustration of tumor invasion and metastasis. (a) The first stage in tumor pathology involves the formation of neoplasm; group of cells characterized by progressive or uncontrolled proliferation. The transformation of normal cells into malignant cells comprises events driven by the acquisition of genetic and/or epigenetic alteration that significantly alters the molecular mechanism of the target cell. (b) The second step involves local proteolysis resulting from the release of specific hydrolytic enzymes synthesized by cancer cells. (c) The further steps involve degradation of the basement membrane and the surrounding region to gain access through intravascular compartments to anatomically distant organ sites and their subsequent adaptation to foreign tissue microenvironments.

The exact mechanism underlying the metastasis is not yet fully understood, however it involves a cohort of actions mediated by the proteases and the inhibitors allowing the cells to detach and enter the bloodstream enabling them to migrate over long distances [46]. During metastasis, a multitude of proteolytic cascades are activated thereby disrupting the attachment of the cells to the basement membrane (BM) and ECM [47]. Therefore a major aim of the anti-cancer therapy is to control the metastatic spread, of which proteolytic enzymes constitute a broad range molecular pathway associated with the malignant progression of tumor.

Therefore the advent of novel anti-cancer drugs should focus on the discovery of treatment to specifically target the genes, proteins, receptors and molecular pathways that regulate the growth, survival and malignant progression rather than to destroy or inhibit the increased rate of synthesis and replication of DNA in tumor cells.

2.1.3 Proteolytic enzymes in metastasis

Proteases are enzymes that degrade polypeptides and proteins. There are five major classes of proteases depending on the site of cleavage: serine, threonine, cysteine, aspartic and metalloproteinases [48]. Owing to the wide array of components that make up the BM/ECM and the substrate specificity of each protease, a number of different proteases are expressed either alone or by a cascade effect, contributing to the overall degradation of matrix[49]. Regulation of the proteases involved in tumor invasion and metastasis is complex and occurs at multiple stages. A number of agents such as growth factors, cytokines, oncogenes and tumor promoter genes can induce the expression of proteases. The key players associated with the metastatic state of cancer are matrix metalloproteinases (MMP-2, MMP-9 etc.), serine proteinases (urokinase type plasminogen activator, plasmin etc.) and lysosomal cysteine proteases (cathepsin B etc.) [50-52].

Over the past, matrix metalloproteinases (MMP) have been the major focus of interest and were considered to play the central role in the physiological processes involved in ECM degradation [53]. However recently cathepsins have gained increased attention for their proposed role in a number of physiological processes such as angiogenesis, apoptosis, ECM degradation and their diversity in a wide array of tumors contributing to neoplastic aggression [54]. There are fewer studies that relate cathepsins to cancer as compared to MMPs or serine proteinases.

2.1.4 Cysteine cathepsins

2.1.4.1 Role in physiology

Cysteine cathepsins (Cc) are a group of proteases with cysteine residues in the catalytic site that are involved in the proteolytic reaction. There are 11 members of the Cc in humans with a common conserved catalytic site formed by cysteine, histidine and asparagine residues [55]. In normal cells lysosomal cysteine proteinases are involved in a number of physiological processes such as antigen presentation, epidermal homeostasis, bone resorption, proenzyme activation, protein degradation/turnover and hormone

maturation [55]. In normal conditions, lysosomal cysteine proteases are regulated at every level of their biosynthesis along with their intracellular distribution and activity [55]. In addition, the cathepsin activity is highly monitored by their endogenous inhibitors, which belong to the cystatin superfamily. However in tumor cells and other malignancies, aberrations are found to occur at three levels, i.e. (i) overexpression of mRNA/protein (ii) abnormal processing and/or intracellular trafficking or (iii) down regulation/altered activity of the endogenous inhibitors [54].

2.1.4.2 Cathepsins in pathophysiology

Cathepsin B (Cat B) is the most extensively studied cathepsin that is found to be involved in numerous pathological conditions such as atherosclerosis, arthritis, osteoporosis and tumors [55]. The role of Cat B in metastasis was first demonstrated in breast cancer in the year 1981 [56], after which numerous studies have explored the role of cathepsin B in tumor and other malignancies. Up regulation of cat B has been reported in a wide array of tumors; breast, colon, esophageal, gastric, lung, ovarian and thyroid carcinomas as well as in gliomas and melanomas [57].

2.1.4.3 Cathepsins: Mechanism of action

In addition to up regulation, tumor cells also show altered phenomenon in Cat B processing, (i) redistribution to different vesicular compartments, (ii) secreted/exopeptidases and (iii) association with the plasma/basal membrane[54]. Cysteine proteases of the family C1 (Cat B) are primarily endopeptidases, however in certain conditions they function also as exopeptidases [58]. Furthermore, the leading edge of the cell exhibits increased expression of Cat B placing the protease at the site of infiltration. It is widely accepted that the Cat B vesicles present at the basal membrane and the cellular periphery (in tumor cells) are secreted and act as exopeptidases thereby mediating the degradation of ECM (fig. 3).

Figure 3: Localization of cathepsin B in tumor cells. In normal conditions, Cat B is primarily secreted as endopeptidases whereas in tumor cells they exhibit altered phenomenon acting as both as endo- and exopeptidases. In tumor cells, vesicles containing Cat B are localized to the apical surface of the plasma membrane, which then releases the secreted proteolytic enzymes responsible for ECM degradation.

Cathepsin B is active over a wide pH range and is known to exhibit proteolytic activity over a wide range of components of the ECM such as type IV collagen, fibronectin and laminin [59, 60]. This being an important step in order to facilitate tumor cell invasion and metastasis process, numerous studies have been focused towards regulation of Cat B production.

2.1.5 Cysteine cathepsin Inhibitors

Cystatins are cysteine protease inhibitors (CPI), which are vital for the regulation of physiological processes by reducing the protease activity[61]. The involvement of cysteine proteases in tumor progression is highlighted by the involvement of CPIs of the cystatin family in tumor invasion and metastasis *in vitro* and *in vivo* [62]. The imbalance between cysteine proteases and cystatin has been correlated with the progression of malignant diseases.

Among the CPIs, cystatin C is the most important and potent inhibitor of cat B[63]. Cystatin C has a molecular weight of approximately 13-14 kDa, with a single chain of approximately 120 amino acid residues and contain two (intrachain) disulfide bonds [64]. The cystatin superfamily is named after chicken cystatin (Cc); the first isolated and characterized CPI of the cysteine proteases of the papain superfamily. It was isolated and purified from chicken egg white [65]. Cc is the prototype of the human cystatin C (hCC) that shares 41% sequence homology and have identical secondary structures [61, 66]. Cc and hCC are natural tight binding reversible inhibitors of cysteine proteases of the papain superfamily, including Cathepsin B, H, J, K, L and S.

The involvement of cysteine proteases in tumor progression is highlighted by the involvement of CPIs of the cystatin family in tumor invasion and metastasis *in vitro* and *in vivo* [62]. A molecular or cellular mechanism gains interest as a novel strategy if a differential expression is observed between normal and malignant tissues. Furthermore it is more important to validate the target using mechanistic studies that determine its biological function and if it plays a crucial role in the formation and progression or inhibition of tumor. Taken together, the identification of mechanism underlying the inhibitory activity of proteases adds valuable information in identifying novel therapeutic target by the design of sophisticated drug delivery agents.

2.1.6 Cathepsin inhibition as a therapeutic target

Alteration in the balance of cystatin C to Cat B is one of the key factors that result in high proteolytic activity and contribute to the invasive ability of tumors [62]. In earlier studies, attempts made to establish the relationship between functional up regulation of cystatin by inhibition of cathepsins confirmed the role of cysteine proteases in tumor progression [62]. On a similar note, several studies have reported the down regulation of cystatin in tumor cells. Furthermore it was recognized that up regulation/overexpression of cystatin by gene delivery (encoding for cystatin) reduced the tumor invasion and metastatic spread [67, 68]. Alternatively silencing of cystatin in metastatic oral cancer by siRNA further aggravated the condition. An increased secretion of cysteine proteases were accompanied by proliferation and invasion of tumor cells [69].

In a second approach targeting the antagonist Cat B, chemical inhibitors directed against cathepsin were able to reduce proteolytic degradation and hindered metastatic cell spreading and tumor progression [70]. Similarly, in another approach to inhibit cathepsin in breast tumor model, RNA mediated knockdown of cathepsin B reduced collagen degradation *in vitro* and limits bone metastasis *in vivo* [71]. Taken together, these studies were focused towards a central theme of tumor progression and their relevance to the role of cathepsin-cystatin complexes.

2.1.7 LbL carriers for protein delivery

In recent years different strategies have been investigated to deliver protein inhibitors to tumor cells [39, 41]. Polymeric micro- and nano particles as a transport system seem to be promising tools in that they taken up quite rapidly into highly

metabolizing cells by means of endocytosis. In addition, incorporation of peptides or proteins in the carriers facilitates protection of the active agent from early decomposition and allows targeted release at the desired site of action. The latter also enhances the therapeutic efficiency and bioavailability of specific drugs. One such molecular target in tumor therapy that is still in the initial stages of development is cysteine protease inhibition.

2.2 Vascular carriers

2.2.1 Introduction

The microencapsulation of substances has been finding many applications in the pharmaceutical, food, cosmetic, paint and print industries [72, 73]. For example, encapsulating pharmaceuticals inside micro-/nano-capsules can help to minimize under- or over- dosing, to protect drugs from degradation, to reduce side-effects, to replace multiple dosing and thus enable better patient compliance [74-76]. Various methods including spray-drying, liposome fabrication techniques, interfacial poly-condensation and emulsion polymerization have been developed to fabricate micro- and nano-capsules [77-79].

2.2.2 Cell as drug vehicles

Although the particulate system based on polymeric materials occupy the field of particulate drug carriers, there are biological carriers that offer an efficient alternative to such systems. Within the different systems of biological carriers, of great importance are cells and cell ghosts, which are both efficient systems and highly compatible, capable of encapsulating a wide array of drugs and active biomolecules [80-82]. Cell systems such as bacterial ghosts, erythrocyte ghosts, polymorphonuclear leukocytes, apoptotic cells, tumor cells, dendritic cells and more recently genetically engineered stem cells are examples of cell systems of diverse nature that have been exploited for drug delivery in various therapeutic applications [80-86].

2.2.3 Cell carriers- Erythrocytes

Erythrocytes are the most readily available and abundant cells within the body that have been extensively studied as cell based drug carriers [87]. Red Blood Cells (RBCs) are the most abundant cells in the human body. They do not possess a nucleus or other intercellular organelles. They do however contain hemoglobin, and carry out the

important function of delivering oxygen to the tissues and bringing CO₂ to the lungs. Human erythrocytes are biconcave discs with an average diameter of 7.8 μm , thickness of 2.5 μm in around and 1 μm in the center, and volume of 85-91 fl (μm^3) [87]. The erythrocyte membrane has a unique structure with special physicochemical properties. The membranes have a high degree of flexibility that enables them to traverse pass through the capillaries with diameters as low as 3-4 μm . The membrane contains basic plasma membrane structure including lipids, proteins and carbohydrates in addition to a unique organization referred to as cytoskeleton, which is a network resulting from the cross-linkage of a number of specialized proteins namely spectrin and erythrocyte actin. This structure enables the membrane to withstand the high shear stress encountered in circulation. Furthermore, when the osmolality of the surrounding medium decreases, the erythrocytes become cup shaped and finally spherical, whereas in the presence of hypertonic medium the erythrocytes shrink, referred to as crenation (fig. 4). This kind of swelling behavior is necessary for most of the methods used for loading the erythrocytes by drugs or other chemicals.

Figure 4: Red blood cell membrane under different osmotic conditions. At isotonic conditions (300 mOsm/kg), the concentration of the dissolved substance is the same as in the fluids the cell is in equilibrium with its environment. At hypotonic conditions (<300 mOsm/kg), where there is lower concentration of dissolved substances than the fluid in the cells, the RBCs undergoes hemolysis and takes in water, swells, and may burst. In hypertonic conditions (>300mOsm/kg), where the concentration of dissolved substances fluid is higher than the cell, it loses water and shrinks, undergoes crenation. (<http://chemistry.about.com/od/imagesclipartstructures/ig/Science-Clipart/Osmosis---Blood-Cells.htm>)

2.2.4 Life-cycle of RBC

The normal life-span of an erythrocyte in systemic circulation is about 120 days. As a result of gradual inactivation of the metabolic pathways of the erythrocyte by aging, the membrane loses its natural integrity, flexibility and chemical composition. These in

turn finally result in the destruction of these cells upon passage through the spleen-trabecules. The other effective site for the destruction of aged or abnormal erythrocytes is the macrophages of the reticuloendothelial system (RES) that include peritoneal macrophages, hepatic kuppfer cells, alveolar macrophages of the lung, peripheral monocytes and vascular endothelial cells. The aging and series of other factors makes the erythrocytes recognizable to phagocytic macrophages by alteration in the chemical composition of membrane [88]. This feature has enabled targeting of erythrocyte based carriers to the RES system.

2.2.5 RBC membrane- “ Regulated portals”

The basis for a number of methods used for encapsulation of drugs in erythrocytes is the fact that when erythrocytes are placed in hypotonic medium, they swell reversibly up to a definite degree of tonicity, to gain about 25% of their original volume. At this point, some transient pores with diameters of 200-500 Å occur in the membrane. If the tonicity of the surrounding medium is restored to isotonic conditions, the pores appear to close. However when the tonicity is decreased beyond certain limits (i.e. less than 20 mOsm/kg, for e.g. water [0 mOsm/kg]), some irreversible ruptures and openings occur in the membrane that lead to complete lysis (fig. 5). The term “erythrocyte ghost” attempts to define the resulting cell-like structure when erythrocytes are subjected to reversible process of osmotic lysis.

Figure 5: Osmotic competence of erythrocyte membrane at various buffer conditions [89].

2.2.6 RBC carriers - Mechanism of Encapsulation

Several methods have been reported for the encapsulation of drugs and other bioactive agents. Erythrocytes ghosts can be obtained by diverse procedures such a

hypotonic dilution, hypotonic pre-swelling, osmotic pulse, hypotonic hemolysis, dialysis, electroporation and chemical methods [87]. Of all the methods, hypotonic dialysis is the most frequently used encapsulation methods. The reasons for it are the simplicity, ease of application for a large number of drugs, enzyme and other substances because this method conserves well the morphological and hematological properties of the erythrocyte ghosts. Hypotonic dialysis is based on the exposure of red cells to the action of a hypotonic buffer, inducing cell swelling and formation of pores that permit the drug to enter the erythrocytes by means of a passive mechanism.

2.2.7 Therapeutic application of EGC carriers

Erythrocyte ghosts are obtained from fresh erythrocytes coming from human blood or different animal species such as rat, mouse, rabbit that are loaded with different types of bio actives such as drugs, peptides, enzymes using different encapsulation methods. For more than thirty years, many studies both *in vitro* and *in vivo* have been carried out to explore the use of erythrocyte ghosts as delivery systems for drugs, proteins and peptides allowing sustained release into the systemic circulation and delivery of these substances to into the reticuloendothelial system (RES) [81].

The potential therapeutic application of erythrocytes as drug carriers covers a wide spectrum of pharmacological biomolecules. Erythrocytes ghost carriers have shown promising results in different types of therapeutic applications. RBCs encapsulated with dexamethasone demonstrated a sustained release *in vivo* and an increased inflammatory response in humans and rabbit [90]. Anti-infective agents such as gentamicin, nocodazole, metronidazole, primaquine were also tested for encapsulation in ghost carriers, and *in vivo* studies using gentamicin encapsulated carriers have demonstrated a sustained release behavior [91]. Erythrocytes were also exploited for the applicability to load biodegradable esteric prodrug. After encapsulation these prodrugs are converted to the parent drug inside the erythrocyte membranes in a controlled fashion that in turn controls the release of the diffusible drug [92].

2.2.7.1 Protein carriers

Erythrocytes have been extensively studied in terms of peptide and protein delivery. For example, enzyme replacement therapy is widely used in different diseases states (e.g. galactosemia, hyperuricemia etc.), for either as detoxification of some compounds or a drug as such. However direct injection of these compounds into blood

stream face several shortcomings such as short plasma half-lives, toxicity, immunologic and allergic reactions in some cases and the need for multiple injections [93].

2.2.7.2 Enzyme replacement therapy

Carrier erythrocytes encapsulated with μ -glucocerebrosidase have been reported for successful treatment in Gaucher's disease [94]. Erythrocyte carriers encapsulated with asparaginase have been successfully used in the treatment of acute lymphoblastic leukemia in pediatrics [95]. The asparaginase encapsulated in the erythrocyte ghost carriers (EGC) exhibited proteolytic activity against asparagine, that is vital for all the neoplastic cells and thereby reduce the remission of neoplasms. Several studies have reported the encapsulation of other enzymes such as hexokinase, alcohol dehydrogenase, uricase, urokinase, urease, brinase, lactate 2-mono oxygenase etc. [87].

2.2.7.3 Acquired Immune Deficiency Syndrome (AIDS)

As mentioned previously RES system appears to be highly desired target for erythrocyte carriers. The application of carrier erythrocytes for delivery of anti-HIV peptides constitutes one of the most successful applications of this carrier system. The monocyte-macrophage system plays an important role in the viral pathology. The cells of the RES system are the first to be affected after the onset of viral infection and after exposure; they are relatively resistant to virus attack and constitute an important pool of reservoir for the virus. Therefore it would be of maximum interest to design a therapeutic system, which is capable of delivering the anti-HIV peptides such as azidothymidine (AZT) and didanosine (DDI) directly targeted to RES system. Since the carrier erythrocytes had the inherent property of RES targeting they were further explored for applications in an animal model. Carrier erythrocytes containing anti-HIV peptides (AZT and DDI) significantly reduced the pro-viral DNA content in macrophages compared to the administration of free peptides in a murine AIDS model [96].

2.2.7.4 Tuberculosis

Another serious infection that usually accompanies HIV-virus is the mycobacterium infection caused by *Mycobacterium avium* (MAC). The MAC is similar to the HIV that finds macrophages as the host system. In order to eradicate the MAC, the drug ethambutol must penetrate the macrophages and the bacterial cell wall. The poor half-life of the free drugs that requires repeated administration, has paved way for the development of alternate drug delivery systems for such complex applications. Carrier

erythrocytes containing prodrugs of the controlled release of AZT and ethambutol have shown excellent antibacterial and antiviral activity under *in vitro* conditions, that demonstrates the versatility of the system [97]. Several other compounds that have been successfully used in conjunction with erythrocyte carriers for macrophage targeting includes dideoxycytidine (ddC), a nucleoside analogue of HIV [98], 9-(2-phosphonylmethoxyethyl) adenine (PMEA) that exhibits antiviral activity against retroviruses, including HIV and herpes virus. This herpes virus is usually accompanied as an opportunistic infection coupled with HIV, and hence a system to target macrophages with both the viruses was of great interest. Carrier erythrocytes encapsulated with PMEA was found to inhibit the activity of both HIV and herpes virus [99].

2.2.7.5 Anti-neoplastic agents

Erythrocytes have also been successful in the delivery of anti-neoplastic agents such as 2-fluoro-ara-AMP (fludarabine) used in the treatment of chronic lymphocytic leukaemia [100] and 5-Fluoro-2'-deoxyuridine 5' monophosphate (FdUMP), a prodrug of 5-fluoro-2'-deoxyuridine (FdUrd) used to target liver metastases in colon carcinomas [100, 101]. These drugs were successfully encapsulated in the erythrocytes and they demonstrated a slow sustained release behavior, which is highly desired for respective applications.

2.2.8 Erythrocytes as multifaceted carriers

Several other drugs of diverse nature have been employed in erythrocyte carriers such as (i) erythropoietin used in certain forms of anemia, which when encapsulated in erythrocytes demonstrated higher stability and slow release behavior [102]; (ii) heparin-loaded carrier erythrocytes used in the prevention of thromboembolism showed prolonged half-life in human and canine models [103]; (iii) dermaseptin S4, that exhibits high antimicrobial activity with reduced hemolytic activity when attached to the erythrocyte membranes to target the bacterial membrane showed potent anti-bacterial activity against *Plasmodium falciparum* [104]; (iv) interleukin-3 that regulates blood-cell production by granulocytes and macrophages [105]; (v) antisense oligonucleotides [106] and (vi) vaccines [107].

Apart from the encapsulation of drugs, erythrocytes have also been explored for other biomedical applications such as (i) oxygen delivery [108]; (ii) paramagnetic

erythrocytes by co-encapsulation of drug (ibuprofen) coupled with ferrofluids such as cobalt-ferrite; (iii) magnetite for targeted delivery guided by external magnetic field [109]; and (iv) encapsulation of MRI contrast agents such as gGadolinium-DTPA that exhibited prolonged tissue enhancement compared to the free form [110].

2.2.9 Drug release and surface modification

The *in vitro* release behavior of the drugs encapsulated in erythrocytes demonstrated different release profiles, which is solely governed by the nature of the cargo molecules. The size and polarity of the drug are considered to be major determinants of the drug release parameters. The *in vitro* release profiles of certain substances encapsulated demonstrated first-order release behavior, which suggests that the molecules permeate through the erythrocyte membranes by passive diffusion [90, 98], whereas in certain cases the drugs do not follow simple diffusion rather membrane lysis. In certain conditions, the drugs can be adhered to the membranes and hemolysis forms a prerequisite for the release kinetics [111]. Moreover zero-order release was observed from carrier erythrocytes encapsulated with enalaprilat [112], whereas rabbit erythrocytes encapsulated with insulin revealed bi-exponential release kinetics demonstrating a complex release process. These results inevitably show that the release behavior is determined by the type of cargo molecule and to a very little extent on the carrier by itself. Nevertheless the carriers were able to sustain and control the release in a slow time dependent manner and prolong the half-life as compared to free drugs; which is highly desired in many therapeutic applications.

2.2.10 Advantages of RBC carrier system

Some of the advantages unique to the RBC carrier systems are mentioned here, that has been instrumental in the rapid advancement of the field. These include

- Biocompatibility and biodegradability
- Inert intracellular environment
- Uniform size and shape
- Longer life-span and increased plasma-half life
- Circulate throughout the body
- Possibility of targeted delivery to RES
- Versatility of encapsulated drugs

Moreover from a therapeutic point of view, the erythrocytes have been successful in diverse applications, such as treatment of lysosomal storage diseases targeted to the RES system [94], treatment of hepatic tumors with antineoplastic agents [101], parasitic infections that includes antibacterial [104], antileishmanial [113] and antiamoebial [114], anti-retroviral therapy [96], treatment in thalassemia for over accumulation of iron by administration of iron chelating agent desferrioxamine (DF) [87] and few other applications.

2.2.11 Surface modification of ghost carriers

Few methods have been demonstrated towards the surface modification of the erythrocyte carriers towards controlled release, of which the membrane cross-linking with glutaraldehyde (GA) represents the most promising till date [115]. Membrane crosslinking with GA increases the membrane stability and their osmotic resistance and hence the resistance to turbulence is increased considerably, which lowers the drug release into the bloodstream. Chemical alteration of the other membranes with substances such as ascorbate/ Fe^{2+} , diamide or band-3 crosslinking agents was found to increase the phagocytic uptake by macrophages [116]. In addition modification of the erythrocyte surface using trypsin, phenyl hydrazine and NHS-biotin was found to increase the phagocytic uptake both *in vitro* and *in vivo* [117, 118]. The other methods of surface modification include pretreatment of carrier erythrocytes to thermal shock, agents reactive to sulfhydryl containing portions of the membrane [119], treatment with neuraminidase have been exploited to increase the RES targeting of the erythrocyte carriers. To summarize, the only surface modification to control the release of bioactive release is membrane crosslinking using glutaraldehyde, whereas the other type of modifications to the membrane predominantly increase their phagocytic uptake, as the antigenic sites on the RBC membrane are altered and thereby unable to evade the immune system.

2.2.12 Drawbacks of RBC carrier system

Although the erythrocytes demonstrated exceptional capabilities for drug delivery applications, there are certain aspects that are yet to be investigated in detail for further advancements. First of all, rapid leakage of certain encapsulated substances and uncontrolled release of cargo molecules are reported, that requires further engineering of these carriers. The solution for this issue could be resolved by introducing controlled release of the cargo molecules by subtle carrier modifications. Controlled release

technologies provide continuous delivery of therapeutic compounds for prolonged durations (hours, days or weeks) depending on the application. Controlled release has been developed in the field of pharmaceuticals to address drug formulation problems arising from drug instability, short *in vivo* half-life, the need for local administration due to systemic toxicity and/or need for high local concentrations, highly potent drugs which exhibit severe peak-concentration related side effects etc.

The most successful methods to achieve controlled release from RBC carriers till date is based on glutaraldehyde cross-linking and no other method have been successful despite being researched for over 30 years. In order to address these drawbacks, we sought to introduce the layer-by-layer technology as a surface modification technique to study the feasibility of controlled release from functionalized erythrocytes. The feasibility of layer-by-layer coating on erythrocytes have been reported almost a decade ago [3], where erythrocytes were merely viewed as a biological template.

Apart from the surface modification methods explained above, the actual hypotonic treatment itself to which erythrocytes are subjected to during encapsulation leads to some membrane changes, which increases their recognition by macrophages. This remains to be another major disadvantage the carrier system, that they are rapidly removed by the RES system. Although this expands the capability of drug targeting to RES, any modification of the membranes leads to rapid clearance by the RES system. The amount of research that focuses on non-RES targeted delivery system is significantly much less as compared to RES systems [87]. However this study is focused towards surface modification using LbL towards controlled release. Once this platform is established, the multilayers could further be engineered to avoid the RES uptake, which is outlined in chapter 7.

One approach recently introduced by Fujimoto and co-workers is to use loaded liposomes as a template core for the LbL deposition [120, 121]. Here, we utilize red blood cells (RBC) ghosts as the template core to fabricate LbL microcapsules. Due to the biocompatibility and biodegradability of RBC, the application of erythrocyte ghost as biological carriers may provide an interesting alternative to other carrier systems such as liposomes or polymeric particles, as there is no toxic compounds released following carrier degradation. Consequently, pre-loaded vesicles could have some advantages as cores for forming LbL microcapsules, which would also eliminate the need to dissolve the core.

In the study reported in chapter 4, we utilized erythrocyte ghosts as the template core to fabricate LbL microcapsules. These vesicles have several potential advantages as template cores for the preparation of LbL microcapsules such as their uniform shape and high mono-dispersity. In brief, erythrocyte ghosts were loaded with lysozyme (14,700 Da) as model compound via hypotonic dialysis [122]. Due to the soft and fragile nature of erythrocyte ghosts, they were chemically stabilized via glutaraldehyde fixation prior to initiating the LbL adsorption. These microcapsules were then characterized in terms of morphology, loading efficiency and release behavior. Following the initial studies, we further refined the carrier system by modifying the method of encapsulation. The new method was hypothesized to have higher loading efficiency and hence the same compound lysozyme was used in further studies as a comparison between the two methods. In addition, we also studied the biological activity of the encapsulated protein, to investigate the fate of the biomolecules after the encapsulation procedure.

2.3 Gene delivery

2.3.1 Introduction

Gene therapy offers a promising alternative for the treatment of genetic disorders. A genetic disorder is a disease caused by abnormalities in individual genetic material (genome). Genetic diseases in human can occur due to a multitude of factors ranging from mutation or deletion of genes impairing the normal metabolic pathways, ligand/receptor function, regulation of cell cycle, structure and function of cytoskeletal or extracellular proteins. The availability of immense information on the complex molecular and cellular mechanisms of these genetic diseases has paved way for the advent of gene therapy. The basic principle of gene therapy is that exogenous genes are delivered to replace defective genes in diseased cells. The course of action of gene delivery to the cells proceeds through the general pathway: (i) formation of DNA containing particles, (ii) uptake into the cell, entrance of the particles into the cytoplasm, (iii) transport of intact DNA to the nucleus and (iv) finally the expression of the delivered gene. The delivery process may fail at any one of the steps, which impairs the transfection efficiency.

2.3.2 Carriers for gene delivery

2.3.2.1 Viral vectors

Generally two different approaches were explored for the delivery of nucleic acids in gene therapy: viral and non-viral vectors. Viral vectors, with their natural ability to

evade the biological barriers have demonstrated excellent transfection abilities over a wide range of cellular targets compared to their counterparts [123]. Although viral vectors demonstrates exceptional properties that enable high transfection they are limited by severe drawbacks such as insertional mutagenesis, immunogenicity and potential oncogenicity [124]; limiting their use in clinical applications. To overcome the disadvantages associated with viral vectors, research has focused on alternative carriers based on non-viral platforms.

2.3.2.2 Non-viral vectors

Development of non-viral gene transfer techniques has been tremendous over the past, particularly the use of several kinds of cationic lipids and cationic polymers such as poly-lysine derivatives, polyethyleneimines, polyamidoamine, dendrimers and so on, which electrostatically form a complex with the negatively charged DNA that can be taken up by the cells. In the field of non-viral vectors, carriers such as lipoplexes and polyplexes, which are colloidal suspensions or complexes of DNA, condensed with cationic lipids and polymers demonstrate good transfection efficiencies.

Although these complexes are highly efficient for *in vitro* transfection, they are limited by several factors under *in vivo* conditions. Some of the major drawbacks of the system are aggregation of complexes, which is caused by the presence of excess positive charge on the DNA-polycation complex reducing the colloidal stability due to the interaction with negatively charged serum proteins [125]; non-specific uptake; poor targeting ability and cytotoxicity [126]. This low *in vivo* efficacy is attributed to the differences in the biology, functionality and complexity between cell cultures and animal models as well as their interaction with cells and biological fluids. The complexes should be delivered exclusively to the target site, where it is subsequently taken up and processed on the cellular level. Taken together, an ideal gene delivery vector should have the following attributes; good stability, biocompatibility, targeting ability, protection of the DNA from extracellular degradation and high transfection efficiency; preferably, these vectors also sustain the delivery of the DNA over several days.

2.3.3 LbL carriers for gene delivery

There has been considerable effort over the past few years in exploring novel carriers to overcome the issues associated with the present drug delivery systems. The layer-by-layer (LbL) technology has recently emerged as an interesting platform for the

development of carrier systems towards various drug delivery applications [127]. The LbL technique is based on the consecutive adsorption of oppositely charged polyelectrolytes on a charged template core [79]. This methodology allows a versatile design for carrier functionalization with active agents in the core and in the multilayer shells; moreover the multilayers can protect the active agents against undesired extracellular degradation. In addition, targeted delivery can be achieved via surface modification of the shells with antibodies [128], polyethylene glycol [129], lipids [130]. This multifunctional design of carrier system enables them to be used in a wide range of applications ranging from controlled drug delivery [131], gene therapy [15], molecular sensing [132, 133].

Furthermore the LbL technology has been promising in tailoring carriers for intracellular applications. In our own previous work as well as that of others, LbL carriers have been used to sense intracellular pH using stimuli responsive dyes [132, 134], deliver anticancer drugs [135] and to deliver proteins for antigen presentation [136, 137]. However in terms of gene therapy, LbL technology is still at the initial stages of development as compared to the delivery of protein or chemical drugs, owing to the numerous barriers. On cellular internalization, the carriers have to escape lysis in the phagosomal/endosomal compartments, reach the cell cytoplasm and finally deliver the DNA to the nucleus.

2.3.4 Previous attempts on LbL carriers for gene delivery

A few initial attempts have reported the potential of LbL carriers in gene delivery; however the studies were mainly focused on the fabrication of DNA encapsulated LbL carriers. The polyionic nature of DNA enables them to be loaded either as a polyanion in the multilayer shell [15, 138] or in the template core [139]. The disulfide-stabilized hydrogen bond-based multilayer capsules on silica templated particles reported by Caruso *et al* have also been reported for encapsulation of nucleic acids such as oligonucleotides, single stranded DNA, double stranded DNA and plasmid DNA (pDNA) [140, 141]. The LbL carriers had high loading amounts and exhibited controlled release patterns with respect to external physiological conditions [142, 143] and even at lower pH [142] to mimic endolysosomes. One major limitation of the carriers is that the intracellular behavior of these capsules is not yet reported.

To date only two studies have reported the biological activity of the carriers at the intracellular level. Reibetanz *et al* demonstrated the successful delivery of DNA from

silica micro particles coated with protamine and dextran sulfate multilayers [15]. In our previous study we employed CaCO_3 templated particles coated with DNA multilayers along with pH sensing functionality to determine the intracellular fate of the ingested particles. The particles were able to deliver the pDNA but however the transfection efficiency was significantly low as compared to LipofectamineTM in both the studies. The poor efficiency was partly attributed to the micron-sized particles which are entrapped in the endolysosomes [132]; other factors which influence the intracellular trafficking are yet to be investigated. However these studies served as pioneering proof-of-principle studies for LbL carriers in gene delivery and further directed us to design carriers with high efficiency. Taken together, the currently applied designs for the transport of nucleic acids across biological membranes with LbL coated carriers are still far from being efficient at the targeted cellular delivery.

Towards this objective, this study seeks to identify the key parameters that govern the intracellular fate of the LbL carriers and tune them for successful gene delivery applications. In our present study we scaled down to nanosized templates, for which hydroxyapatite nanoparticles (<200nm) were chosen. Hydroxyapatite nanoparticles have the advantage of high binding affinity to a variety of biomolecules; these particles have been explored in the delivery of genes [144], proteins [145] and various drugs [146] due to their excellent biocompatibility [147, 148]. The study reported here is the first attempt to employ HA nanoparticles for LbL assembly. Future studies will address the possibility of multifunctional carrier agents using core and shell functionalized nanoparticles.

Our first objective was to study the possibility of functionalizing these carriers with layer-by-layer coated polyelectrolytes to be used as nanocarriers. The carriers were functionalized with nucleic acid (plasmid DNA) and model proteins (BSA and lysozyme). The cellular uptake and the intracellular processing of the LbL coated nanoparticles was the focus of our investigation as many of the particulate systems fail to overcome the complex intracellular environment to deliver the active molecules. Therefore to get insights into the intracellular fate and associated mechanism, pDNA encoding for reporter fluorescent protein was chosen and studied as a function of two different multilayer pairs; pARG and PLL. The incorporation of pDNA with two different multilayer pairs allowed us to investigate the transfection efficiency as a function of the layer materials.

The second objective was to investigate the intracellular trafficking of this system in order to identify the molecular pathways associated with the uptake and intracellular

processing. To elucidate the mode of cellular uptake, we first compared the effects of different metabolic inhibitors blocking different types of endocytic pathways and effect of temperature if the uptake followed an energy independent process. The defoliation pattern of the multilayer shells was studied by incorporation of the pDNA at different layer positions. To summarize, the study encompasses the fabrication; functionalization and mechanistic investigations of LbL coated HA nanoparticles towards intracellular drug delivery applications

2.4 Targeted drug delivery

2.4.1 Introduction to targeted delivery

Targeted drug delivery has been classified into two major subgroups, active and passive targeting. Active targeting aims to increase the delivery of drugs to target by utilizing specific interactions at the target sites where pharmacological activities are required [149, 150]. The specific interactions include antigen-antibody, ligand-receptor, and cell/tissue specific signal markers. In addition physical signals such as magnetic field, temperatures that are externally applied to the target sites are utilized for active targeting. In active targeting specific ligands (e.g. anti-bodies, peptides, polysaccharides, aptamer), are coupled to the surface to enhance the interaction of these carriers with the specific target cell to allow a localized delivery within the intracellular compartments such as cytoplasm, nucleus etc. [149, 150].

On the other hand passive targeting is focused to prolong the circulation times of these colloidal carriers in circulation and selective target localization [149, 150]. In particular, it is the methodology to increase the target/non target ratio of delivered drugs by adjusting physical and chemical properties of carriers to physiological and histological characteristics of the target and non-target tissues, organ and cells [149, 150].

2.4.2 Challenges in passive targeting

After intravenous (IV) administration, all colloidal systems, indeed, dramatically interact with plasma proteins, especially with immunoglobulins, albumin, the elements of the complement activation pathway, fibronectin, etc. [151]. The process of opsonization is a crucial parameter in dictating the subsequent fate of colloidal particles.

2.4.3 Mononuclear phagocyte system (MPS) – a major barrier to passive targeting

The propensity of macrophages for rapid endocytosis of the particulate drug carriers has been a challenging task in the field of particulate technology. In cases where the monocytes and macrophages in contact with blood are not the desired target, it's a huge challenging task to overcome the phagocytic barrier imposed by the immune system. Following intravenous injection of the of particulate systems, they are rapidly cleared by the liver (Kuppfer cells) and spleen macrophages from the blood circulation [152]. This site specific but passive mode of clearance is a facet of the immune cells' primary scavenging role for particulate invaders and self-effete products. The current stealth technologies employed in particulate systems to evade the mononuclear phagocyte system (MPS) are only able to delay the phagocytic uptake. Therefore the advent of more sophisticated design methodology is needed in the future design of carriers to avoid the MPS uptake.

Figure 6: Barriers to the intravenous delivery of particulate systems in drug delivery applications [151].

The particles have to overcome the five major barriers in order to reach the target region (fig. 6). These include hemolysis, which involves lysis of the red blood cells in the circulation by non-specific interaction with the RBC membrane, thrombogenicity, which involves platelet activation, and the sequential steps, which are highly undesirable. Considerable attention has been paid to these two parameters mentioned above and

further design was carried out with materials that were blood compatible, i.e. absence of hemolytic and thrombogenic activity. However the more serious and inevitable steps were plasma protein adsorption and complement activation, which often leads to the enhanced opsonophagocytic uptake by the MPS system.

2.4.4 Surface modification- a tool to evade the MPS system

The successful approach for prolonging the circulation of colloidal particles in the blood stream is to create a steric barrier to evade the immune system. The suppression of nonspecific interactions with the defense system i.e. decreased interactions with blood components (opsonization) inducing activation of the complement system, leads to reduced blood clearance of drug carriers and conjugates, which is known as the ‘stealth’ effect.

There is a vast array of nano/micron scale particulate technologies such as liposomes, polymeric micelles, colloidal particles, dendrimers, gold particles, quantum dots, solid lipid nanoparticles etc. that are capable of delivering drugs in different applications. Predominant research using these particulate technologies have been widely limited to studies on physicochemical properties, drug loading, cytotoxicity tests and relatively simple *in vivo* tests. Further down, surface modification of the carriers was envisioned as an additional functionality that was often necessary to control their properties in a desired fashion. However the surface modification was directed much towards active targeting, which includes targeting of cell specific receptors, ligands within the confined perspective of intracellular delivery as the main goal. On the other hand, the surface modifications towards passive targeting include longevity and stability in the circulation, alteration of bio distribution, targeting effect; stimuli (pH or temperature sensitive) and contrast properties were significantly less owing to the challenging barriers.

Towards this goal, attention was given to focus on the surface composition of the colloidal carriers. The most prominent and early approach for long-circulation carriers was based on the natural red blood cells, using red blood cells as carriers. However with the rapid advancement and development of diverse particulate systems, alternate methods to induce prolonged half-life were developed, of which polyethylene glycol (PEG) based modification remains at the top of the list.

2.4.5 Advent of passive targeting

Passive targeting has received only considerable over the past as compared to active targeting which could be attributed to the lack of thorough knowledge on the underlying biological mechanisms. The main reason for this being the advent of monoclonal antibody that are highly specific to the target cells and demonstrated possibilities of drug conjugation for targeted delivery applications. However the limitations of monoclonal antibodies were realized only during the translation of *in vitro* to *in vivo* studies, where numerous biological barriers and non-specific uptake has to be accounted for. The specificity of antibody towards the target tumor cells in an *in vitro* model was not comparable to the tumor accumulation of antibody-drug conjugate in an *in vivo* model. This was then realized that the concept of target delivery is not a single step translation from *in vitro* to *in vivo* model; rather requires a complex design of the carrier system.

In this chapter our focus on the first part remains to be on the review of most successful synthetic approaches (based on polyethylene-glycol, PEG) taken towards passive targeting to prolong the circulation time of particulate systems such as polymeric carriers, liposomes etc. and their associated advantages and disadvantages. In the second part, the application of natural carriers based on red blood cells as long-circulating carriers will be introduced together with the advantages as well as advancements towards passive targeting. Finally the concept of using red blood derived vesicles is proposed as a parallel strategy to be used in synchrony with the colloidal LbL system towards the design of particulate system for passive targeting.

2.4.6 “State of the art” technology in passive targeting –PEG system

To prolong the circulation of nanoparticles by evading the immune system, surface modification with polyethylene glycol, a non-ionic hydrophilic polymer with ‘stealth’ behavior has been the gold standard till date [153]. The majority of conjugated drugs as well as liposomal and micellar formulations in therapeutic applications or the advanced clinical trials that are commercialized are PEG containing products.

2.4.6.1 Mechanism of PEG- formation of steric layer

The surface bound PEG chains are known to form a steric barrier around the particulate system that extend into the aqueous physiological environment and repel proteins, decrease antibody formation and increase the circulation time of the drug carrier

in the blood stream. The formation of an efficient steric hindering cloud on the surface of particles is not only dependent on the material composition, but is also influenced by other factors such as the molecular weight of PEG, the surface density, and the conformation of PEG molecules attached to the surface (e.g. brush-like or mushroom-like)[154]. PEG-modification is often referred to as PEGylation, a term that implies the covalent binding or non-covalent entrapment or adsorption of PEG.

PEG has minimal interfacial free energy with the water molecules and hence high hydrophilicity, high surface mobility and steric stabilization effects. In addition the molecular conformation of the PEG surface groups in water forms a dense distribution of oriented molecular pattern that prevents protein adsorption [155]. Moreover, the water molecules in contact with the PEG surface are hydrogen bonded to the ether oxygen of PEG thereby forming a protective steric barrier around the particles.

2.4.6.2 Significance of PEG system

PEG also offers the advantages of being certified by United States Food and Drug Administration (FDA) for human use in therapeutic application [153]. Polyethylene glycol (PEG) is a hydrophilic and uncharged linear polyether diol ($\text{HO}-(\text{CH}_2-\text{CH}_2-\text{O})_n-\text{H}$ where n represents the degree of polymerization). The stealth function of PEG is mainly attributed to the formation of a dense, hydrophilic cloud of long flexible chains on the surface of the colloidal particle that reduces the hydrophobic interactions with the RES. Many reports support the pharmacokinetic improvements achieved with the PEGylation of nanocarriers, however there are some studies that have reported the undesirable side effects associated with PEG system.

2.4.6.3 PEGylated particulate carriers

Liposomes were the first particulate carriers to be functionalized with the PEG. The PEGylated liposomes, that were 50-300 nm in diameter were able to achieve a circulation time of 45 h as compared to conventional liposomes with life times of few hours[156]. PEGylation increased the plasma-half life of the carrier that enabled drug delivery to the desired tissues and organs and could successfully evade the MPS system. Initial studies using drug encapsulated liposomes showed increased circulation times of peptides and proteins such as interleukin-2 [157] and vasopressin [158]. Following successful outcome with liposomes, the PEG system was translated to the polymeric particulate systems with surface display of PEG chains for stealth behavior. This

approach eventually turned out to be successful widening the prospects of PEG for further applications, which had made them the most desired agent towards passive targeting till date [153]. Beside PEG, several other polymers were also considered for passive targeting such as semitelechelic poly(N-2-hydroxypropyl) methacrylamide)s (ST-PHPMA)[159], synthetic polymers of vinyl series such as polyacrylamide (PAA)[160], poly(vinyl pyrrolidone) (PVP) [161], polysialic acids [162], heparin [163], dextran [164] that showed long-circulating properties. However the studies pertaining to these polymers were significantly less, inconclusive and were not as successful as the PEG system.

2.4.7 Disadvantages of PEG system

While PEGylation has improved to prolong the circulation lifetime of particulate carriers, it has several limitations that includes the adverse immunological reactions and associated side effects.

Several researchers have reported the immunological response and fast clearance of PEGylated liposome following repeated doses. The long circulation capacity of PEGylated liposomes are significantly altered following repeated intravenous doses mediated by specific antibodies directed against the PEG, leading to the accumulation in liver [165]. Kiwada *et al* reported that the injection of PEGylated liposomes in rats elicits PEG-specific IgM that favours the rapid elimination of a second dose of PEGylated liposomes. A detailed analysis of the underlying mechanism towards the antibody response showed that the splenic B-cells secrete anti-PEG IgM in a T-cell independent (TI) manner. The PEG has a repeating O-CH₂-CH₂ subunit in the structure, which is typical of to the TI antigens found on highly repetitive structures, such as capsular polysaccharide [166]. A similar behavior was observed in other types of PEGylated particulate carriers such as polymeric micelles [167] , solid lipid nanoparticles(SLN) and polymeric nanoparticles [168].

Chemical modification of proteins with PEG constitutes another class of therapeutic system, which is designed to take advantage of the PEG stealth layer to increase the circulation half-life of the therapeutic protein. Ganson et al reported a similar type of immune response with activation of IgG and IgM antibodies in patients treated for chronic gout with PEGylated-uricase [169]. Similarly, Armstrong et al reported the rapid clearance of PEGylated-asparaginase due to production of anti-PEG antibodies in leukemic patients [170].

Although PEG was initially considered to be non-immunogenic in nature, further studies reported hypersensitive reactions along with rapid clearance of PEGylated liposomes for the delivery of oligodeoxynucleotides [171]. Other carriers that are known to cause allergic reactions include PEGylated liposomal doxorubicin in humans [172] and pigs [173].

2.4.8 Natural vs. synthetic system

From the above discussion it is very clear that the PEG system has several potent disadvantages and hence the advent of new carrier systems are necessary. The reason for the advancement in the PEG system till date is mainly due to the lack of a equivalent competitor on par with PEG in evasion of the MPS system. The other system with equal advantages comprises the red blood cells and their derived carriers. The following section discusses the approaches and advancement in the design of carriers based on red blood cells for passive targeting.

A detailed review on the application of red blood cell carriers has been discussed in section 2.2. Hence in this section we will introduce the design methodology and types of red blood cell derived carriers and other interdisciplinary approaches based on red blood cells. The potential advantages of the following carriers in terms of pharmacokinetic and immunogenic profiles are detailed in the discussion of this chapter.

2.4.9 RBCs: A natural passive targeted carrier

Healthy erythrocytes evade the macrophages of the immune system and fulfill their function of transporting oxygen with a life span of 110 to 120 days. A multitude of physicochemical and physiological factors are believed to control the life span of red blood cells. These include surface characteristics (e.g. surface charge, membrane phospholipid composition, surface antigens) as well as bulk properties (e.g. shape and their extent of deformability). Nevertheless the circulation lifetime of RBC carriers have always been higher as compared to the synthetic systems. Moreover the RBC carriers are non-immunogenic, although the membrane changes induced during the encapsulation procedures significantly alters the lifetime that facilitates RES mediated clearance.

The circulation lifetimes of various carriers in mice is shown for the sake of consistency of the comparison (data for humans are not available for many drug delivery systems).

Table 1: Circulation time of various drug delivery carriers used for passive targeting applications [174].

Drug delivery carrier	Size (nm)	Shape	Half-life in blood
RBC	5,000–7,000	Biconcave disc	10–15 days
PEG-Liposomes	50–500	Spheres	3–6 hours
Polymersomes	50–500	Spheres	10–20 hours
Polymer micelles	20–300	Spheres	0.1–6 hours
Proteins and conjugates	5–5,000	Irregular spheres	10 min – 6 hours

It could be clearly observed that the RBC carriers have a circulation half-life, which is much higher as compared to the polymeric systems, owing to the natural endogenous property. However due to a lack of scale up facilities and automation technologies, their presence in clinical trials were hampered significantly. Apart from the natural carriers, some other approaches using the inherent properties of RBCs have been envisaged for long circulating capabilities. These include nanoerythrocytes (nE), membrane vesicles, hitchhike nanoparticles and RBC membrane camouflaged nanoparticles.

2.4.10 Nanoerythrocytes- derivative of RBCs

2.4.10.1 Design of nanoerythrocytes

Nanoerythrocytes (nE) are nano-vesicles prepared from erythrocyte membranes with a size of 100 nm in diameter. Nanoerythrocytes are prepared by three methods extrusion, sonication, and electrical breakdown. Of all the three methods, extrusion was found to be optimal that yields highly uniform vesicle formation. They are prepared by consecutive extrusion of erythrocyte ghosts under nitrogen pressure through polycarbonate filter membrane to produce nano-sized vesicles with an average diameter of 100nm (fig. 7). In an approach to study the efficacy of nanoerythrocytes as drug carriers, daunorubicin (DNR) conjugated nanoerythrocytes were tested for their anti tumor effect.

Figure 7: Preparation of nanoerythrosomes (nE) from RBC. (a) Photomicrograph of normal erythrocytes (b) photomicrograph of erythrocyte ghosts, and (c) SEM of drug-loaded nEs [175].

It was found that daunorubicin conjugated to nanoerythrosomes was found to have higher antineoplastic index than the free drug and increased plasma half-life [176].

2.4.10.2 Pitfalls of nanoerythrosomes

Although the nEs linked with drug (DNR) exhibited a higher anti-neoplastic index, their pharmacokinetic profile was a big downturn. *In vitro* studies have shown that the nanoerythosome-daunorubicin complex is rapidly adsorbed and phagocytized by macrophages [177]. A similar profile was reflected under *in vivo* conditions; following intravenous administration, nanoerythrosomes (nE) were rapidly removed from blood circulation (< 30 min). Liver, spleen and lungs were the predominant organs for the accumulation of these vesicles [178]. The carriers with the nano-size range was expected to exhibit increased circulation half lives as compared to its precursor erythrocytes, however the entire scenario was different. The reason for this is mainly attributed to the procedures involved in the preparation of nE which involves high pressure extrusion under N₂, which might induce some irreversible membrane changes that is different from the RBC membrane.

2.4.11 Reverse biomembrane vesicles (RBVs) - derivative of RBCs

One another derivative of erythrocytes are the reverse biomembrane vesicles (RBV), prepared from erythrocyte ghost membranes. The vesicles were prepared by spontaneous vesiculation of ghost membranes using vesiculation medium that yield reverse bio membrane vesicles, i.e. an inverted membrane orientation as compared to the parent ghost membrane [179]. The vesicles were encapsulated with doxorubicin (Dox) as a model drug and the *in vivo* pharmacokinetic profiles were compared with the free drug and drug encapsulated in RBVs (Dox-RBV). Free doxorubicin (Dox) was cleared quickly from circulation, whereas the blood concentration of Dox-RBVs remained high

for a long period. The half-life of the drug was enhanced about 5.7 folds for Dox-RBVs and 3 times lower body clearance compared to free drug [179].

Following the initial successful results, further studies were carried out to study the therapeutic efficiency in a tumor model. In addition to the RBV-Dox vesicles, another carrier included the folate-RBV-DOX, which were prepared by attachment of folic acid, as targeting ligand. The folate receptors are overexpressed in tumor cells and hence folate was used as a targeting ligand for the RBVs. The antitumor activity of vesicles was studied in mice tumor model bearing murine leukemia L-1210. The cytostatic activity was assessed and found that IC_{50} (half maximal inhibitory concentration) was decreased to 3.2 and 2.1 times for folate-Dox-RBV and Dox-RBV respectively compared to free drug. Of all the three formulations the folate-Dox-RBV showed the highest cytotoxic activity followed by Dox-RBVs and the free drug [180].

Taken together the carriers demonstrated exceptional capabilities for tumor drug delivery, but till date there has been no follow up study reporting the bio distribution of the carriers and in-depth mechanistic investigations. Moreover there was no rationale for using the reverse membrane vesicles, with an inside-out membrane orientation compared to the parent RBC membranes.

2.4.12 Interface of particulate systems and RBC

2.4.12.1 Hitchhike nanoparticles

In a different approach to prolong the circulation lifetime of nanoparticles rather than by surface modification of the carriers, they were anchored to the surface of red blood cells (RBCs) by non-covalent attachment, termed as “hitchhike nanoparticles” (fig. 8).

Figure 8: Schematic illustration of hitchhike nanoparticles. The circulation lifetime of nanoparticles are increased by attachment of the particles to the RBC surface. (<http://www.sciencedaily.com/releases/2007/06/070627084654.htm>)

The strategy employed here is based on the pathogen-host interaction where some bacteria adhere to the surface of blood cells to evade the immune system. The particles were linked to RBC by non-covalent interaction, that includes ‘van der Waals’ force, electrostatic, hydrogen bonding, and hydrophobic forces between polystyrene particles and the RBC (fig. 9).

Figure 9: Electron micrographs of red blood cells carrying hemobartella or polystyrene nanoparticles. (a) RBCs carrying hemobartella (bacteria), as reference. The image is taken from (b) RBCs carrying 220 nm polystyrene particles. (c) RBCs carrying 450 nm polystyrene particles. (d) RBCs carrying 1.1 μm polystyrene particles [181].

The attachment of polymeric nanoparticles to red blood cells combines the advantages of the long circulating lifetime of the red blood cell, and their abundance, with the robustness of polymeric nanoparticles for multifunctional drug delivery applications. Although the approach was a breakthrough in terms of design, their pharmacokinetic profile *in vivo* was a big disappointment, which led to the downfall of the system and no further research using these carriers.

Particles of different sizes (220,450,800 and 1100 nm) were tested for the *in vivo* circulation behavior (fig. 9). The circulation time of the erythrocyte bound nanoparticles were found to depend on the particle size; of all 220 and 450 nm particles exhibited the longest circulation times. The time required for eliminating 95% of the injected dose of 220 nm and 450 nm diameter particles was greater than 7 h, whereas particles larger than 450nm were rapidly removed from circulation. This circulation profile is comparatively much lesser to the erythrocytes carriers as well as the PEGylated carriers. A detailed analysis on the mechanistic investigation behind the fast clearance revealed that the particles eventually detach from red blood cells due to shear forces and cell-cell interactions and are subsequently cleared in the liver and spleen [182].

2.4.12.2 Nanoparticles-camouflaged with RBC membrane

Very recently in 2011, a study reported the surface coating of nanoparticles with RBC membranes. The biomimetic approach here was particle functionalization by coating biodegradable polymeric nanoparticles (PLGA) with erythrocyte membranes, to prolong the circulation time of nanoparticles.

Figure 10: Schematic illustration of the preparation process of the RBC-membrane-coated PLGA nanoparticles (NPs) [183].

RBC ghost membranes were prepared from human blood, which was then subjected to sonication and membrane extrusion to yield 100 nm membrane vesicles [183]. Co-

extrusion of the preformed RBC derived vesicles with polymeric PLGA particles (70nm), resulted in particles cloaked with a lipid bilayer (fig. 10).

The pharmacokinetic profiles of the membrane-functionalized nanoparticles revealed superior circulation half-life as compared to control particles coated with the state-of-the-art synthetic stealth (PEGylated nanoparticles) materials, with particle retention in blood for 72h in a mice model. However, the particles employed were devoid of any drugs and hence there is no data available on the drug release profiles of the proposed carriers.

2.4.13 Concluding Remarks

From the above discussion, it is obvious that the amount of research based on blood cells for passive targeting is very less compared to the synthetic system despite having great potential. Herein we propose a carrier that combines the advantage of both colloidal system and natural biological system. For particle engineering, we have taken a lead from nature i.e. exploring membrane orientation of red blood cells that plays the major determinant role in circulation to evade macrophage recognition.

The first step involves the design of a surface coating material and the second step involves introduction of the material LbL coated multilayer shells. Here we have developed membrane vesicles with inside-out and right-side-out orientation and their respective characterisation steps. The further steps involve the study of the phagocytic uptake of the vesicles by monocytes in order to investigate the influence of membrane orientation on phagocytic uptake by MPS system. The last step involves the surface functionalization of the vesicles in the multilayers of LbL coated silica particles and analysis of the membrane orientation of the vesicles following coating.

Chapter 3: Fabrication and functionalization of CaCO₃ particles for protein delivery-therapeutic application in breast tumor

OVERVIEW

This study aims to design a delivery system for intracellular cathepsin inhibition and thereby control tumor progression. The first part focuses the selection of carrier for encapsulation of cystatin, following which their cellular interaction is studied. Herein we demonstrate the design of a drug carrier system, which was generated by the stepwise adsorption of oppositely, charged polyelectrolytes by LbL self-assembly on cystatin C loaded CaCO₃ particles. Following LbL assembly, CaCO₃ core was dissolved leaving behind cystatin loaded LbL microcapsules. The efficiency of the microcapsules for molecular suppression and selective therapeutic inhibition of cathepsin B was studied in breast tumor cells (MCF-10A neoT). Following cellular internalization, the intracellular activity of cystatin was studied using cat B specific fluorogenic substrate Z-Arg-Arg-MR and the cytotoxic effect by the MTT assay.

3.1 MATERIALS AND METHODS

3.1.1 Materials

Calcium chloride, sodium carbonate, poly-L-arginine hydrochloride (pARG) (>70,000 Da), protamine sulfate sodium salt from herring (5~10 kDa), lysozyme from chicken egg white (14.7 kDa), *Micrococcus lysodeikticus*, bovine serum albumin (BSA), chicken egg cystatin were purchased from Sigma Aldrich. Dextran sulfate sodium salt (36 ~ 70kDa) was purchased from MP Bio. Phosphate buffered saline (PBS) was used for all the experiments. PBS containing 137 mM sodium chloride; 2.7 mM potassium chloride and 10mM phosphate buffer was prepared from a 10x PBS stock solution.

3.1.2 Preparation and characterization of CaCO₃ particles

Calcium carbonate crystals were prepared by colloidal aggregation after mixing equal volume of 0.33 M calcium chloride and 0.33 M sodium carbonate. The mixing was carried out under vigorous shaking for 3 minutes to control the crystal growth within the size range of 5 -7 μ m. The solution was left for one minute for the crystal growth and washed to stop the crystallization. Subsequently, the particles were subjected to 3 washes with millipore water.

3.1.3 Characterization of CaCO₃ particles

The morphology of the micro particles was characterized using analytical scanning electron microscope (JOEL JSM-6390 LA). A platinum layer was first coated onto the sample surface using JOEL JFC-1600 auto fine coater operating at 20mA for 120 seconds and observed under the microscope

3.1.4 LbL coating of CaCO₃ particles

Protamine sulfate sodium salt (PRM) and dextran sulfate sodium salt (DXS) were dissolved in 0.22 μ m-filtered 0.1M NaCl to a final concentration of 3mg/ml. The particles were incubated in PRM solution for 10 minutes with gentle shaking by a thermo mixer (Eppendorf) for adsorption of polymer onto the particle surface. The suspension was then pelleted using a centrifuge (Eppendorf 5424) at about 5000 rpm at room temperature for 1 minute for micro particles. Following the removal of the polymer solution supernatant, the pellet was re-suspended in 0.1M NaCl to remove excess polymer not adsorbed to the particles. A brief vortexing was done to ensure total dispersion of particles in solution. Centrifugation with the same conditions was carried out to retrieve the pellet from the washing step. After three washing steps, the particles were then incubated in the next oppositely charged polymer solution. The preceding steps were repeated until the desired number of layers was adsorbed on the particles. For protein encapsulation in the multilayers, the polyelectrolyte solutions were replaced by the respective protein solutions and the supernatant collected after each coating step for protein quantification.

3.1.5 Core encapsulation of Lysozyme in CaCO₃ particles

Lysozyme was loaded in the core by the method of co-precipitation [14]. In this method 5 μ l of protein solution at a concentration range from 1~ 4 μ g/ μ l was added to 500 μ l of 0.33M calcium chloride followed by the addition of 500 μ l of sodium carbonate to initiate crystal formation. The mixing was carried out under vigorous shaking for 3 minutes to control the crystal growth within the size range of around 5 μ m. The solution was left for 60 seconds for the crystal growth and washed to stop the crystallization. Subsequently 3 washes were done with millipore water. The supernatants were collected during the washing steps and analyzed for protein by MicroBCA (Thermo scientific, USA) for quantification of the unencapsulated protein

3.1.6 Lysozyme activity assay

The bioactivity of the encapsulated protein, lysozyme was assayed using the conventional microbial assay [184]. *Micrococcus lysodeikticus* were cultured and grown in agar slants. The most important factor for influencing the cell growth is the choice of medium. The ingredients of the agar were 0.5% of peptone, 2% of agar by weight percent and remaining required volume of water is added to make complete growth medium. The cells were cultured in the agar plate by adding cell suspension to it. Cell suspension was prepared by dissolving strain (*Micrococcus lysodeikticus*) in M/15 phosphate buffer at the concentration of 1 mg/ml. The cells were grown in petridish for 48 hours at 37°C. The bacterium was extracted from the agar plates by washing with M/15 phosphate buffer and a uniform cell suspension was prepared. The cell suspension was diluted with buffer to an optical density of 0.75 to 1.0 at 600nm. The primary step is the preparation of standards with different concentration of enzyme (lysozyme) solution. 25 μ l enzyme solution is added to cell suspension. Once the enzyme solution is added to the cell suspension, it was stirred for uniform mixing of the enzyme and the substrate. Transmittance was read at 450nm for every 30 seconds interval for a total duration of 5 minutes. A standard graph was prepared with the change in transmittance at 450nm (ΔT) as a function of different protein concentrations. The activity of the protein release from the carriers was compared with native protein.

3.1.7 Preparation of cystatin doped CaCO₃ particles

Blank calcium carbonate crystals were prepared by colloidal aggregation after mixing equal volume (500 μ l) of 0.33 M calcium chloride and 0.33 M sodium carbonate. For cystatin encapsulation, 5 μ l of protein solution in a concentration range from 1~ 4 μ g/ μ l were added to CaCl₂ solution during the crystal formation. The mixing was carried out under vigorous shaking for 3 minutes to control the crystal growth within the size range of ~ 5 μ m. The solution was left for 60 seconds for the crystal growth and washed to stop the crystallization. Subsequently 3 washes were done with millipore water. The supernatants were collected during the washing steps and analyzed for protein by Micro BCA assay kit (Thermo scientific, USA) for quantification of the unencapsulated protein.

3.1.8 Preparation of polyelectrolyte microcapsules (PEMC)

Poly-L-arginine hydrochloride (pARG, MW > 70000 Da, Sigma Aldrich) and dextran sulfate sodium salt (DXS, MW 36,000-50,000 Da, MP bio) were used as polyelectrolytes

for the multilayer coating. The polyelectrolytes (pARG, DXS) were dissolved in 0.1M NaCl solutions at a concentration of 0.5 mg/ml. The coating was conducted with CaCO₃ particles at a working concentration of 1mg/ml in 1 ml of polyelectrolyte solution of positive pARG, or negative DXS. Incubation under gentle shaking was conducted for 10 minutes; after which centrifugation at 6000 rpm for 1 min at room temperature. Following incubation, three washings in 0.1M NaCl were performed to remove the non-adsorbed polyelectrolyte solutions. The LbL steps were repeated until the desired number of bilayers was deposited followed by core dissolution. The particles were dispersed in 0.2M EDTA solution for 15 minutes under gentle shaking followed by three washing steps to obtain the microcapsules.

The electrophoretic mobilities of the polyelectrolyte coated CaCO₃ particles were measured using a Zetasizer (Malvern zetasizer Nano, UK). The electrophoretic mobility was converted into the respective ζ -potentials using the Smoluchowski relationship.

Analytical Scanning Electron Microscope (JOEL JSM-6390 LA) was used to characterize the morphology of the fabricated microcapsules. A platinum layer was first coated onto the sample surface using JOEL JFC-1600 auto fine coater operating at 20 mA for 120 seconds, and observed under the microscope.

3.1.9 Tumor model

MCF-10A neo T cells (ATCC) that are ras transformed human breast epithelial cells were used as tumor model system. The cells were cultured in MEGM growth medium (Lonza) supplemented with cholera toxin (Sigma Aldrich) at a concentration of 100ng/ml at 37°C in a humidified atmosphere containing 5% CO₂. The cells were grown as a monolayer until 80% ~ 90 % confluence and prior to use in the assay, cells were detached from culture flasks with 0.05% trypsin-0.5mM EDTA solution and sub cultured. The viability of cells in the experiments were at least 95%, as determined by staining with trypan blue.

3.1.10 Immunocytochemistry

The localization of intracellular cathepsin B in MCF-10A neo T cells was visualized by immunochemical staining. Cells were grown on glass coverslips, fixed with methanol (-20°C) for 5 min, and permeabilized in 0.01% Triton X-100 in PBS, pH 7.4 for 10 min. Nonspecific binding was blocked with 3% BSA in PBS, pH 7.4 for 1 h. Cells were further incubated for 2 h with monoclonal anti-cathepsin B antibody (Sigma aldrich), washed with PBS, and incubated with goat antimouse secondary antibody labeled with FITC

(Sigma Aldrich), for 45 min. Controls lacked the primary antibody. Coverslips were mounted on chambers and visualized under confocal laser scanning microscope (Zeiss LSM510 META) at a laser excitation of 488nm(argon laser) to detect the fluorescence intensity distribution of FITC.

3.1.11 Internalization of PEMCs

The cellular uptake behavior of PEMCs by MCF10A cells was investigated using fluorochrome labeled microcapsules to enable particle localization. The CaCO_3 particles were encapsulated with fluorescent-labeled PAH (PAH^{FITC}) during the crystal formation followed by the LbL coating and core dissolution.

The coating pattern is as follows: $\text{CaCO}_3^{\text{PAH-FITC}}/(\text{pARG/ DXS})_3/\text{pARG}$

In order to visualize the cellular uptake at the intracellular level, the MCF-10A cells were incubated with particles at a ratio of 1:5 for 4 hours in transfection medium OptiMEM (Invitrogen). Following 4-hour incubation period, the cell membrane was stained with Cell mask Orange (Invitrogen) and observed under confocal microscope. A laser excitation of 488nm (argon laser) was used to detect the fluorescence intensity distribution of FITC emitted from the microcapsules and 543nm (helium/neon laser) to visualize the red fluorescence intensity of the stained cell membrane.

The cellular uptake profile of polyelectrolyte microcapsules was studied using FACS Calibur (Beckton Dickinson). Cells were seeded in 12 well plates (2.5×10^5 cells/well) and allowed to grow for 24h at 37°C. After cell attachment, the cells were treated with capsules at different concentrations (1:1 ~1:20) for 4h at 37°C. Following incubation, the cells were trypsinized and washed twice with Dulbecco's PBS and analyzed by FACS. 10 μ l of trypan blue (0.4%) was added to 300 μ l of cell suspension in order to quench the fluorescent signal from the carriers adsorbed on the outer surface of the cell. The fluorescence intensity of the FITC from the microcapsules is detected in the FL-1 channel that uses a 530/30nm band pass filter. The data (10^4 events) were acquired for forward scatter intensity, side scatter intensity and for fluorescence intensity (FL-1) in logarithmic mode. WinMDI software was used to analyze the results.

3.1.12 Intracellular proteolysis inhibition assay

The inhibition of cat B mediated by the cystatin C released from the PEMCs was visualized by a specific cell membrane permeable fluorogenic substrate, Z-Arg-Arg-MR

(MP bio). Enzymatic cleavage of one or both arginine residues by cathepsin B converts the molecule into a red fluorescent product. Cells were grown in an 8-well chambered cover glass system (Nalgene-Nunc, Labtek) for 24 h for initial attachment. Prior to the assay, the cell growth medium was replaced with transfection medium (Optimem). The cells were treated with cystatin-loaded capsules; in addition equivalent amounts of free cystatin and blank capsules (hollow core) were used as positive controls whereas untreated cells served as negative control. After overnight incubation, the culture medium was replaced with fluorogenic substrate (Z-Arg-Arg-MR, 10 μ M) and monitored for fluorescent product after 10 min under confocal microscope. A 516- to 555-nm single band filter was used for excitation and a 574- to 648-nm barrier filter for monitoring the red fluorescence.

3.1.13 Cell proliferation assay

The cytotoxicity of the PEMCs towards the tumor cells was determined by the 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) colorimetric assay. Viable cells (5×10^4), suspended in 200 μ l of growth medium were seeded in 96 well micro-titer plates containing PEMCs at different concentrations. Equivalent amounts of free cystatin and blank capsules (hollow core) were used as positive controls whereas untreated cells served as negative control. After overnight incubation at 37°C and 5% CO₂, 10 μ l of MTT (5mg/ml) was added to cells and incubated for 4 h. 100 μ l of dimethylsulfoxide (DMSO) was added to dissolve the formazan crystals formed. The absorbance of the solubilized crystals was measured at 595nm with UV spectrofluorophotometer microplate reader (Perkin Elmer Victor 1420, USA). Cell viability was expressed by Eq. (1) where $A_{\text{test cells}}$ and $A_{\text{control cells}}$ denotes the absorbance of formazan determined for cells treated with different concentrations of nanoparticles and non treated control cells, respectively:

$$\text{Cell viability (\%)} = (A_{\text{test cells}} / A_{\text{control cells}}) \times 100 \quad (\text{Eq. 1})$$

3.2 RESULTS AND DISCUSSION

3.2.1 Characterization of CaCO₃ particles

3.2.1.1 Morphology

Calcium carbonate particles were prepared by the principle of colloidal aggregation. CaCO₃ particles are biocompatible and biodegradable, inexpensive, easy to prepare. The porous structure of the micro particles enables encapsulation of bio-functional macromolecules allowing a local, low dose and time-controlled targeted drug delivery into specific cells and tissues. The morphology of the micro particles was examined by means of Scanning Electron Microscopy (SEM) and Field Emission Scanning Electron Microscope (FESEM).

Figure 11: Electron micrograph of CaCO₃ micro particles. The SEM image (a) depicts a size distribution of $5 \pm 7 \mu\text{m}$, (b) whereas single particle at a higher magnification (7500X) indicates the porous structure of the micro particles, (c) The Field Emission Scanning Electron Microscope (FESEM) image of a particle taken at 30000X further highlights the porous surface.

Calcium carbonate micro particles with a size distribution in the range of $5 \pm 2 \mu\text{m}$ were prepared (fig. 11a). The magnified FESEM image (fig. 11c) reveals a porous topology of the particles that enables encapsulation of macromolecules in the core by the method of co precipitation [14]. Calcium carbonate particles, which were monodispersed, uniformly sized, spherical and of the required size were further used for layer adsorption.

3.2.1.2 Zeta potential

The particles were coated with alternate layers of positive and negatively charged polyelectrolytes, which are held together by means of electrostatic interaction. The absolute value and nature of the surface charge of colloid is characterized by the ζ -potential measurement. The ζ -potential of a dispersion system is defined as the potential

between the tightly bound surface liquid layer of a dispersed particle and the bulk phase of the solution. LbL assembly on the CaCO_3 particles was tested by electrophoresis; a net charge reversal at each step was measured by the ζ -potential.

Figure 12: Zeta potential of CaCO_3 particles coated with PRM/DXS. The ζ - potential is plotted as a function of layer number. Layer number 0 corresponds to bare CaCO_3 particles. The odd layers correspond to PRM (polycation) deposition and the negative layers correspond to DXS (polyanion) deposition. The ζ - potential measurements were made in 0.01M NaCl at pH 7.4. The error bar represents standard deviation from three triplicate measurements.

The ζ -potential measurements show the stepwise layer-by-layer assembly of polyelectrolytes on CaCO_3 micro particles. The ζ - potential measurements of the bare CaCO_3 particles (fig. 12) point out a negative surface charge of -35mV; therefore the LbL assembly was started with positively charged polyelectrolyte. The odd layers represent the particles with cationic layers as the outer layer and the even layer number with DXS as the outer layer. A net reversal of surface charge between positive and negative values is evident for each polyelectrolyte layer, suggesting the successful coating of PE layers on the micro particles.

The advantage of using CaCO_3 micro particles is that the core can be dissolved by complexation with EDTA thereby forming stable microcapsules. The particles can be functionalized in two different ways, where the macromolecules could be encapsulated in the (i) core by the method of co-precipitation and (ii) in multilayers based on electrostatic attraction.

3.2.2 Functionalization of CaCO₃ particles

3.2.2.1 Protein encapsulation in multilayers

3.2.2.1.1 Qualitative analysis

Since the adsorption of the polyelectrolyte layers by means of the LbL method is mainly driven by electrostatic interaction, the insertion of charged proteins into the multilayers is possible. In this study the coating of proteins into the multilayers based on the surface charge was investigated. Bovine Serum Albumin (BSA) was introduced as a negative layer whereas in a second approach, lysozyme constituted the positive layer. The multilayer buildup was followed by ζ -potential measurements after each layer.

Figure 13: Zeta potential of CaCO₃ micro particles consecutively coated with (a) lysozyme replacing the cationic layer and (b) BSA replacing the anionic layer. The charge reversal observed after each layer confirms the assembly of the proteins in the multilayers. The error bar represents standard deviation from three triplicate measurements.

Both LbL preparations were started with PRM/DXS coating pair prior to the protein encapsulation. Lysozyme being positively charged at neutral pH, exhibited strong electrostatic interaction towards DXS layer where the zeta potential of -44mV representing the second DXS layer was increased to -17mV by means of lysozyme coating (fig. 13a). All further DXS layers showed a slightly increased zeta potential of ~ -35mV and the lysozyme layers ~ -15mV. The shift observed in the ζ -potential values confirms the coating of lysozyme.

BSA was used to replace the anionic layer as shown in fig. 13b. Since BSA is negatively charged at neutral pH, it had a strong affinity towards PRM and was coated as the 4th layer after PRM. The PRM layer exhibited a ζ -potential of 11mV, which was

shifted to -14mV after the BSA coating. All further layers showed a net difference of 10mV between the PRM and the BSA layer. This net charge reversal obtained after each layer confirms the multilayer build up.

3.2.2.1.2 Quantitative analysis

Protein quantification in the multilayers was performed in the supernatant after each coating step by means of Micro BCA assay kit.

Figure 14: Encapsulation efficiency of lysozyme and BSA coated on CaCO₃ micro particles. The coating patterns are as follows

CaCO₃/PRM/DXS/Lys/DXS/Lys/DXS/Lys/DXS-

CaCO₃/PRM/DXS/PRM/BSA/PRM/BSA/PRM/BSA-

CaCO₃/pARG/DXS/pARG/BSA/pARG/BSA/pARG/BSA-

The encapsulation efficiency (E.E) is calculated based on the following equation,

$$E.E(\%) = \frac{(\text{Initial amount of feed protein} - \text{amount of unencapsulated protein})}{\text{Initial amount of feed protein}} \times 100$$

As shown in figure 14, the encapsulation efficiency (E.E) of lysozyme was high with almost ~ 96% in the first layer and the subsequent two layers around 80%, whereas BSA had an efficiency of 75-85%. Since the encapsulation is mainly driven by electrostatic interaction, we also studied whether the polyelectrolytes used influenced the protein loading amounts. Therefore in this study the cationic polyelectrolyte PRM was replaced with polyarginine hydrochloride (pARG) and the protein BSA was used as a negative layer. The loading amounts of the protein calculated in terms of the particle

counts based on a relatively monodisperse population. The particle count was done with a haemocytometer and the total amount of protein held by the particles in three layers is shown in table 2. The encapsulation efficiency of the first BSA layer was only ~38% and the other two layers reached a encapsulation efficiency of ~65%, whereas the loading amounts of lysozyme and BSA were ~22 pg between the PRM/DXS layers and the loading amounts of BSA using the pARG/DXS pair was only ~14 pg.

Table 2: Loading amounts of BSA and lysozyme in the layers of CaCO₃ micro particles.

Coating pattern on CaCO ₃ micro particles	Loading amount (pg/particle)
CaCO ₃ /PRM/DXS/ Lys /DXS/ Lys /DXS/ Lys /DXS	22.61±0.34
CaCO ₃ /PRM/DXS/PRM/ BSA /PRM/ BSA /PRM/ BSA	21.40±0.10
CaCO ₃ /pARG/DXS/pARG/ BSA /pARG/BSA/pARG/ BSA	14.50±0.16

Both model proteins, lysozyme and BSA characterized by different molecular weights and different surface charge showed high encapsulation efficiency and loading amounts with the PRM/DXS pairs denoting the versatility of the system, which can be extended to other proteins in real time applications. Another advantage of these carriers is that the protein loading can still be increased by scale up of the layer numbers without an increase in the particle count.

3.2.2.2 Protein encapsulation in template CaCO₃ particles

CaCO₃ particles also had the possibility of encapsulation of biomolecules by the technique of co-precipitation. The protein to be encapsulated, lysozyme was mixed with CaCl₂ and Na₂CO₃ solution to facilitate the protein encapsulation during the crystal formation. The loading amounts were varied to test the maximal loading of protein in the core particles.

Table 3: Encapsulation efficiency of lysozyme in CaCO₃ particles.

Loading amount of lysozyme (µg)	Encapsulation efficiency (%) (Mean ± S.D)
5	100
10	78.62 ± 2.26
20	36.48 ± 2.70

Lysozyme was successfully loaded in the particles and a trend was observed with increasing loading amounts (table 3). At 5 μg loading amount the E.E was 100%, which suggests that the particles still have the ability to accommodate more protein, whereas at 10 μg , a saturation effect was observed with E.E reaching about 78%. On further increasing the loading amount to 20 μg , the E.E was drastically reduced to 36%, which clearly confirms the saturation effect at 10 μg .

The above-mentioned experiments clearly confirm the versatility of the LbL- CaCO_3 carrier system. The ability of multilayer protein coating is of prime importance in cases of surface functionalization that employs proteins like monoclonal antibodies as targeting ligands [19]. Furthermore core loading can be applied for the production of microcapsules filled with functional biomolecules in the hollow cavity. Besides the cargo functionality, retention of the biological activity is a key trait for a successful drug carrier. Several carriers have been demonstrated to possess the ability of encapsulation, however their biological activity is hampered by several factors involved in the carrier fabrication steps. Towards this we sought to investigate the stability of proteins during encapsulation procedure using lysozyme as a model protein.

3.2.3 Bioactivity of Encapsulated Protein

Proteins are relatively large molecules whose function largely relies on the three-dimensional structure. Any damage induced to the protein during or after encapsulation will significantly reduce their activity and therapeutic effectiveness [185]. The therapeutic function of a polypeptide is not only governed by their primary chemical structure but also by its secondary, tertiary and in some cases quaternary spatial structures [186]. Hence any subtle changes imparted to the protein during their encapsulation procedure render them ineffective. The activity of the lysozyme released from the carriers was tested by standard microbial lysozyme assay.

Figure 15: Schematic illustration of enzymatic assay of lysozyme using *Micrococcus lysodeikticus* as a substrate. Lysozyme exhibits hydrolytic activity against mucopolysaccharide present in the cell wall of *Micrococcus lysodeikticus*.

Lysozyme exhibits hydrolytic activity against a bacterial strain, *Micrococcus lysodeikticus* (fig. 15). Lysozyme hydrolyzes β -(1-4)-glucosidic linkages between N-acetylmuramic acid (NAM) and N-acetyl-D-glucosamine (NAG) residues present in the mucopolysaccharide cell wall of *Micrococcus lysodeikticus* [184]. The absorbance noted at 450 nm in bacterial suspension is attributed to the light scattering that takes place at the interface between cells and medium. The addition of lysozyme to the bacterial suspension induces changes in bacterial cells wall owing to the hydrolytic activity exerted. As a result of which, the cells are destroyed and dispersed marked by a sharp decrease in absorbance (increase in transmittance). The assay principle is based on the measurement of the rate at which the lysozyme samples can clear up turbid suspensions of *M. lysodeikticus* cells.

The activity of the protein released from the CaCO_3 carriers was compared with the native protein. The enzymatic activity towards the cell lysis is monitored for 5 minutes and the change in the transmittance (ΔT) is monitored at 450 nm. The activity of the encapsulated lysozyme vs. the activity of an equal amount of native protein was quantified. CaCO_3 particles were coated with three layers of lysozyme and stored at 37°C for three days. The protein sample released from the micro particles after 3 days were quantified and the activity was found to be ~96% as compared to the native protein. Similarly, for the protein released from the CaCO_3 particles encapsulated with lysozyme in the core, had an activity retention of ~92%. The activity of the protein is retained during the encapsulation procedure as the entire process is done at mild conditions and no harsh conditions such as solvents are used during the carrier fabrication procedure [187].

The three dimensional network of proteins can be disrupted by chemical, physical and environmental factors [185]. The chemical degradation occurs by the action of harsh chemicals that may disrupt the structure by inducing hydrolysis oxidation or any other undesired modification at the expense of the biological activity [185]. Physical degradation involves the effect of salt, pH, temperature, organic solvents, solutes, detergents and mechanical processing (stirring and shear forces) [188]. Since the causes of protein denaturation have been well studied, the simplest approach in the future design is to simply avoid them or to minimize them as much as possible. The efficiency of the LbL carrier is mainly attributed to simple fabrications steps that does not involve any harsh conditions as listed above that alters the nature of protein molecules, which were also confirmed experimentally. These studies demonstrate that these biocompatible

carriers with high encapsulation efficiency, loading amounts and activity retention constitute a powerful tool for drug delivery applications.

The first part of the study involved the design of protein loaded CaCO_3 particles and their associated physicochemical characterization steps. The next part involves the application of these carriers for targeted drug delivery in tumor therapy. The aim of the study was to design a carrier for intracellular delivery of cystatin C (therapeutic protein) to inhibit cathepsin B (overexpressed in tumor cells), which eventually inhibits cell growth and induces cell death.

3.2.4 Fabrication of cystatin loaded calcium carbonate (Cys- CaCO_3) particles

A schematic illustration of the steps involved in the preparation of cystatin loaded polyelectrolyte microcapsules is shown in figure 16. Cystatin was encapsulated in the CaCO_3 particles by the method of co-precipitation (fig. 16a). The colloidal particles are incubated with cationic polyelectrolyte (pARG) to facilitate layer formation onto the negatively charged CaCO_3 particles (fig. 16b). Subsequently the unabsorbed polyelectrolytes are removed leaving behind the positively charged template. The next step, (fig. 16c) involves the introduction of anionic polyelectrolyte layer (DXS) to the colloidal templates. The positively charged particles now adsorb the negatively charged polyelectrolyte based on electrostatic self-assembly. Additional layers were deposited by repeated deposition cycles, making use of the surface charge reversal that occurs upon adsorption of each layer (fig. 16d). Following LbL coating, hollow capsules are produced by subsequent removal of the template using EDTA as a chelating agent to remove the CaCO_3 templates (fig. 16e). Finally hollow microcapsules are obtained that are formed by a meshwork of interconnected polyelectrolyte thin films held together by electrostatic assembly (fig. 16f).

Figure 16: Schematic illustration for the preparation of cystatin loaded polyelectrolyte microcapsules. The steps involved are as follows; (a) CaCO_3 particle was prepared by mixing solutions of CaCl_2 and Na_2CO_3 in the presence of therapeutic protein cystatin. The cargo molecules are encapsulated in the porous template. (b) The encapsulated CaCO_3 particles are then coated via consecutive assembly of oppositely charged polyelectrolytes. Poly-arginine-hydrochloride (pARG) was used as a cationic layer and (c) dextran sulfate sodium salt (DXS) as the anionic layer (d) LbL coating steps were repeated until desired number of multilayers is formed. (e) After coating with desired number of multilayers, the CaCO_3 particles were dissolved using EDTA. (f) Finally microcapsules containing cystatin surrounded by thin polyelectrolyte films are obtained.

CaCO_3 based colloidal template was chosen for the synthesis of polyelectrolyte microcapsules. The particles prepared were in the size range of $5 \pm 2 \mu\text{m}$. In order to investigate the maximum loading capacity of CaCO_3 particles, different amounts of cystatin solutions ($5 \sim 20 \mu\text{g}$) were added to a fixed volume of CaCl_2 and Na_2CO_3 during crystal formation. The supernatants were collected during the particle preparation steps to quantify the encapsulated protein. Table 4 summarizes the encapsulation efficiency of cystatin in the chosen template particles.

The encapsulation efficiency (E.E) is calculated based on the following equation,

$$\text{E.E}(\%) = \frac{(\text{Initial amount of feed protein} - \text{amount of unencapsulated protein})}{\text{Initial amount of feed protein}} \times 100$$

Table 4: Encapsulation efficiency of cystatin in CaCO_3 micro particles.

Loading amount of cystatin (μg)	Encapsulation efficiency (%) (Mean $\pm$ S.D)
5	100
7	81.34 ± 3.69
10	54.30 ± 3.80
20	26.25 ± 1.24

Although a loading amount 5 μg showed 100% efficiency, the particles still had the ability to encapsulate few more molecules and hence the loading amount of 7 μg was chosen to be optimal and used for subsequent particle preparations. The Cys- CaCO_3 particles were coated with alternate layers of positive (pARG) and negatively charged polyelectrolytes (DXS), which are held together by means of electrostatic interaction. LbL assembly on the CaCO_3 particles was tested by electrophoresis; a net charge reversal at each step was measured by the ζ -potential.

Figure 17: Zeta potential of CaCO_3 particles coated with pARG/DXS. The ζ - potential is plotted as a function of layer number. Layer number 0 corresponds to bare CaCO_3 particles. The odd layers correspond to pARG (polycation) deposition and the even layers correspond to DXS (polyanion) deposition. The ζ - potential measurements were made in 0.01M NaCl at pH 7.4. Each error bar represents standard deviation from three triplicate measurements.

The ζ -potential measurements show the stepwise layer-by-layer assembly of polyelectrolytes on CaCO_3 micro particles (fig. 17). Blank particles (without cystatin) were slightly negative with a ζ -potential of (- 5mV), whereas cys- CaCO_3 turned slightly positive to (+2 mV). The slight increase in the surface charge is attributed to the cystatin incorporation, which are positively charged at neutral pH. The odd layers represent the particles with cationic layers as the outer layer and the even layer number with DXS as the outer layer. A net reversal of surface charge between positive and negative values is evident for each polyelectrolyte layer, suggesting the successful coating of PE layers on the micro particles.

After coating sufficient number of bilayers the particles were subjected to core dissolution. The core was dissolved by treatment with EDTA by a simple and quick process. Following core dissolution, the microcapsules filled with cystatin are obtained. In order to investigate the stability of the LbL shells after capsule formation, SEM was used to visualize the capsules after core dissolution with EDTA.

Figure 18: SEM image (10,000X, insert for 8500X) of polyelectrolyte microcapsules. The microcapsules consisting of $(\text{pARG/DXS})_3$ multilayer shells were prepared by means of core dissolution of LbL coated CaCO_3 particles by EDTA.

The SEM image (fig. 18) depicts the stability of the microcapsules following core dissolution. Shell rupture or disassembly of the multilayer films could not be observed indicating a high stability of the bio-polymer capsules. Although some dissolution methods using templates such as melamine formaldehyde are described as causing instability [189], EDTA seems to be a mild method. Moreover it was found that there

was no release of cystatin molecules entrapped in the core during the LbL coating and core dissolution procedure.

The selection of a suitable template is of prime importance as they play a main role in the design of microcapsules. The stability of the multilayer shells is well dependent on the nature of polyelectrolytes used. pARG/DXS multilayer pair demonstrated good stability and the capsules were monodisperse and no aggregation was found as observed under light transmission microscope. Although relatively higher concentration of Ca^{2+} ions are released in the interior of the shell, they rapidly diffuse through the multilayer shells and does not affect the integrity of the shell as shown in figure 18.

3.2.5 Localization of Cathepsin B

The localization of cat B in MCF-10A neo T cells was detected using monoclonal Anti-cat B. A highly fluorescent region found in the perinuclear region coupled with cytoplasmic staining corresponds to the vesicular distribution of cat B (fig. 19A).

3.2.6 Cellular internalization of PEMC

The cellular uptake of PEMCs was monitored using confocal microscopy. The last layer was chosen to be cationic pARG since the positively charged outer layer is known to enhance cellular uptake by a factor of two as compared to an anionic outer layer (DXS) [15]. The fact that both the cellular membrane and the microcapsules are seen in the same confocal plane indicates that the microcapsules are effectively taken up by cells and not merely sticking to the cell surface by electrostatic interactions (fig. 19B). The CLSM images shows that the PEMCs were associated in the cell cytoplasm after incubation, which confirms the internalization of capsules by the cells. This interaction was further corroborated by the quantitative analysis of cells by flowcytometry.

Figure 19: Confocal microscopy images of MCF10A neo T cells. (A). Immunocytochemical localization of cathepsin B permeabilized MCF-10A neoT cells by anti-cathepsin monoclonal antibody. Vesicular perinuclear distribution of cathepsin with peripheral cytoplasmic staining was observed. (B) Cellular internalization of polyelectrolyte microcapsules. For particle-staining PAH^{FITC} was encapsulated in CaCO₃, followed by coating 3 bilayers of pARG/DXS and cell membrane visualized with CellmaskTM membrane stain.

The optimal concentration of microcapsules for the use of tumor inhibition in MCF-10A neoT cells was determined by studying the cellular uptake profile of PEMCs. LbL microcapsules with a coating scheme of (CaCO₃^{PAH-FITC}/(pARG/DXS)₃/pARG was incubated with cells to investigate their endocytic capability. Cells were incubated with varying concentration of microcapsules, with cell: capsule ratio of 1:1, 1:5, 1:10 and 1:20. The positive charge of the outermost pARG layer facilitates the cell uptake by non-specific electrostatic interaction with the negatively charged cell membrane. The simultaneous recording of fluorescence emitted from labeled PEMCs and forward scattering intensity allows quantification of the capsules taken up by the cells. An unequivocal distinction between internalized and surface adherent capsules were made possible by trypan blue quenching. The plasma membranes of living cells are impermeable to trypan blue and therefore, only the fluorescence of those capsules that are attached to the cell surface from the bulk will be quenched.

An analysis of the uptake profile revealed that about 36% of the cells have taken up capsules at a cell: capsule ratios of 1:1, whereas it further increased to 78% at a ratio of 1:5. At higher feed concentrations (cell: capsule ratio) of 1:10 and 1:20, the internalization of polyelectrolyte microcapsules by cancer cells were found to be 88% and 95% respectively. For illustrative purpose, the steady increase in the fluorescence intensity as a function of microcapsule concentration is shown in figure 20 as the

fluorescent intensity from the cells is directly proportional to the amount of internalized capsules.

A linear dose-dependent relationship was observed over the concentration range of 1:1 to 1:20 (fig. 20). No saturation effects were observed over the tested range and hence for future experiments a range of 1:5 to 1:40 was selected for cell viability assays. It should however be noted that the internalization experiments were designed primarily to select the concentration range of microcapsules to be used in further intracellular assays.

Figure 20: Cellular uptake profile of polyelectrolyte microcapsules. Flowcytometry of FITC labeled PEMCs internalized into MCF-10A neo T cells as a function of different concentration. The coating configuration is $\text{CaCO}_3^{\text{PAH-FITC}}/(\text{pARG/DXS})_3/\text{pARG}$, with last layer being positive pARG to enhance cell uptake. The cells were tested in a concentration range with cell: capsule ratio ranging from 1:1 to 1:20. Cells untreated with PEMCs were used a control, to compensate for auto fluorescence. Representative histogram of three independent experiments is shown.

A major attribute for the microcapsules to be efficient delivery vehicle for intracellular application is good cellular internalization followed by cargo release at the target site. Several studies have reported the uptake of microcapsules in a variety of cell types. The microcapsules have demonstrated good cellular uptake despite their large size [132, 135]. The positive surface charge has been shown to facilitate the uptake of cells even without the presence of specific ligands on the surface. pARG/DXS microcapsules employed in our study demonstrated good cellular uptake and the cellular uptake profile

showed a dose dependent relationship. The arginine residues present on the outermost surface are also related to the cellular uptake of the microcapsules. Arginine residues are found to be the key players in the viral coat proteins that facilitate the cell uptake and rapid multiplication of the HIV-virus and in other cell penetrating peptides [190]. This unique property of arginine sequence have also been explored by using arginine/DNA complexes alone as such and in surface modification of a few other drug delivery carriers such as chitosan, dendrimers [191, 192] to facilitate cellular uptake and intracellular delivery of therapeutic molecules.

It should also be noted that the uptake pathway determines the intracellular trafficking and fate of the encapsulated cargo molecules [18]. Previous studies have reported the localization of microcapsules to the endolysosomal compartments [18], and it is of direct advantage as the cystatin is to be targeted to the Cat B located in the lysosomes. Microcapsules comprising pARG/DXS multilayers were found to be taken up via macro pinocytosis in dendritic cells. Following cellular uptake the multilayer shells were degraded mediated by the acidification of the lysosomal proteases [193]. This signifies the use of biopolymers such as pARG/DXS rather than the synthetic polyelectrolytes PAH/PSS that are non-biodegradable. Furthermore the degradation of multilayers facilitates the release of cargo molecules to the target site.

3.2.7 Intracellular proteolysis inhibition assay

A schematic illustration of the intracellular action of polyelectrolyte microcapsules is shown in figure 21. Polyelectrolyte microcapsules encapsulated with cystatin were treated with the tumor cells that overexpress derogatory proteolytic enzyme, cathepsin B (fig. 21a). The microcapsules are internalized via endocytic pathways and subsequently the layers defoliate mediating the release of the encapsulated cystatin molecules. The released cystatin thereby inhibits the antagonist molecule cathepsin B (fig. 21b). Successful inhibition of the cathepsin B by the cystatin induces cell death and therefore eradicates the primary tumor cell (fig. 21c).

Figure 21: Intracellular inhibition of cathepsin by cystatin loaded polyelectrolyte microcapsules. (a) MCF-10a neoT cells, breast tumor cells which overexpression the cysteine protease Cat B was treated with cystatin encapsulated LbL microcapsules. (b) Following cellular internalization, the endolysosomal compartments degrade the LbL shells, which favors the release of cystatin from the inner cavity. (c) Cystatin released thereby inhibits the cathepsin B and induces death of tumor cells.

Particulate system offers the possibility to target highly specific molecular pathways in a number of malignant conditions. The ability of the microcapsules to inhibit Cat B in the cells was tested using the fluorogenic substrate Z-Arg-Arg-MR. The assay principle is based on the activity of intracellular Cat B; when one or both arginine residues are split off via enzymatic activity, the molecule turns fluorescent allowing the identification of intracellular proteolytic activity of cat B.

A cell: capsule ratio of 1:20 was used as a fixed concentration for the quantitative study of cat B activity using confocal microscopy (fig. 22). In addition to cystatin loaded microcapsules, free cystatin and hollow capsules were used as additional controls

Figure 22: Intracellular proteolysis inhibition assay. CLSM images to demonstrate cathepsin B activity in MCF-10A neoT cells imaged by degradation of Z-Arg-Arg-MR substrate. Red fluorescence present in the perinuclear region shows the presence of cathepsin B. (A) Untreated control cells (B) Cells treated with cystatin (116 nM) equivalent to the amount present in microcapsules. (C) Blank (hollow) capsules ($\text{CaCO}_3/(\text{pARG}/\text{DXS})_3\text{pARG}$) treated to cells at a cell: capsule ratio of 1:20. (D) Cystatin loaded microcapsules ($\text{Cys-CaCO}_3/(\text{pARG}/\text{DXS})_3\text{pARG}$) treated to cells at a cell: capsules ratio of 1:20. The substrate concentration was $10\mu\text{M}$ and incubation time of 10 minutes. The instrument settings and image analysis parameters were kept constant.

Figure 22a represent the control population, i.e. untreated cells, which show the intracellular distribution of cathepsin B in bright fluorescent red spots. In contrast under the same conditions, cystatin incorporated in microcapsules completely abolished the red fluorescence, showing the inhibition of cathepsin B in fig. 22d. Figure 22b represent the

cells treated with cystatin (equivalent to amount present in test population), which resemble the control cells with bright red fluorescence. Cystatin being impermeable to the cell membrane has no effect on intracellular Cat B. Further hollow capsules were used as additional controls to show that the inhibitory activity is mainly attributed only to the encapsulated cystatin and not the multilayer shells. As observed in fig. 22c, the inert behavior of the capsules was confirmed by the bright red fluorescence of Cat B activity that closely resembled the control cells. Interestingly the capsules too appeared to have red fluorescence, which is linked to the secreted extracellular cathepsinB bound to the surface of the capsules involved in the proteolytic reaction. These results clearly show that cathepsin B was strongly inhibited by cystatin that has been released from the PEMCs and the inhibition of intracellular proteolytic activity was successful.

3.2.8 Cell proliferation studies

Cystatin loaded microcapsules were tested for possible proliferative activity of MCF-10a neoT cells following Cat B inhibition. The cytotoxicity was compared against free cystatin (equivalent to the amount in PEMCs) and blank capsules (hollow core). The cellular viability is expressed in terms of percentage relative to the control, untreated cells. As shown in figure 23, the microcapsules efficiently reduced the cellular viability to 60% at cell: capsule ratio of 1:40, whereas free cystatin had no effect even at such high concentrations. The cells were treated with different cell: capsule ratios ranging from 1:5 to 1:40. The cell viability reduced to 90% at a concentration of 1:5, which further reduced to 80%, 70% and 60% at 1:10, 1:20 and 1:40 respectively. A dose dependent relationship was observed over the tested range, i.e. as the capsule concentration increased the cellular viability decreased. Neither free cystatin nor blank capsules had any effect on the cellular viability, which clearly indicates the significance of the biocompatibility of LbL capsules. These results inevitably confirm that cathepsin B was strongly inhibited by cystatin that has been released from the PEMCs and thereby induce cell death.

Figure 23: Tumor inhibition by cystatin loaded polyelectrolyte microcapsules. Cell viability was determined by MTT assay. Control population represents the untreated cells. Results are presented as percentage of viability relative to control cells. The test samples were treated with different concentration of cystatin loaded microcapsules and their cytotoxicity compared against equivalent amount of free cystatin and hollow microcapsules. Statistical analysis was performed using Student's t test, and a value of $p < 0.05$ was considered to be statistically significant.

A detailed discussion on the advantage of polyelectrolyte microcapsules to unveil the therapeutic target of cathepsin B inhibition is explained in detail below. In particular, the importance of cysteine proteases in pathophysiology and the previous attempts towards cysteine protease as a target are explained; and finally the significance of the proposed system is drawn towards the conclusion.

Previous research over the past 25 years was focused entirely on the matrix metalloproteinase (MMP) as anti-cancer targets, however a majority of them were not successful in clinical trials due to the complex interaction within themselves. Inhibition of certain MMPs was shown to further aggravate the disease due to a lack of knowledge on the physiological and regulatory roles of the respective enzymes. These studies highlighted the significant effort needed to delineate the specific roles of proteases that contribute to malignant progression state [194, 195] and necessitate the role of other proteinases involved in the pathology of the disease. Consequently several groups have focused on the role of cysteine cathepsins as a potential target and their

pathophysiological roles have served as invaluable information in the identification of therapeutic targets. Cathepsin B is one of the most extensively studied cysteine cathepsins [54] and their respective inhibition is envisaged here as a potential target for future approaches.

Cysteine proteases are proteolytic enzymes secreted by cancer cells that play a major role in degradation of basement membrane and extracellular matrix proteins in metastasis and angiogenesis [54]. Cystatins are cysteine protease inhibitors that are vital for the regulation of physiological processes by reducing the protease activity. The imbalance between cysteine proteases and cystatins has been correlated with the progression of malignant diseases. Consequently a number of recent works have suggested that enzyme inhibitors like cystatin that are able to inactivate proteases as a promising strategy to lower tumor growth [196]. However cystatin is impermeable to cell and hence their delivery requires drug carriers that can efficiently cross the membrane barrier and deliver them at the intracellular environment.

The concept of cathepsin B inhibition as a therapeutic invention to stop metastasis is an old topic of research; however the search for an appropriate inhibitor is still an active field of research and development. Since the natural endogenous inhibitor, cystatins are impermeable to the cell membrane; small molecule chemical inhibitors have been the focus of research for cathepsin inhibition. Several reports have implicated the role of cathepsin inhibition as a potential target for controlling metastasis [62, 197]. The cysteine protease inhibitor E-64, specific to cathepsin B was shown to limit the extracellular matrix degradation and reduce the invasive potential of human bladder cancer cells [198]. CA-074, another inhibitor specific for cathepsin B was shown to significantly reduce the pulmonary and bone metastasis in breast cancer, whereas a broad specific cysteine cathepsin inhibitor JPM-OEt did not exhibit similar effect [71].

Although the inhibitors were effective in tumor suppression and helpful in elucidating the key role of cathepsins among the pool of proteolytic enzymes, their application is limited by a major disadvantage. In the case of a breast tumor model, cathepsin inhibitor CA-074 was effective in suppression of metastasis but did not have any effect on the primary tumor [71]. This is due to the mode of action of the inhibitor, which is impermeable to the cell membrane and the enzymatic action limited to the extracellular cathepsins secreted as exopeptidases. Therefore inhibition of cathepsins at the intracellular level could possibly lead to changes in the tumor cellular environment,

decreased cell survival and spreading, thereby not only to reduce the metastatic onset instead remove the tumor cells as such. Furthermore an ideal treatment is that one that targets the eradication of tumor cell, rather than to attenuate the disease.

Towards this approach, we devised a drug carrier encapsulated with cystatin, the natural endogenous inhibitor for intracellular Cat B inhibition. A challenging task is the choice of a subtle carrier, which facilitates good encapsulation, cellular uptake, and intracellular release of the cargo molecule at the desired site. To our knowledge, this study reports for the first time the successful inhibition of cat B within the tumor cells. One previous attempt reported the use of PLGA particles encapsulated with cystatin for cathepsin inhibition, however it was not successful due to the inherent cytotoxic activity of the carrier particles as such. The blank PLGA particles and the cystatin particles were cytotoxic to the same extent on the tumor cells [199]. Although PLGA is known to be biocompatible and biodegradable, the particles exhibited cytotoxicity, which is attributed to the polyvinyl alcohol used in the preparation of carriers.

To circumvent the issues associated with cytotoxicity of carriers, a precise selection of materials is highly desired for cellular applications. Towards this goal, the evolution of LbL carriers has opened up great opportunities in the field of drug delivery owing to its versatility in the system design. In this study, cystatin encapsulated in microcapsules comprising pARG/DXS multilayers successfully inhibited intracellular cathepsin B and exhibited a dose-dependent response. Taken together, the utilization of microcapsules for the delivery of cystatin to inhibit Cat B is considered as highly promising and novel strategy to target them at the intracellular level.

3.3 SUMMARY

This chapter dealt with the fabrication and functionalization of CaCO_3 using LbL technology for protein delivery applications. The first part explained the general methods for production of protein loaded LbL- CaCO_3 particles. Two model proteins, BSA and lysozyme were used to demonstrate the feasibility of encapsulation of proteins in the multilayers despite the charge and size differences. The carriers demonstrated high loading amounts; it was also found that the encapsulation of proteins was also dependent on the nature of the multilayer used. Besides the encapsulation, protein (lysozyme) encapsulated in the multilayer shells retained 96% of the biological activity whereas the protein encapsulated in the core had 92% activity retention. For CaCO_3 particles, the bioactive agents had the possibility to either be encapsulated into the interior or packaged in the multilayer shells by electrostatic interactions, with the option to tailor the properties of the carriers to the specific needs of the material and its therapeutic applications.

The second part explained the application of polyelectrolyte microcapsules fabricated by the LbL technique in the tumor therapy via a novel therapeutic target. Cathepsin B is a proteolytic enzyme, overexpressed in tumor cells are known to play a major role in tumor progression and metastasis. Cystatin, a natural endogenous inhibitor is down regulated in tumor cells; and hence exogenous delivery of cystatin for cathepsin B inhibition was envisaged as a potential therapeutic target. However cystatin C, a large protein (13 kDa) is impermeable to the cell membrane and necessitated a drug carrier to cross the biological barrier. CaCO_3 particles encapsulated with cystatin were prepared by co-precipitation followed by coating alternate layers of polycation (poly-arginine-hydrochloride) and polyanion (dextran sulfate). After coating seven layers, the CaCO_3 core was dissolved leaving behind the cystatin-loaded microcapsule. The intracellular release of cystatin was investigated by inhibition of the proteolytic activity using a fluorogenic substrate specific for cathepsin B. Finally the cytotoxic activity of the fabricated microcapsules on tumor cells were studied using the MTT assay. A dose dependent relationship was observed between the microcapsule concentration and cell viability whereas free cystatin or blank microcapsules (hollow core) had no effect on the tumor cells. Our study reported is regarded as an essential step towards the design of carriers for intracellular cysteine cathepsins inhibition in tumor therapy. This study shows the proof-of-principle for the clinical application of LbL engineered capsules in therapeutic conditions to unlock novel molecular targets much beyond the conventional drugs. Future studies using the LbL carriers are focused on the surface functionalization of

the multilayer shells with ligands that enables them to target the tumor cells with high specificity.

Chapter 4: LbL engineered vascular carriers –An approach towards controlled release of bioactive molecules

OVERVIEW

The use of erythrocytes as drug carriers has developed tremendously over the past two decades for various therapeutic applications. One significant drawback that is yet to be solved is the rapid leakage of drugs from the carriers, due to the lack of an efficient surface modification technique to achieve sustained release behavior. This work reports the fabrication of layer-by-layer (LbL) coated erythrocyte ghost carriers (EGC) that provide a simple mean for controlling the burst and subsequent release of bioactive agents. Red blood cell (RBC) ghosts were loaded with fluorescently labeled lysozyme via hypotonic dialysis. The loaded vesicles were then successfully coated with the biocompatible polyelectrolytes, poly-L-arginine hydrochloride and dextran sulfate. After establishment of the LbL-EGC carrier platform, we further enhanced the carrier system with subtle modifications in the encapsulation procedure. The modified erythrocyte carriers were then coated by LbL system and in addition to the controlled release; we studied the bioactivity of the encapsulated protein, lysozyme using the conventional microbial assay.

4.1 MATERIALS AND METHODS

4.1.1 Materials

Poly-L-arginine hydrochloride (PAG) (70,000-130,000 Da), poly-L-lysine hydrobromide (PLL) (30,000-70,000 Da), poly-L-glutamic acid sodium salt (PGA) (50,000-100,000 Da), 25% glutaraldehyde solution, chitosan (medium molecular mass), alginic acid sodium salt (viscosity 20-40 cp), and protamine sulfate (5000-10,000 Da) were purchased from Sigma-Aldrich. Dextran sulfate (DXS) (36,000-50,000 Da) was purchased from MP Bio. PBS containing 137mM sodium chloride, 2.7mM potassium chloride and 10mM phosphate buffer was prepared from a 10x PBS stock solution. PBS with different osmolality was prepared from 10x PBS stock solution by adjusting the water volume. The osmolality of solutions was measured using an Advanced Micro Osmometer (Advanced Instruments, Inc. Norwood Massachusetts).

4.1.2 Preparation of erythrocyte ghosts

Blood samples were withdrawn by venipuncture from healthy volunteers, with informed consent approved according to the procedures of the institutional review board and transferred to EDTA coated tubes. After centrifugation at 1800g for 10 min at 4°C, the plasma and buffy coat were removed and the remaining packed erythrocytes were washed three times with isotonic PBS. 1 ml of packed erythrocytes were washed thrice in 10ml of 150 mOsm/kg hypotonic PBS via centrifugation at 20,000g at 4°C followed by another three washings of the ghost membrane pellet in 10ml of 100 mOsm/kg hypotonic PBS. The resulting ghost membranes were then gently dispersed in isotonic PBS and subsequently incubated at 37°C for 1 h to allow the membranes to reseal and to form erythrocyte ghosts (EG).

4.1.3 Loading of erythrocyte ghosts

The EG were re-dispersed in 50 mOsm/kg PBS containing FITC-lysozyme at the appropriate concentrations and transferred into dialysis bag with 5000~10000 Da molecular mass cut off for FITC-lysozyme. The EG were then dialyzed against 30 mOsm/kg hypotonic PBS for 2h at room temperature in order to facilitate loading of the erythrocyte ghosts. Subsequently, the 30 mOsm/kg PBS was changed to isotonic PBS and dialyzed for another 2h at 37°C to reseal the EG. The EG were then washed 4 times with isotonic PBS to separate the loaded erythrocyte ghost carriers (EGC) from free, un-encapsulated macromolecules.

4.1.4 Polymer coating of erythrocyte ghosts

Prior to the LbL adsorption, EGC were fixed with 2.5% glutaraldehyde solution for 1h at 4°C. For the LbL adsorption on EGC, polyarginine hydrochloride (pARG) and DXS salts were used as cationic and anionic polyelectrolytes. EGCs were added to a solution containing 0.5mg/ml of the respective polyelectrolyte in 0.1M NaCl. The EGC were allowed to incubate with the polymer solution for 10 minutes at room temperature, following which the EGC were washed twice in isotonic PBS to remove non-adsorbed polyelectrolyte before the coating of vesicles with next polyelectrolyte layer. Layer-by-layer (LbL) consecutive adsorption of positive and negative charged polyelectrolytes was carried out by repeating these steps in sequence until the desired layer number was achieved.

4.1.5 Characterization of LbL-microcapsules

In order to quantitatively study the amounts of FITC-lysozyme loaded into the bare EGC, aliquots of EGC suspension were dispensed into 1.5 ml eppendorf tubes and 1% Triton X-100 was added to completely lyse the EGC. In order to quantify the amount of lysozyme, the supernatant was placed in black 96-microwell plates and the intensity of each well was read at excitation and emission wavelength of 485 and 535 nm, respectively, using a UV spectrofluorophotometer microplate reader (Perkin Elmer Victor 1420, USA).

4.1.6 Microscopic techniques

Confocal Laser Scanning Microscope (CLSM) (Zeiss LSM 510) was used to qualitatively study the distribution of FITC-lysozyme in EGC. Scanning electron microscopic measurements (JSM-5310, Jeol Ltd) were carried out to study and compare morphological differences between erythrocytes, EG and EGC. Standard procedures for preparing the samples for scanning electron microscopy (SEM) were employed. In brief, one drop of the sample suspension was put onto a clean silicon wafer and dried at 37°C and coated with a fine gold film and observed under a 5kV accelerating voltage.

4.1.7 Electrophoretic mobility measurements

In order to monitor the layering process, the electrophoretic mobilities of the bare EGC and coated EGC were measured using a Malvern Zetasizer (Malvern Zetasizer Nano, UK). The electrophoretic mobility was converted into the respective ζ -potentials using the Smoluchowski relationship.

4.1.8 Release of macromolecules from LbL-microcapsules

The release of FITC-lysozyme was studied and compared between bare EGC and EGC coated with 3, 5, 7 and 9 PE-layers. Equal amounts of coated and uncoated EGC were added to 500 μ l PBS and kept at 37°C. At defined time interval, the samples were centrifuged and 500 μ l of supernatant was collected in order to analyze for the fluorescence intensity and the EGC were resuspended in 500 μ l of fresh isotonic PBS.

4.1.9 Modified method of loading in erythrocyte carriers

The isolated erythrocyte were re-dispersed in 50 mOsm/kg PBS containing FITC-lysozyme at the appropriate concentrations and transferred into dialysis bags with

5000~10,000 Da molecular mass cut off. The whole set were then dialyzed against 30 mOsm/kg PBS for 6h at 37°C. Subsequently, the 30 mOsm/kg PBS was changed to isotonic PBS and dialyzed for another 2h at 37°C to reseal the erythrocyte. Thereafter the loaded erythrocytes (i.e. erythrocyte carriers) were washed 4 times with isotonic PBS to separate them from free, un-encapsulated macromolecules.

4.2 RESULTS AND DISCUSSION

4.2.1 Preparation and characterization of Erythrocyte ghost carriers

Erythrocytes have been used as carriers for drug delivery applications over the past two decades. However controlled release from these carriers has been a challenging task. Here the LbL system was used to coat the erythrocyte carriers and modulate the release by varying the layer numbers.

A schematic illustration for the preparation of erythrocyte carriers is shown in figure 24. The erythrocytes were isolated from human blood by centrifugation to remove buffy coat and plasma. The isolated packed erythrocytes were washed twice in isotonic PBS prior to the encapsulation procedure (fig. 24a). The erythrocytes were then subjected to hypotonic lysis by introducing them in hypotonic buffer; as a result of which the RBC membranes swell and undergo hemolysis (fig. 24b). Repeated washing steps in hypotonic buffer release the membrane bound hemoglobin yielding white RBC membranes termed as 'RBC ghosts' (fig. 24c). At this stage, the membranes have pore openings that facilitate the cellular entry of foreign molecules. The ghost membranes are placed together with the cargo molecules to be encapsulated in a dialysis membrane to facilitate encapsulation of macromolecules (fig. 24d). Following incubation, the ghost membranes were washed in isotonic PBS to remove the unbound molecules and closure of membrane pores that open in hypotonic solution. The membranes of the erythrocyte carriers are then cross-linked with glutaraldehyde (GA) to increase the membrane strength prior to LbL coating (fig. 24e). The erythrocyte carriers are incubated with cationic polyelectrolyte (pARG) to facilitate layer formation onto the negatively charged templates (fig. 24f). Subsequently the unabsorbed polyelectrolytes are removed leaving behind the positively charged ghost membranes. The next step, involves the introduction of anionic polyelectrolyte layer (DXS) (fig. 24g). The positively charged particles now adsorb the negatively charged polyelectrolyte based on electrostatic self-assembly. Additional layers were deposited by repeated deposition cycles, making use of the surface charge reversal that occurs upon adsorption of each layer (fig. 24h).

Figure 24: Schematic illustration for the preparation of erythrocyte ghost carriers. (a) Erythrocytes were isolated from human blood samples and resuspended in isotonic PBS. (b) The erythrocytes were subjected by hypotonic lysis by placing them in hypotonic, as a result of which they start to swell, release hemoglobin and opening of membrane pores. (c) Repeated washing steps in hypotonic buffer yields hemoglobin free RBC membranes termed as RBC ghosts. (d) RBC ghosts are now placed with the cargo molecule in dialysis bag to facilitate encapsulation within the ghost membranes via the membrane openings. (e) After incubation, the ghost membranes are washed in isotonic PBS in order to close the membrane openings and reseal the membrane to retain the encapsulated molecules intact. (f) Following encapsulation in erythrocytes, the membranes were cross-linked with glutaraldehyde to increase the membrane strength prior to LbL coating. (g) The erythrocyte carriers are then introduced to cationic polyelectrolyte solution (pARG) to facilitate deposition of thin film on the surface of ghost carriers. (f) The unbound polyelectrolytes are removed by repeated washing steps and are further introduced in the oppositely charged anionic polyelectrolyte (DXS). (g) The LbL coating steps are repeated until the desired number of multilayers is formed [131].

The erythrocytes were first subjected to hypotonic lysis, where the hemoglobin is removed and the so formed cells referred to as Erythrocyte Ghost (EG). The morphology of the erythrocyte ghosts was compared with the native RBCs by means of SEM.

Figure 25: SEM image of erythrocytes and EG :(a) Erythrocytes (b) well resealed EG (c) enlarged view of well resealed EG and (d) poorly resealed EG. The arrows in (fig 22b) highlight the folds and crevices observed on the ghost membranes [131].

The native human erythrocytes show the typical discoid shape and have a flat surface (fig. 25a). In contrast, EG appear flat with several folds and creases on the surface (fig. 25c). In order to investigate the effects of the osmolality on the EG shape, erythrocytes were also suspended in hypotonic PBS having a much lower osmolality of 20 mOsm/kg followed by resealing in isotonic PBS. As shown in figure 25d, most of these EG cannot retain their shape and integrity. It can be concluded that a reduced osmolality below a certain limit causes irreversible ruptures and openings of the membrane.

4.2.2 Protein encapsulation

FITC-lysozyme was loaded into EG via hypotonic dialysis (fig. 26). To visualize the distribution of FITC-lysozyme in the EGC, loaded EGC were investigated by means of confocal laser scanning microscopy (CLSM).

Figure 26: CLSM image of erythrocyte ghost loaded with FITC-lysozyme.

Lysozyme was chosen as a model protein for the encapsulation. As can be seen in figure 26, EGC were filled completely and uniformly in ghosts whereas in some ghosts, they are trapped in the membrane as observed by green rings. The encapsulation efficiency for lysozyme was found to be $12 \pm 2.3\%$. Following macromolecule encapsulation, the ghosts are subjected to isotonic conditions for membrane resealing.

4.2.3 LbL coating of EGC

In order to facilitate a time-controlled release of the encapsulated drug, EGC were coated with the pARG/DXS multilayers. Prior to the LbL adsorption, EGC were fixed with 2.5% glutaraldehyde solution for 1h at 4°C. Zeta potential measurements were performed to determine successful layer coating.

Figure 27: ζ -potential of EG fixed with glutaraldehyde as a function of the number of polyelectrolyte layers formed by the consecutive adsorption of pARG and DXS. The odd layers correspond to pARG (polycation) deposition and the even layers correspond to DXS (polyanion) deposition. The ζ - potential measurements were made in 0.01M NaCl at pH 7.4. Each error bar represents standard deviation from three triplicate measurements [131].

As shown in figure 27, EGC have a negative ζ -potential of -50 mV, which changed to a positive value (+38 mV) after the deposition of the first cationic polyelectrolyte layer (pARG). This indicates that the surface charge of EGC was overcompensated by the pARG molecules. The charge overcompensation resulting from pARG facilitated the subsequent deposition of the anionic polyelectrolyte layer (DXS). A net reversal of surface charge between positive and negative values is evident for each polyelectrolyte layer, suggesting the successful coating of EGC with the PE layers.

4.2.4 Release kinetics of lysozyme from EGC

The release profiles of the encapsulated FITC-lysozyme are shown in figure 28. Release behavior of FITC-lysozyme was compared between unlayered EGC and EGC coated with different number of layers. The release of lysozyme was found to decrease with increasing layer numbers. Within ~ 290 hours, about 86% of the lysozyme has been released from bare RBC ghosts whereas the release of ghosts coated with nine polyelectrolyte layers is reduced to 39%.

Figure 28: Release profile of FITC-lysozyme from RBC ghosts as a function of the time and number of polyelectrolyte layers [131].

The release profiles also reveal that the polyelectrolyte layers prevent or reduce the initial burst release. Lysozyme shows a burst release of around 40% from bare vesicles within the first two hours. For lysozyme, the addition of polyelectrolyte layers suppresses the burst significantly (table 5). With nine layers, the burst is reduced to about 20% ($\sim 3.5\times$) for lysozyme. In addition to reducing the burst release, it is also desirable that a drug carrier maintains a slower, controlled release rate following the initial burst release.

The release kinetics of the encapsulated substances was analyzed over the region following the burst as shown in table 5.

Table 5: Release parameters of FITC-lysozyme [131].

Number of layers	Burst duration (hrs) ^a	Burst release (%) ^b	release rate (ng/hr) ^c	n-value ^d
0	28.5	75.8	3.6±0.13	0.09
3	33	47.3	5.0±0.62	0.23
5	32	45.2	4.6±0.24	0.22
7	30	29.3	5.1±0.88	0.28
9	29.5	20.1	4.3±0.72	0.25

^a Time point where the initial burst release ends estimated from figure 23.

^b Amount released during the initial burst release.

^c Release rate following the initial burst release.

^d n-value from a power law fit ($y=kt^n$) of the release profile following the initial burst release.

A comparable reduction of the initial burst release is achieved with seven or nine polyelectrolyte layers and subsequent release rates are hardly affected by the number of polyelectrolyte layers. Nevertheless, these results clearly indicate that the concept of polyelectrolyte-encapsulated vesicles allow the time-controlled release of a wide range of bioactive molecules.

The kinetics of the drug release from erythrocyte carriers depends on the size and polarity of the encapsulated agents. In general three different patterns of drug release can be observed: 1) the drug release occurs by rapid diffusion through the membrane for lipophilic drugs such as primaquin [114], methotrexate[200], 2) the drug release occurs by cell lysis for polar drugs such as enalaprilat [112] or gentamicin [201]; 3) the release profile is intermediate between the first two patterns, as observed for erythropoietin[102] and isoniazid [202]. Among the different strategies to control the release of drugs from erythrocytes, cross-linking with glutaraldehyde seems to be one of the most successful to date. This study demonstrates a new method for controlling the release of macromolecules from erythrocyte carriers by varying the shell thickness, independent of

the nature of the macromolecules. By suitable choice of the number and type of polyelectrolyte layers, it is possible to fine-tune the entire release curve, without depending on lysis of these carriers to control the release.

Following the success of the initial experiments, we further sought to refine the system based on erythrocyte carriers. We further studied a modified method for encapsulation, hypotonic dialysis, which obviates the step of hypotonic lysis. In addition we also studied the biological activity of the proteins that were encapsulated in the erythrocyte carriers. The protein activity was studied using *Micrococcus lysodeikticus*, substrate for lysozyme as detailed in section 3.1.6.

4.2.5 Modified hypotonic loading method

A schematic illustration of the modified hypotonic loading method is explained in figure 29. The erythrocytes were isolated from human blood by centrifugation to remove buffy coat and plasma (fig. 29a). The erythrocytes were then subjected to hypotonic dialysis, by introducing them in hypotonic buffer together with the macromolecules to be encapsulated (fig. 29b). In the modified loading method, the hypotonic lysis and subsequent loading step are combined to one step namely hypotonic dialysis. In this way, the hemolysis of the erythrocytes is significantly reduced and the shape of the erythrocytes is well retained. Following incubation, the ghost membranes were washed in isotonic PBS to remove the unbound molecules and closure of membrane pores that were open in hypotonic solution (fig. 29c). Prior to LbL coating, the membranes were cross-linked to glutaraldehyde. The erythrocyte carriers are then introduced to cationic polyelectrolyte solution (poly-L-lysine hydrobromide, PLL) to facilitate deposition of thin film on the surface of ghost carriers (fig. 29d). The unbound polyelectrolytes are removed by repeated washing steps and are further introduced in the oppositely charged anionic polyelectrolyte (dextran sulfate sodium salt, DXS) (fig. 29e). Finally, the LbL coating steps are repeated until the desired number of multilayers is formed (fig. 29f).

Figure 29: Schematic illustration of preparation of erythrocyte carriers coated with multilayer polyelectrolyte shells. The steps involved are as follows; (a) Erythrocytes were isolated from human blood and resuspended in isotonic PBS; (b) The erythrocytes were subjected to hypotonic dialysis. In brief, the erythrocytes are resuspended in hypotonic buffer together with the macromolecules to be encapsulated. Following incubation, (c) the erythrocytes encapsulated with active molecules were washed with isotonic PBS to remove the surface bound molecules. Prior to LbL coating, the membranes were cross-linked to glutaraldehyde. (d) The erythrocyte carriers are then introduced to cationic polyelectrolyte solution (poly-L-lysine hydrobromide, PLL) to facilitate deposition of thin film on the surface of ghost carriers. The unbound polyelectrolytes are removed by repeated washing steps and are further introduced in the oppositely charged anionic polyelectrolyte (dextran sulfate sodium salt, DXS) (e). Finally, (f) the LbL coating steps are repeated until the desired number of multilayers is formed [203].

Rhodamine-B-isothiocyanate labeled lysozyme (RITC-lysozyme) was loaded into erythrocyte via hypotonic dialysis method as schematically illustrated in figure 29. As shown in SEM images (fig. 30), the hypotonic dialysis loading method for RITC-lysozyme encapsulation results in some morphological/topological changes in erythrocyte where the unloaded normal cells have typical biconcave discoid shape (fig. 30a) and the loaded erythrocyte have a more flat surface (fig. 30b). To visualize the distribution of RITC-lysozyme within the erythrocyte carriers, confocal laser scanning microscopy (CLSM) pictures of the erythrocyte carriers were taken in several planes (fig. 31). As shown, most of erythrocyte carriers are completely and uniformly filled with RITC-lysozyme. As indicated by the CLSM observation, considerable encapsulation efficiency (41.6%) and loading amount (12.7pg/cell) was also achieved.

(a)

(b)

Figure 30: Morphology of erythrocyte and erythrocyte carriers. SEM images of (a) native erythrocyte (b) erythrocyte loaded with RITC-lysozyme and coated with 3 bilayers of PLL/DXS [203].

Figure 31: CLSM images of RITC-lysozyme loaded erythrocyte carriers [203].

Among all the methods employed to prepare erythrocyte carriers, hypotonic dialysis method has been the most commonly used procedure [20]. This is because hypotonic dialysis is the method that best preserves the morphology and physiological characteristics of erythrocytes resulting from this process, while at the same time it can achieve a considerable loading efficiency [20]. Fig. 30b shows that the RITC-lysozyme loaded and polyelectrolyte-coated erythrocytes still retain their shape like the native erythrocyte (fig. 30a), indicating that the loading and coating procedure does not induce major morphological changes. Besides the successful morphology maintenance, the encapsulation efficiency obtained in this study was considerable increased from the previous hypotonic lysis method.

The activity of the released RITC-lysozyme was also investigated. **Table 6** provides the enzymatic activity of the lysozyme in the released medium after 10 hours. In comparison to the activity of the native protein, both the bare ghost and the layered EGC preserved ~100% activity of the protein which indicates that the activity was completely preserved during the carrier fabrication procedures.

Table 6: Enzymatic activity of lysozyme in the released sample[203].

Sample	Enzyme activity (%) (mean $\pm$ S.D, n=3)
Control	99.2 $\pm$ 0.19
Unlayered EGC	98.91 $\pm$ 0.69
6 layer coated EGC	99.75 $\pm$ 1.32

Control: Lysozyme (0.4mg/ml) in release medium (1X PBS) stored at 37°C.

The activity of the encapsulated protein is of utmost concern in the selection of particulate systems for drug delivery approaches. The concentration of glutaraldehyde used for the cross linking is one major parameter that influences the activity of the encapsulated protein. Glutaraldehyde being a bi functional cross-linking reagent it is highly possible that glutaraldehyde might crosslink the encapsulated protein along with the erythrocyte membrane, which renders the protein biologically inactive. Glutaraldehyde at a concentration of 0.125% was found to be optimal to crosslink the erythrocyte membranes and preserve the activity of the encapsulated protein. It was also reported that glutaraldehyde at this concentration had no derogatory effects on β -

galactosidase encapsulated in erythrocyte ghosts [37], which shows that this system could further be explored for other therapeutic molecules without any activity loss. Following encapsulation and cross linking, the LbL coating steps were carried out at mild buffer conditions unlike other well established biocompatible and biodegradable particulate system such as PLGA fabrication steps which requires harsh conditions such as ultrasonication, freeze drying and exposure to toxic solvents, where there is a substantial loss in the activity of the encapsulated protein [38-39]. Moreover the polyelectrolytes used for LbL coating do not impair the activity of the protein as shown in table 6. These studies clearly demonstrate that the biological activity of the proteins is preserved in its native form using EGC carriers and in addition the LbL coating serves to control the release of the encapsulated molecules predominantly by membrane diffusion.

4.3 SUMMARY

This chapter explained the fabrication and functionalization of erythrocytes carriers using the LbL technology for controlled release applications. The study employed pre-loaded RBC ghosts as template cores for the fabrication of polyelectrolyte multilayer capsules via LbL deposition, simplifying the preparation of loaded LbL microcapsules. Erythrocytes ghost carriers were prepared by encapsulating lysozyme by hypotonic dialysis method. The encapsulation was visually confirmed by confocal microscope, whereas the morphology of the carriers and native RBC were obtained using scanning electron microscope. Following encapsulation, the EGC carriers were coated with alternatively charged polyelectrolytes in an LbL fashion, which was further confirmed by ζ -potential measurements. The release kinetics of the encapsulated biomolecule, lysozyme was studied as a function of layer number. Applying the layer-by-layer polyelectrolyte coating technique onto erythrocyte carriers successfully suppressed the burst and subsequent release of lysozyme. It is demonstrated that the release profiles of the encapsulated molecules can be regulated over a wide range by adjusting the number of polyelectrolyte layers. After establishment of the LbL-EGC carrier platform, we further enhanced the carrier system with subtle modifications in the encapsulation procedure. These carriers with the modified method had increased encapsulation efficiency from 12 % to 41%, less time consuming and morphological resemblance close to the native RBCs. The erythrocyte carriers prepared by the modified method were then coated by LbL system with biocompatible polyelectrolytes, poly-L-lysine hydrobromide and dextran sulfate. In addition to the controlled release, we studied the bioactivity of the encapsulated protein, lysozyme using the conventional microbial assay. The carrier fabrication methods were further optimized in order to retain the biological activity as a result of which protein retained all of its activity similar to the native form. In conclusion, these novel LbL microcapsules have several unique properties such as ease of preparation, tunable release, biocompatibility, biodegradability, and the possibility of multi-functionality. The concept of erythrocyte ghost carriers coupled with polyelectrolyte multilayers is therefore a promising alternative to other carrier systems and should be further explored for suitable drug delivery applications.

Chapter 5: Fabrication and functionalisation of HA nanoparticles by LbL technique for gene delivery applications

OVERVIEW

A fundamental goal in the field of gene therapy is the development of safe and efficient non-viral vectors for therapeutic applications. This work demonstrates the fabrication and functionalization of layer-by-layer (LbL) coated hydroxyapatite (HA) nanoparticles (<200nm) for intracellular gene delivery applications. A non-viral gene vector was prepared via the LbL technology, i.e. by the alternate deposition of polycation and polyanion on HA nanoparticles. The encapsulation strategy employed in this work involves the integration of plasmid DNA (pDsred) encoding for red fluorescent protein and model proteins (BSA and lysozyme) in the multilayer shells of the nano-carriers. The intracellular processing of the HA nano-carriers with the encapsulated pDNA was studied with HEK293T cells employing two different multilayer pairs poly-L-arginine hydrochloride (pARG)/dextran sulfate (DXS) or poly-L-lysine hydrobromide (PLL)/DXS. The cytotoxicity of the nanoparticles towards HEK293T cells was analyzed using MTT assay. Flowcytometry and confocal laser scanning microscopy were used to investigate the cellular uptake and intracellular processing of the particles. Further studies were performed to delineate the intracellular trafficking of this system in order to identify the molecular pathways associated with the uptake and intracellular processing. To elucidate the mode of cellular uptake, we first compared the effects of different metabolic inhibitors blocking different types of endocytic pathways, and effect of temperature if the uptake followed an energy independent process. We also report the defoliation pattern of DNA from the multilayer shells at the extracellular and intracellular level, by incorporation of the pDNA at different layer positions.

5.1 MATERIALS AND METHODS

5.1.1 Materials

Hydroxyapatite nanoparticles (<200 nm), polyallylamine hydrochloride (PAH, MW 70 000 Da), fluorescein isothiocyanate (FITC), poly-L-lysine hydrobromide (PLL) (30,000-70,000 Da), poly-L-arginine hydrochloride (pARG, MW > 70000 Da), bovine serum albumin (BSA), lysozyme, 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) was purchased from Sigma Aldrich (Singapore) and dextran sulfate sodium salt (DXS, MW 36,000-50,000 Da) from MP biomedical. All the chemicals were used as received. Cell transfection medium (Optimem), cell membrane stain (Cell mask Orange) was purchased from Invitrogen (Singapore) and plasmid DNA (pDsRed) was purchased from Clontech. HEK293T/17 cells were obtained from ATCC and Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) was purchased from PAA Laboratories.

5.1.2 Preparation of LbL nanoparticles

The HA particles were alternately incubated in pARG or PLL and DXS solution (0.5 mg/ml, 0.1M NaCl) for 10min under constant shaking. After each coating step the particles were centrifuged and washed 3 times in 0.1M NaCl. Plasmid DNA (55 ng/μl) and proteins (0.5mg/ml) were coated in 0.1 M NaCl solution and incubated for 10 min followed by three washings with 0.1M NaCl. After each coating step the supernatant was collected in order to quantify the unencapsulated protein and DNA via the Micro BCA kit and Nanodrop (Nanodrop1000, Thermo Fisher Scientific Instruments) respectively.

5.1.3 Electron Microscopic studies

Field-emission scanning electron microscopy (FESEM, JSM-6700F, Japan) was employed to study the morphology of polyelectrolyte coated nano carriers. Standard procedure for preparing the sample was employed. In brief, one drop of the sample suspension was put onto a clean silicon wafer and dried at 37°C. It was then coated with a fine gold film and observed under a 5kV accelerating voltage.

5.1.4 Zeta potential measurements

The electrophoretic mobilities of the polyelectrolyte coated HA particles were measured using a Zetasizer (Malvern zetasizer Nano, UK). The electrophoretic mobility was converted into the respective ζ -potentials using the Smoluchowski relationship.

5.1.5 Layer-by-layer coating on HA nanoparticles monitored by FACS

The LbL growth on the HA nanoparticles were monitored by flow cytometer using fluorescent dye (RITC) labeled poly-arginine-hydrochloride (pARG^{RITC}). The labeling of pARG with RITC was followed from previous reports [27]. The particles were coated with varying number of fluorescent-labeled layers (pARG^{RITC}) and their fluorescence intensity monitored as a function of number labeled layers. The coating scheme of particles with labeled layers is as follows

5.1.6 Cell viability studies

The viability of the cells treated with nanoparticles was determined by the MTT colorimetric assay [204]. Viable cells (5×10^4), suspended in 200 μl of growth medium, were seeded in 96 well micro-titer plates containing LbL coated HA nanoparticles at different concentrations. The coating configuration of the nanoparticles is HA/pARG/DXS/pARG/DXS/pARG. After incubation for 24h at 37°C and 5% CO₂, 10 μl of MTT (5mg/ml) was added to the cells and incubated for 4 h at 37°C. Thereafter 100 μl of dimethylsulfoxide (DMSO) was added to dissolve the formazan crystals formed. The absorbance of the solubilized crystals was measured at 595nm with a UV spectrofluorophotometer microplate reader (Perkin Elmer Victor 1420, USA). The cell viability is expressed by the equation:

$$\text{Cell viability (\%)} = (A_{\text{test cells}} / A_{\text{control cells}}) \times 100$$

where $A_{\text{test cells}}$ and $A_{\text{control cells}}$ denote the absorbance of formazan determined for cells treated with different concentrations of nanoparticles and of non-treated control cells, respectively.

5.1.7 Confocal Laser Scanning Microscopy (CLSM)

The CLSM images were obtained with a Zeiss LSM-510 Meta Laser Scanning Microscope equipped with a Plan-Apochromat 63x/1.4 oil objective. For the FITC fluorescence, the Ar/Kr laser and for Cell mask orange, the cell membrane stain, and DsRed fluorescence the He/Ne laser was used. All images were obtained in sequential mode between frames to separate between green and red emission.

5.1.8 Flow cytometry (FACS)

Fluorescence intensities of the cells treated with HA nanoparticles were investigated by flowcytometry (FACS Calibur, Beckton Dickinson, USA) with a laser excitation wavelength of 488nm (FITC detection in FL-1 and DsRed detection in FL-2). 10^4 events in the gated region of the cells were detected in each measurement and were analyzed by WinMDI 2.9 software.

5.1.9 Particle/Cell interaction

The cellular internalisation of particles by HEK293T cells were visualised by CLSM. HA nanoparticles were coated with FITC labelled PAH (FITC-PAH) as the innermost layer for particle tracking. Either PLL or pARG were employed as cationic layers whereas DXS was used for the anionic layers with the two coating patterns, HA/FITC-PAH/DXS/PLL/DXS/PLL/DXS/PLL and HA/FITC-PAH/DXS/pARG/DXS/pARG/DXS/pARG. For a qualitative analysis of the uptake, the cell membranes were stained with cell mask Orange and investigated via CLSM. For quantitative information, particle/cell experiments were performed in 12-well chamber slides containing 2.5×10^5 HEK293T/17 cells seeded one day before particle incubation. After incubating the particles for 4 hours at 37°C they were investigated by means of flowcytometry. For a quantitative analysis of the uptake, the cells were trypsinized and washed twice with Dulbecco's PBS and analyzed by flowcytometry. 10 μ l of trypan blue (0.4%) was added to 300 μ l of cell suspension in order to quench the fluorescent signal of non-internalized carriers.

5.1.10 pDNA expression

The expression of the reporter fluorescence protein mediated by the nano carriers was visualized by means of CLSM. HA nanoparticles were coated with FITC-PAH as the innermost layer and with anionic and cationic polyelectrolytes as described in the previous paragraph while the outermost DXS layer was replaced with pDsRed. HEK293T cells were incubated with these particles in optimum for 4 h at 37°C. Thereafter the medium was replaced with complete growth medium (DMEM + 10%(v/v) FBS). The gene expression was investigated qualitatively after incubation for three days via CLSM.

Quantitative information on the transfection efficiency of HA nanoparticles was obtained via flowcytometry. HA nanoparticles were coated with either PLL or pARG as

cationic layers and DXS for the anionic layers while the outermost DXS layer was replaced with pDsRed. Cells were seeded in 12 well plates as described above and allowed to grow for 24h at 37°C. Thereafter the medium was replaced with Optimem containing the desired particle concentrations. After an incubation of 4h, the medium was replaced with complete growth medium (DMEM+10% FBS) and the gene expression was investigated quantitatively after an incubation time of three days at 37°C and 5% CO₂.

Lipofectamine2000TM (Invitrogen) was used as a positive control for transfection experiments. The DNA/Lipo solutions were incubated with the cells for 4h at 37°C with standard Lipo/DNA concentrations as per the manufacturer's protocol, after which the medium was replaced with complete growth medium (DMEM+10% FBS) and allowed to grow for transgene expression. After three days, the cells were trypsinized and washed twice with Dulbecco's PBS and analyzed by FACS.

5.1.11 Cellular uptake pathway

For quantitative information on cellular uptake, the HA nanoparticles were coated with 1 layer PAH^{FITC} for particle tracing. The coating patterns are as follows; HA/PAH^{FITC}/DXS/pARG/DXS/pARG/DXS/pARG. HEK 293T cells were seeded into 12-well plates (2x10⁵ cells/well) and allowed to grow for initial attachment. The cells were then incubated for 4 hours with the LbL-HA nanoparticles in serum free medium (optimem). Prior to incubation with LbL coated HA nanoparticles, HEK 293T cells were pre-incubated with endocytic inhibitors; chlorpromazine (an inhibitor of clathrin-mediated endocytosis, 28 µM) [205], methyl-β-cyclodextrin (depletes cholesterol from the plasma membrane inhibiting pathways dependent on lipid rafts MβCD 20 µM [205] , dynasore (an inhibitor of dynamin, 160 µM) [206], amiloride hydrochloride hydrate (an inhibitor of macropinocytosis, 50µM) [207], cytochalasin D (an inhibitor of F-actin elongation involved in caveolar pathway and macropinocytosis, 10 µM) [205] prepared in Optimem (1 h, 37 °C). The medium was then removed and substituted with medium containing particles. Following incubation for 4 h, the cells were trypsinized and washed twice with Dulbecco's PBS and analyzed by FACS (FACS Calibur, Beckton Dickinson, USA). 10µl of trypan blue (0.4%) was added to 300µl of cell suspension in order to quench the fluorescent signal from the carriers adsorbed on the outer surface of the cell. The internalization of nanoparticles was calculated as the mean fluorescence value and being normalized. The 100% uptake was determined from cells incubated with the particles in the absence of endocytic inhibitors.

5.1.12 Defoliation pattern of DNA from LbL-HA nanoparticles

The defoliation of plasmid DNA from LbL-HA particles coated with two different configurations was studied for in vitro release behavior and in the intracellular level. The coating pattern is as follows ;

HA/pARG/DXS/pARG/**DNA**/pARG (x=1) and

HA/pARG/**DNA**/pARG/DXS/pARG (x=3)

where (x=n) denotes the number of polyelectrolyte layers on top of the DNA layer.

5.1.11.1 In vitro DNA release:

To quantify the DNA released, the plasmid DNA was fluorescent labeled using the Label IT[®] fluorescein labeling kit (Mirus Bio). The labeled DNA was then encapsulated in the multilayers shells of HA nanoparticles as explained previously. Equal amounts of LbL-HA particles coated with different configurations were added to 200 μ l PBS and kept at 37°C. At defined time intervals, the samples were centrifuged and 200 μ l of supernatant was collected in order to analyze for the fluorescence intensity and the particles were resuspended in 200 μ l of fresh PBS. The supernatant was placed in black 96-microwell plates and the intensity of each well was read at excitation and emission wavelength of 485 and 535 nm, respectively, using a UV spectrofluorophotometer microplate reader (Perkin Elmer Victor 1420, USA).

5.1.11.2 Intracellular release behavior

HEK293T cells (5×10^4) were seeded with varying amounts (0-30 μ g) of LbL- HA nanoparticles coated with two different configurations. The cells were allowed to grow for gene expression and the number of fluorescent cells was counted on day 4 and day 5.

Statistical Analysis: All data are expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using Student's t test, and a value of $p < 0.05$ was considered to be statistically significant.

5.2 RESULTS AND DISCUSSION

The size of the particles is a major concern in different drug delivery applications such as gene delivery and other forms of intracellular delivery. To date, in the field of LbL based carriers, microcapsules have been extensively studied [133, 208, 209]. This work aims to develop nanoscale LbL carriers based on commercially available hydroxyapatite (HA) nanoparticles (<200 nm).

A schematic illustration of the steps involved in the functionalization of HA nanoparticles by LbL technique is depicted in figure 32. Hydroxyapatite nanoparticles (<200 nm) was chosen as template for the LbL coating (fig. 32a). The colloidal particles were incubated with cationic polyelectrolyte (pARG) to facilitate layer formation on the template particles (fig. 32b). Subsequently the unabsorbed polyelectrolytes are removed leaving behind the positively charged template. The next step involves the introduction of anionic polyelectrolyte layer (DXS) to the colloidal templates (fig. 32c). Additional layers were deposited by repeated deposition cycles, making use of the surface charge reversal that occurs upon adsorption of each layer (fig. 32d). The conformation of LbL carriers offers the possibility to encapsulated functional molecules within the multilayer shells based on electrostatic interactions. For example in the case of DNA, that is negatively charged is replaced in the place of anionic polyelectrolyte (fig. 32e). On a similar note, proteins can also be used to replace the polyelectrolytes based on electrostatic interactions. Finally, HA nanoparticles coated with multilayer shells that represents a functional scaffold are obtained that are used as carrier agents for intracellular delivery applications (fig. 32f).

Figure 32: Schematic illustration for the preparation of LbL coated HA nanoparticles. (a) HA nanoparticles (<200nm) chosen as templates for fabrication of nano size LbL carriers. The HA particles are then coated via consecutive assembly of oppositely charged polyelectrolytes. (b) Poly-arginine- hydrochloride (pARG) was used as a cationic layer and (c) dextran sulfate sodium salt (DXS) as the anionic layer. (d) LbL coating steps were repeated until desired number of multilayers is formed. (e) Bioactive molecules such as plasmid DNA (pDNA) and proteins are encapsulated in multilayer shells either as polycation/polyanion based on electrostatic attraction. (f) Finally, HA nanoparticles coated with multilayer shells that represents a functional scaffold are obtained that serve as carrier agents for intracellular delivery applications.

5.2.1 Physicochemical characterization of LbL HA nanoparticles

The first step lies in the selection of the polyelectrolytes for the LbL assembly. The adsorption of the polyelectrolytes on the HA nanoparticles is the predominant step for carrier fabrication. The LbL approach is based on the alternate deposition of charged materials through electrostatic interactions. The first step lies in the selection of a suitable polyelectrolyte pair for the LbL assembly. Polyarginine hydrochloride (pARG)/poly-L-lysine hydrobromide (PLL) and dextran sulfate (DXS) were found to be optimal for LbL assembly with the least aggregation. Figure 33 depicts the FESEM picture of hydroxyapatite (HA) nanoparticles coated with 2 bilayers of pARG/DXS. The FESEM image is used to demonstrate the monodisperse and uniform nature of the nanoparticles after the coating steps.

Figure 33: FESEM image of HA nanoparticles coated with 2 bilayers of pARG/DXS.

The multilayer assembly was monitored by zeta potential measurements after coating each layer. As shown in figure 33, HA nanoparticles are slightly positive with a zeta potential of $\sim 0.77\text{mV}$ and the first layer was started with DXS. The net charge reversal obtained after every layer clearly confirms the multilayer buildup on the nanoparticles.

Figure 34: Zeta potential of HA nanoparticles consecutively coated with polycation pARG (0.5mg/ml) and polyanion DXS (0.5 mg/ml). The charge reversal obtained after every layer confirms the multilayer formation on the HA nanoparticles. Each error bar represents standard deviation from triplicate measurements.

Besides ζ -potential measurements, the layer build up was monitored by flowcytometry using fluorescent-labeled polyelectrolyte layers. The fluorescence intensity of nanoparticles coated with different number of pARG^{RITC} layers were analyzed by flowcytometry.

Figure 35: LbL coating on HA nanoparticles. Histograms of flow cytometric measurements of HA nanoparticles coated with RITC labeled pARG multilayers. The fluorescence intensity of the particles was monitored after coating each 1 (red curve), 2 (green curve) and 3 (blue curve) layers of pARG^{RITC}.

A linear increase in the fluorescence intensity was observed as the pARG^{RITC} layer number increased, which unequivocally confirms the layer buildup on the nanoparticles (figure 35).

5.2.2 Functionalization of LbL-HA nanoparticles

The multilayered architecture of the shells allows the design of shells with functionalized active agents for therapeutic purposes.

5.2.3 LbL nanoparticles as protein carriers

Besides conventional polyelectrolytes as layer materials, model proteins BSA and lysozyme were employed to replace the respective polyelectrolyte based on the surface charge. Figure 36 demonstrates the qualitative confirmation of protein encapsulation in the multilayer shells as monitored by the zeta potential measurements. The first two layers were pARG and DXS, followed by the proteins of appropriate opposite charge. BSA being negatively charged at neutral pH, was used to replace the negative layer following the pARG layer as shown in figure 36.

Figure 36: Zeta potential of HA nanoparticles consecutively coated with (a) lysozyme replacing the cationic layer and (b) BSA replacing the anionic layer. The charge reversal observed after each layer confirms the assembly of proteins in the multilayers. Each error bar represents standard deviation from triplicate measurements.

The pARG layer had a ζ -potential of +38mV, which was shifted to -10mV following BSA coating (fig.36b). In a second approach, lysozyme was employed to replace the positive layer. As shown in figure 36a, the ζ -potential of lysozyme layer shifted to -13mV from -38mV of the previous DXS layer. The shift in the surface charge observed after each layer confirms the protein assembly in the multilayers.

The encapsulation efficiency of the 2 proteins followed different patterns.

Figure 37: Encapsulation efficiency of BSA and lysozyme coated on HA nanoparticles. The coating pattern is as follows
 HA/pARG/DXS/Lys/DXS/Lys/DXS/Lys/DXS -
 HA/pARG/DXS/pARG/BSA/pARG/BSA/pARG/BSA -

As demonstrated in figure 37, lysozyme had a uniform efficiency of ~68% for all the 3 layers whereas BSA showed different trends in encapsulation. The first layer of BSA had an efficiency of 90%, whereas the second layer dropped down to 66% and the third layer further down to 40%. This could be attributed to the weak interaction between the pARG and BSA. The amount of protein held in the three protein layers per unit amount of nanoparticles is denoted in terms of the loading amount (table 7).

Table 7: Loading amounts of BSA and Lysozyme in HA nanoparticles.

Coating pattern on HA nanoparticles	Loading amount ($\mu\text{g}/\text{mg}$ of particles)
HA/pARG/DXS/Lys/DXS/Lys/DXS/Lys/DXS	991 ± 12.56
HA/pARG/DXS/pARG/BSA/pARG/BSA/pARG/BSA	1027.41 ± 11.59

The prime advantage of the LbL encapsulation method is that it does not involve any covalent linkages or any modification that alters the therapeutic molecules as the entire process is driven by electrostatic interactions. Moreover increasing the layer numbers without an increase in the particle count can increase the protein loading. In addition, the coating steps and protein encapsulation were carried out at mild buffer

conditions unlike other biodegradable particulate systems such as PLGA. The fabrication steps using PLGA requires harsh conditions such as ultrasonication, freeze-drying and exposure to toxic solvents, where there is a substantial loss in the activity of the encapsulated protein [210, 211].

5.2.4 Cellular interaction of LbL-HA particles

5.2.4.1 Cell viability

The influence of the LbL nanoparticles on the cell viability was evaluated using the MTT assay. HEK293T cells were treated with different concentration of nanoparticles coated with PLL/DXS and pARG/DXS multilayer pairs to assess the cell viability. The results are presented as percentage viability as compared to the control cells (figure 38).

Figure 38: Cell viability of HEK293T cells treated with HA nanoparticles coated with pARG/DXS and PLL/DXS multilayer shells. Cell viability was determined by MTT assay. Measurements were corrected for O.D of the background. Results are presented as percentage of viability relative to control (Equation 1). Each error bar represents standard deviation from triplicate measurements. The coating configuration of the particles is as follows HA/pARG/DXS/pARG/DXS/pARG.

The nanoparticles showed no cytotoxic effects even at concentrations twice as high as ones used for transfection experiments as the polyelectrolytes chosen were biocompatible and biodegradable [137]. The biodegradability and biocompatibility of the carrier materials is a major determinant in the design of particulate carriers for therapeutic applications. Some of the carriers such as gold nanoparticles, quantum dots, and carbon

nanotubes serve as excellent drug delivery carriers for *in vitro* purposes; however, they are limited in the *in vivo* applications due to potential cytotoxicity [212-214]. The development of functional biocompatible and biodegradable systems is a significant ongoing challenge in particulate systems.

5.2.4.2 Intracellular processing of LbL particles

A schematic illustration of the intracellular processing of nanoparticles is demonstrated in figure 39. On internalization, the multilayer shells on the nanoparticles are supposed to defoliate which mediates the release of the bioactive molecules in the intracellular milieu. Following release, the molecules are to be translocated to their desired target organelles. In our application, the DNA released from the shells should reach the nucleus in order to achieve the desired transfection and expression of the fluorescent protein.

Figure 39: Schematic illustration of gene delivery using LbL nanocarriers demonstrated in 3 steps.

1. HA nanoparticles with functionalized multilayer shells are treated with HEK293T cells.

2. Intracellular uptake and delivery of cargo molecule. The DNA in the LbL shells of the nanoparticles are released following cellular uptake and delivered to the desired intracellular target organelle, nucleus.

3. Gene expression. The DNA (pDsred) delivered by the nanocarriers induces the expression of the red fluorescent protein.

5.2.5 Confocal Microscopy

5.2.5.1 Cellular uptake and intracellular processing

One of the major limitations of gene carriers is poor cellular uptake, which can be enhanced by suitable choice of surface functionality. To study the cell/particle interaction, plasmid DNA pDsred as well as FITC labeled polyelectrolyte layer were incorporated into the multilayer as a reporter and the successful transport and release of the plasmid

DNA was tested by the expression of DsRed. Confocal microscopy was used to investigate the particle uptake by the cells and to examine subsequent intracellular delivery of the reporter plasmid. The last layer was chosen to be cationic pARG/PLL since the positively charged outer layer is known to enhance cellular uptake by a factor of two as compared to an anionic outer layer (DXS) [15]. Figure 40 shows CLSM images of the cellular uptake of the labeled nanoparticles (fig. 40a) as well as the expression of the fluorescent proteins (fig. 40b), clearly proving the successful cellular uptake and release of the intact plasmid DNA molecules.

Figure 40: Confocal Laser Scanning Microscopy (CLSM) of HEK 293T to demonstrate cellular uptake of LbL nanoparticles and their subsequent DNA delivery. The images correspond to the overlap of FITC and RITC signal from the CLSM. (a) The HA nanoparticles are coated with FITC- labeled polyelectrolyte layer and the cell membrane stained using cell mask orange membrane stain, which demonstrates the cellular uptake of the LbL nanoparticles. (b) CLSM image to demonstrate the protein expression mediated by nanoparticles. The nanoparticles are labeled with FITC labeled polyelectrolyte layer and DNA (pDsRed) encapsulated in the multilayer. Successful defoliation of the DNA at the intracellular compartments induces the expression of the reported protein DsRed, presumably after nuclear penetration.

5.2.6 Flow cytometry

5.2.6.1 Cellular internalization of LbL-HA nanoparticles

Quantitative measurements via flowcytometry reveal that nanoparticles coated with pARG and DXS demonstrate a significantly higher uptake as compared to PLL and DXS multilayers (fig. 41), which in both cases increases linear with the particle concentrations. The simultaneous recording of fluorescence emitted from labeled LbL-HA nanoparticles and forward scattering intensity allows quantification of the particles

taken up by the cells. Unequivocal distinctions between internalized and surface adherent particles were made possible by trypan blue quenching. The plasma membranes of living cells are impermeable to trypan blue and therefore, only the fluorescence of those particles that are attached to the cell surface from the bulk will be quenched. Analysis of the cellular uptake parameters further reveals that only about ~30% of the PLL/DXS coated particles are taken up by the cells as compared to pARG/DXS, which is probably due to the cell penetrating properties of poly-arginine [215].

Figure 41: Cellular uptake profile of LbL coated of HA nanoparticles as a function of particle concentration. The G.mean (fluorescence intensity) of the nanoparticles taken up by the cells is plotted against concentration of LbL coated HA nanoparticles.

The nanoparticles were coated with 2 different multilayer pairs and the coating pattern is as follows

HA/ PAH^{FITC}/DXS/ pARG/DXS/pARG /DXS/pARG -
 HA/ PAH^{FITC}/DXS/PLL/DXS/PLL/DXS/PLL -

5.2.6.2 Transfection efficiency of LbL-HA carriers

Apart from the cellular uptake it is important to understand the intracellular trafficking and chemical environment to which the functional molecules are exposed. The therapeutic effect of most bioactive molecules occurs at the targeted organelles, so these molecules must preserve their functionality inside complex intracellular milieu. The ingested particles may be taken up inside various intracellular compartments apart from the desired targets. In our study we investigated the intracellular delivery of reporter

pDNA encapsulated within the LbL coated nanoparticles. For successful delivery of the pDNA, the ingested carriers should evade the endosomal compartments and reach the cytosol, and then release the pDNA to the target site, nucleus.

The encapsulation efficiency of pDNA in the nanoparticles was $\sim 96\%$ and the loading amount was $\sim 10.6 \mu\text{g}/\text{mg}$ of nanoparticles, for both the multilayer pairs (pARG/DXS and PLL/DXS). The pDNA was coated as a negative layer and a final top layer of cationic polyelectrolyte was coated on the DNA layer to enhance the cellular uptake. CLSM image of the reporter protein expression mediated by the DNA loaded nanoparticles is shown in figure 40b. For quantitative information on the transfection efficiency using these non-viral vectors, FACS was employed.

To study the cell/particle interaction quantitatively, pDsRed was incorporated into the multilayers and the transfection efficiency was compared with the commercially available transfection reagent Lipofectamine 2000TM via flowcytometry. Figure 42 shows the relative transfection efficiency of nanoparticles coated with two different multilayer pairs. Although the DNA loading amount is similar for pARG/DXS and PLL/DXS multilayer pairs, the transfection efficiency of PLL/DXS is significantly lower as compared to pARG/DXS, which is consistent with lower cellular uptake of PLL/DXS coated particles (fig. 41).

Figure 42: Evaluation of the transfection efficiency of three different types of vectors in HEK293T cells. Percentage of transfected cells over 3 days after incubation of HA nanoparticles coated with 2 different multilayer pairs and LipofectamineTM as positive control. The transfection efficiency is plotted as function of a DNA delivered by means of 3 different vectors. The coating pattern on HA nanoparticles are as follows

HA/ pARG/DXS/pARG /**pDsRed**/pARG -
HA/ /PLL/DXS/PLL/**pDsRed**/PLL -

5.2.7 LbL-HA nanoparticles- significance over polyplexes

In order to investigate the significance of the LbL-HA nanoparticles, we sought to test the polyplexes composed of PLL and pARG at various N/P ratios and associated cytotoxicity. The transfection efficiency of the polyplexes was analyzed at varying N/P ratio of the cationic polyelectrolytes (poly-L-arginine hydrochloride and poly-L-lysine hydrobromide). N/P ratios of 2,5,10,15,20,30,40 and 80 were tested for transfection efficiency and cytotoxicity analysis. It was interesting to note that the complexes exhibited very low transfection efficiency compared to the LbL-HA particles. In the case of pARG/DNA complexes, the highest transfection efficiency was ~3% at a N/P ratio of 15 whereas for PLL/DNA complexes it was found to <1% at all N/P ratios with the highest (~ 0.8 %) at N/P ratio of 40.

The same polycations used in LbL approach exhibited different transfection patterns, which is mainly attributed to the packaging mechanism employed. In the case of complex formation, there is a random alignment of the DNA/polycation complex that may not favor the cell uptake and intracellular gene expression. On the other hand, the LbL methodology aids in facile packing of the components in the multilayer system, for e.g. the outermost cationic layer helps in the cell internalization after which the underneath DNA is released for gene expression.

In order to further compare the efficacy of the system apart for transfection efficiency, we studied the cell viability of HEK 293T cells towards the polyplexes. The aim of the study was to investigate whether the complexes were cytotoxic at particular N/P ratios compared to transfection efficiencies.

Figure 43: Cytotoxicity of the polyplexes comprising of polyelectrolyte-DNA complexes at various N/P ratios. Cell viability was determined by MTT assay. Control population represents the untreated cells. Results are presented as percentage of viability relative to control cells.

The polyplexes, pARG/DNA and PLL/DNA exhibited different cytotoxic patterns, with pARG/DNA the cell viability reduced to ~88% at N/P ratio of 40 and further down to ~61 % at N/P ratio of 80. In the case of PLL/DNA complexes, the cell viability was reduced to ~78% at N/P ratio of 80 (fig. 43). The higher cytotoxic activity of pARG complexes is in a way attributed to the cell penetrating activity of the arginine residues compared to the lysine residues in the PLL complexes.

Taken together these studies serve to further exemplify the significance of the LbL technology as compared to the conventional methods for gene delivery and further studies on investigating the intracellular trafficking pathways are reported in the next part of this chapter.

5.2.8 pARG vs. PLL: Role in transfection

In a different study employing the comparison of internalization of arginine-rich Tat peptide and lysine rich NLS (Nuclear localization Signal) peptide, the lysine-rich peptide sequences had a much lower degree of internalization compared to arginine rich residues [215]. It was reported that the arginine rich moieties present in the RNA binding proteins of viruses such as HIV-1 (Tat peptide), flock house virus (FHV) are the key players in cell membrane translocation and intracellular uptake [215, 216]. Our results are in good agreement with the previous studies and a similar profile is observed in our study where the arginine rich pARG/DXS pair had higher uptake when compared to the nanoparticles coated with PLL/DXS (fig. 41). Several other researchers have also

explored the cell penetrating activities of the arginine sequence by using arginine/DNA complexes alone as such and in surface modification of a few other vectors such as chitosan, dendrimers [191, 192] to increase the transfection efficiency.

Apart from the cellular uptake, the choice of the cationic layers employed for DNA complexation does have a major impact at the intracellular level. Engineering the multilayers for endosomal escape is fundamental for gene delivery in particulate systems. In our system, endosomal uptake plays a negligible role since both lysine and arginine residues have inherent property of endosomal evasion [217]. In this aspect, once released from the endosomal compartments the polyelectrolyte complex should subsequently dissociate from the pDNA, which can then enter the nucleus for efficient transgene expression.

The decondensation of the packed DNA in the cytosol is a crucial factor for efficient delivery. Formation of a tight complex with the carrier prevents DNA release at the intracellular environment that renders the carriers invalid as delivery agents merely serving as encapsulating agents. Although lysine and arginine contain the same positive charges, the molecular mechanism that underlies the DNA complex formation is quite different. In lysine, all the positive charges contribute to the DNA complex and binds more tightly to the DNA owing to the highly branched nature, on the other hand only partial cationic residues from arginine are involved in the interaction with DNA [218]. This packaging mechanism in lysine residues further induces more loss of conformational entropy of the DNA molecule due to the subsequent folding and bending during complex formation [219]. These findings support the fact that the high cellular uptake and facile release of DNA from the arginine multilayers aid in high transfection efficiency compared to its counterpart PLL.

Furthermore, arginine and lysine residues have been shown to entirely follow different condensation and release patterns *in vitro*. The lysine residues condense DNA into monomolecular and multimolecular structures whereas arginine residues predominantly form multimolecular structures and completely release the DNA [217], which correlates with the high transfection efficiency observed with pARG/DNA compared to PLL/DNA complex (fig. 37). Although the above mentioned nanocarriers are not as efficient as lipofectamine in transfection efficiency, liposomes are limited by certain factors such as poor serum stability [220] and possibly toxicity issues [221].

The exploitation of LbL technology in gene delivery applications is still in its infancy. Several studies have been reported on the fabrication of DNA functionalized LbL carriers [143, 222-224], however the release (i.e., defoliation) parameters have only been studied under different conditions (e.g. ionic strength, salt concentrations) and only *in vitro* [143, 224]. So far only two studies have been reported at the cellular level. The biological activity of the DNA at the intracellular environment was reported by Reibetanz *et al* [15] using silica particles and CaCO₃ particles coated with PRM/DXS multilayers in our previous study [132]. However transfection efficiency using the carriers were very low (3~5%) [15]. The particles used were in the micron size range, which tends to accumulate in the endolysosomes, where they are exposed to degrading enzymes that prevent the intracellular delivery of the pDNA [132]. Besides the choice of the core, the LbL shell also plays a pivotal role in the intracellular drug delivery applications, since the encapsulation and release of the bioactive molecules is directly dependent on the multilayer components. Here in our study we demonstrated highly efficient gene carriers designed on the LbL platform in the nano size range with transfection efficiency ~ 40 % and further refinement of the multilayer system will result in highly sophisticated carriers for drug delivery applications.

5.2.9 Mechanisms of cellular uptake

5.2.9.1 Plasma membrane- “Regulated portals for cellular entry”

Eukaryotic cells possess a complex uptake system that comprises plasma membrane and associated proteins that allow them to internalize macromolecules/particles from the extracellular space through distinguished pathways to further shuttle them to various intracellular compartments to afford their metabolic utilization. Nanoparticles are internalized into cells by a variety of mechanisms and their intracellular fate is usually linked to the entry mechanism. The intracellular organelles and the membrane trafficking routes connected with the plasma membrane must together be seen as one complex system that allows the intracellular transport and material processing towards the desired outcome (fig. 44). Therefore, the successful design of a non viral gene delivery system requires a deep understanding of gene/carrier interactions as well as the mechanisms involved in the interaction of the systems with the target cells.

Figure 44: Schematic illustration of an LbL-HA nanoparticle interacting with lipid bilayer of the cell membrane. The multilayer shells interacting with the cell membrane guides the cell to chose the specific uptake pathway for intracellular processing.

In this study we aim to delineate the intracellular trafficking of this system in order to identify the molecular pathways associated with the uptake and intracellular processing. From the initial studies employing LbL functionalized HA nanoparticles for gene delivery, we found that particles comprised of pARG/DXS multilayers to be the best for gene expression, with almost 50% transfection efficiency as compared to Lipofectamine™. Based on our initial studies on using pARG as a layer material we further proceed to study the molecular mechanism governing the cellular interaction of the nanoparticles coated with pARG multilayers.

5.2.10 Pathways of cell uptake

The dynamic formation of membrane invagination/domains within the matrix of lipid bilayer has been considered to be an important regulator of molecular interactions in membrane trafficking. The plasma membrane has a complex secretory pathway by which cells can sequentially and in a temporally regulated way initiates signaling mechanisms and synthesize proteins; those which regulates the mechanism by which the compartments or vesicles undergo fusion and fission with each other to exchange the cargo they bear. Two modes of uptake mechanism exists of which endocytosis is the inherent natural and predominant pathway whereas non-endocytic pathways have recently

evolved as strategy to cross the cellular barrier (fig. 45). Endocytosis comprises of the events that lead to the internalization and trans barrier delivery of macromolecules.

Figure 45: Schematic illustration of the various pathways involved in the cellular uptake of macromolecules [225].

Endocytosis is subdivided into the process of phagocytosis and pinocytosis. Phagocytosis is the ingestion of relatively large particulates such as microorganisms by specialized cell types such as macrophages, dendritic cells, neutrophils etc. that are involved in the body's defense mechanism. On the other hand, pinocytosis occurs in all cells that refer to the internalization or invagination of plasma membrane domains from the cell surface to internalize cell bound molecules and any associated fluid to the cell. Pinocytosis includes clathrin mediated, caveolae mediated, macropinocytosis and clathrin/caveolae independent endocytosis. The endocytic pathway differs with regard to the size of endocytic vesicles, nature of carriers (ligands, receptors and lipids) and the mechanism of vesicle formation. On the other hand, non-endocytic mechanism involves energy independent process such as cell membrane penetration, membrane fusion, microinjection and electroporation that facilitates entry to the cell.

5.2.11 Endocytic pathways

To elucidate the mode of cellular uptake, we first compared the effects of different metabolic inhibitors blocking different types of endocytic pathways. Further elucidation of these pathways can be used to identify the bottlenecks associated with the system that enables them for an enhanced efficiency based on the LbL system.

Figure 46: Multiple portals for cellular entry of LbL-HA nanoparticles. Nanoparticles may enter cells via two major mechanism namely endocytosis dependent and non-endocytic mechanism. The endocytotic pathway can further be divided into phagocytosis and pinocytosis. Pinocytosis is prevalent in all the cell types whereas phagocytosis is restricted to specialized cell types such as macrophages. Pinocytosis includes clathrin mediated, caveolae mediated, macropinocytosis and clathrin/caveolae independent endocytosis. The endocytic pathway differs with regard to the size of endocytic vesicles, nature of carriers (ligands, receptors and lipids) and the mechanism of vesicle formation. Non-endocytic mechanism involves energy independent process such as cell membrane penetration, membrane fusion, microinjection and electroporation that facilitates entry to the cell.

5.2.12 Inhibition of endocytic pathways

A quantitative assessment of the contribution of each endocytic pathway to the overall cellular uptake is essential for elucidating the corresponding intracellular behavior of the drug carrier. Specifically this may allow one to relate the endocytic pathway to the overall fate, ranging from uptake of a gene carrier to its intracellular routing and associated transfection efficiency (fig. 46).

Figure 47: Influence of metabolic inhibitors on the cellular uptake of LbL-HA nanocarriers. Cells were pretreated with specific endocytic inhibitors; amiloride (inhibits macropinocytosis [207]), methyl- β -cyclodextrin (MBCD) (inhibit caveolar pathway by depletion of cholesterol [205]), cytochalasin D (inhibitor of caveolae and macropinocytosis by actin depolymerization [205]), chlorpromazine-Hcl (specific inhibitor of CME [205]) and dynasore (inhibits dynamin, involved in the vesicle formation process in CME[206]). Subsequently fluorescent-labeled LbL-HA nanoparticles were added and incubated for 4 h at 37°C followed by FACS analysis. 10^4 cells were analyzed in each measurement and the fluorescent intensity of control cells (without inhibitors) was set as 100%. The percentage uptake of cells subjected to inhibitor treatment is expressed relative to control cells.

Figure 47 represents the internalization of LbL-HA nanoparticles by HEK 293T cells in the presence of inhibitors of various endocytic routes; clathrin, caveolar and macropinocytic pathways. Pretreatment of cells with amiloride (an inhibitor of macropinocytosis) and methyl- β -cyclodextrin (that inhibit caveolar pathway by depletion of cholesterol [226]) had minor effects, where the cellular uptake reduced to about ~76% and ~69% respectively. Furthermore cytochalasin D which is known to inhibit actin polymerization, reduced the uptake to about ~43%. By contrast chlorpromazine (an specific inhibitor of clathrin mediated endocytosis (CME) and dynasore (inhibitor of dynamin, involved in the vesicle formation process in CME) had a major effect on the internalization reducing the uptake to ~19 % and ~22% respectively. These datas are fully compatible with a major role of clathrin-mediated endocytosis in the internalization of LbL-HA particles.

Brief interpretations of the results are explained above; a detailed analysis of the uptake pathways and their implications in intracellular fate of the cargo molecule are explained further. Fundamental questions in the intracellular trafficking of NPs with cells include whether and how, NPs map onto these pre-existing cellular uptake mechanisms.

Despite the marginal effect of other inhibitors on NPs cellular uptake, blocking the CME pathway resulted in almost 80% drop reduction in the particle uptake.

The events involved in the endocytic pathways and the mechanism of the inhibition are explained in the following subsection; finally the discussion outlines the preexisting and desired uptake pathway of the future.

5.2.12.1 Clathrin Dependent Pathway

Clathrin mediated endocytosis (CME) involves the clustering of the ligand-receptor complexes in clathrin coated pits on the plasma membrane. The clathrin coated vesicles then depolymerizes, resulting in early endosomes, which fuse with each other or with other preexisting endosomes to form late endosomes that further fuse with lysosomes (fig. 46). Particles entering via this pathway will rapidly experience a drop in pH from neutral to pH 5.9 to 6.0 in the lumen of early endosomes, with a further reduction to pH 5 during progression from late endosomes to lysosomes. Within the endosomes, ligands and receptors are sorted to their appropriate cellular destinations, such as lysosomes, Golgi apparatus, the nucleus, or the cell surface membrane. In our study, chlorpromazine was employed as a chemical inhibitor for CME. Chlorpromazine is a cationic amphiphilic drug which inhibits clathrin-coated pit formation by a reversible translocation of clathrin and its adapter proteins from the plasma membrane to intracellular vesicles [227].

On the other hand, the vesicles involved in the CME pathway, are pinched off in a controlled mechanism that involves several proteins including dynamin. Dynamin is a GTPase that forms a dynamin ring at the stem connecting the nascent vesicle to the plasma membrane. Dynamin's contraction and twisting results in the scission of the endocytic vesicle. Dynasore, a dynamin GTPase inhibitor that prevents the scission of dynamin-dependent endocytic vesicles [228], The GTPase dynamin is required for the CME as it collaborates with coat constituents in mediating vesicle budding [229]. Dynasore was used alongside with chlorpromazine to investigate the role of CME pathway.

5.2.12.2 Caveolae dependent pathway

Caveolae/raft mediated endocytosis involves the formation of small, flask shaped invaginations termed as caveolae, at the plasma membrane that are hydrophobic membrane micro domains rich in cholesterol and glycosphingolipid. These are in fact the components essential for the formation and stability of caveolae. Depletion of cholesterol

from the plasma membrane using chemical agents such as methyl- β -cyclodextrin, nystatin prevents caveolae formation [226], that are useful in blocking certain endocytic pathways. The vesicles segregate from caveolin and are sorted out of caveosomes for delivery to the intracellular organelles (fig. 46). The caveosomes are nonacidic and neutral in pH as compared to the endosomes involved in CME [230]. Methyl- β -cyclodextrin a cyclic oligomer of glucopyranoside that inhibits cholesterol dependent endocytic processes by reversibly extracting the steroid out of the plasma membrane [231].

5.2.12.3 Macropinocytosis

Macropinocytosis is a non-selective endocytic mechanism that involves the formation of membrane protrusions or ruffles generated by actin driven invagination of the plasma membrane. Ruffling is dependent on actin cytoskeleton; therefore drugs that disrupt the actin cytoskeleton can inhibit. Although actin cytoskeleton is inevitable for CME in yeast, the disruption of actin filaments in mammalian cells have a partial effect on the clathrin coated vesicle formation [232, 233]. In addition arginine rich peptides have also been shown to induce the F-actin organization and hence the disruption of actin cytoskeleton has a significant effect on the cellular uptake pathway [234]. This particular effect of cytochalasin D on the CME pathway is related to the significant drop in the cellular uptake of LbL-HA particles, where the cells pretreated with cytochalasin exhibited significant drop in the particle uptake, 43 % (fig. 47).

5.2.13 Discussion on endocytic pathways

Having demonstrated that dynasore and chlorpromazine was capable of blocking endocytosis of LbL-HA nanoparticles to a major extent, CME was found to be the predominant uptake pathway. However CME is not the desired route of uptake for many applications, as the particles have to endure the endosomal compartments that may rather render the biomolecules inactive and prevent them from reaching the cytoplasm. Exploring and targeting new receptors that can be internalized by clathrin independent mediated endocytosis are likely to provide high efficient systems as they are relatively unaffected by lysosomal degradation. On the contrary, caveolar pathway is desired as the intermediary vesicles, caveosomes are neutral in pH that is inert to the bio actives and permits intracellular delivery of macromolecules. After summarizing the different pathways of cellular internalization and their association with LbL-HA nanoparticles, we now consider the available knowledge with the current research in the design of next generation carriers. Besides the nature of material employed, another interesting factor

was found to affect the cellular uptake.

5.2.14 'Size does matter' for endocytic pathways

The uptake pathways have been linked partially to the size of the carrier molecules despite the material composition. Orgis et al demonstrated that polyplexes of DNA/PEI (>500nm) were more efficient in gene transfection as compared to small polyplexes. Although the polyplexes were of the same material, the higher transfection efficiency in larger molecules are mainly attributed to their size [235]. On a similar note, a study by Rejman *et al* studied the size dependence effect on the cellular uptake of ligand-devoid particles. Microspheres (<200nm) were found to be internalized via CME and translocated to the endolysosomes, whereas particles (500nm) entered the cells via caveolar pathway and thereby evading the endosomal compartments [236]. The results are in agreement with our findings reported using HA nanoparticles (<200nm), and hence it is hypothesized that increase in the template size by a few hundred nanometers result in higher transfection by evading the CME pathway.

Understanding the mechanisms that regulate uptake of these nanoparticles will probably help to extend the utility of these carriers in intracellular targeted drug delivery applications. The combined effects of complex size, composition on internalization pathways requires additional investigation, as knowledge of these interactions is inevitable for design of effective transfection vectors. Having reviewed on the mode of endocytic pathways and their associated mechanism, we now move on to discuss the non-endocytic pathways involved in the uptake LbL-HA nanoparticles.

5.2.15 Non-endocytic mechanisms

Endocytosis is the major pathway for the cellular uptake of particulate drug carriers. It is a temperature dependent pathway and is usually suppressed at low temperature. Therefore it can be strongly inhibited by lowering the temperature to deplete the ATP pool [237]. Recently a new class of compounds termed as membrane permeable peptides (or) cell penetrating peptides (CPP) has recently evolved as carrier molecules. CPPs have the ability to cross the plasma membranes of mammalian cells in an apparently energy and receptor-independent/non-endocytic fashion, the characteristics of which were utilized for the delivery of macromolecules into cells. Consequently there has been a rapid development over the past few years in the potential of CPPs to act as vectors for the delivery of chemically conjugated biomolecules such as peptides or

oligonucleotides.

In order to find out the existence of a non-energy uptake pathway; the particles were treated with control cells (at 37°C) and 4°C to quantify the differences in the uptake profile.

Figure 48: Cell penetrating property of LbL-HA nanoparticles. Quantitative comparison of the uptake of nanocarriers treated with cells at normal physiological conditions at 37°C (*green curve*) and at 4°C (*red curve*). Cells were pre-incubated for 1h at 4°C, and subsequently treated with the nanoparticles, also at 4°C for 4 h, followed by FACS analysis. Representative histogram of three individual experiments is shown.

Figure 48 demonstrates the differential uptake profiles of cells that have taken up particles via energy independent and independent ways respectively. It was interesting to note that the particles were taken up at 4°C, which elicit the existence of cell translocation activity. The cells at 37°C had a G.mean of ~115 a.u, whereas the cells at 4°C had a significant drop in the cellular uptake of particles with the G.mean value of ~33 a.u. From these results it could be interpreted that approximately 29% of the particles are taken up by non-endocytic pathway. Almost all the studies reported on CPPs employ only qualitative studies by using fluorescence microscopy to detect the cellular uptake/cell penetration at 4°C and hence there is a lack of information to match them with our quantitative datas. However there was difference between the two suggesting only a minor role-played by the membrane translocation activity, whereas the endocytic pathways play the significant role. The membrane translocation activity exhibited here is reported for the first time using LbL carrier, which is attributed to the arginine residues present on the outer layer. A very recent study explained the cell penetration activity of

arginine residues by employing transmission electron microscopy (TEM) to image the endocytic pits formed during cellular uptake of nanoparticles (250 nm) with different surface coating such as polylysine, polyethylenimine (PEI) and polyarginine. It was interesting to observe that the nanoparticles modified with polylysine and PEI were entering the cell through endocytic pathways whereas polyarginine coated particles appeared to transcytose across the cell membrane in the absence of independent endocytic engulfment [238]. Cell penetrating moieties have recently evolved and hence a brief introduction on their origin and their interference with the polyelectrolyte poly-arginine are explained further.

5.2.16 Introduction to cell penetrating peptide (CPP)

The concept of CPP originated from the Tat peptide, a basic peptide derived from human immunodeficiency virus (HIV)-1 Tat protein (48-60) was reported to have the ability to translocate through cell membranes and accumulate in the nucleus. One of the premier studies employing Tat peptide was the Tat- β -galactosidase fusion protein with a molecular weight of 120 kDa, which resulted in the delivery of protein with β -galactosidase activity to various tissues in mice and even in the brain with the ability to cross the blood brain barrier [239]. This pioneering study opened the way to the protein transduction technology, thus widely broadening the potential for therapeutic application of proteins to intracellular targets.

5.2.17 Properties of CPP

Besides the potential of Tat-peptide as a drug carrier, the internalization mechanism was also intriguing whether the cellular uptake follows the endocytic pathway or through any undefined mechanism. For example, Tat peptide (GRKKRRQRRRPPQ) is a highly basic and hydrophilic peptide, which contains 6 arginine and 2 lysine residues in its 13 amino acid residue sequence. The peptide was able to translocate the cell membranes even within 5 min at a low concentration of 0.1 μ M, and the uptake was not inhibited even at 4°C [240]. The key properties of cell penetrating peptides were that of (i) an ability to act as a carrier for exogenous proteins and various membrane-impermeable molecules, (ii) rapid internalization within a few minutes, (iii) high extent of nuclear accumulation, (iv) entry into cells at 4°C with almost the same efficiency as at 37°C.

5.2.18 “Key players” of CPP – arginine residues

In order to find the key players in the Tat peptide sequence that help in the translocation, various analogs of the peptides were synthesized and tested for membrane translocation. These include the native Tat peptide (48-60), D-Tat its D-amino acid substituted analog, arginine-substituted analog (R_9 -Tat) where the residues corresponding to positions 49-57 were replaced with arginine, along with peptide sequences (rich in lysine residues) corresponding to nuclear localization sequences (NLS) derived from simian virus 40 and nucleoplasmin as references. It was interesting to find out that the D-Tat and R_9 -Tat were internalized into the cells as efficiently as native Tat peptide whereas the simian virus 40 and nucleoplasmin derived peptides showed much lower degree of internalization. These results proposed the fact that the arginine residues were the key components and the NLS sequences used as references ruled out the importance of lysine residues in the membrane translocation [215]. Subsequently a variety of RNA binding peptides derived from virus proteins such as HIV-Rev 1, flock house virus coat proteins, and the DNA binding peptides such as c-Fos and c-Jun were found to exhibit similar translocation ability as the native Tat peptide. The only common feature that existed among these peptides is that the sequences are rich in arginine, and the guanidine groups in the arginine are responsible for the cell penetrating ability [216, 241].

These findings were further extended by synthesizing peptides composed of arginine residues alone termed as polyarginine (R_n) and were tested for the translocation efficiency as a function of chain length (n). Interestingly the polyarginine peptides too demonstrated cell penetrating ability comparable to the Tat peptide, however the octarginine (R_8) residues demonstrated the highest translocation efficiency [215]. Based on these finding, we confirm the role of arginine residues in the pARG to impart the cell penetrating ability to the LbL-HA carriers.

It should however be noted that the particles exhibited the CPP property owing to the polyelectrolyte layer (pARG) in addition to the natural endocytic mechanism, and the CPP property also may have contributed to the increased cellular uptake of LbL-HA particles coated with pARG as compared to PLL (fig. 36). Although the Tat peptides (13 amino acid residues) had excellent cell penetrating ability, the properties were considerably altered after cargo fusion that induces different modes of cellular uptake into mammalian cells. The complexes or fusion of quantum dots (20 nm) and further down to globular proteins were mainly endocytosed whereas small non-globular proteins are taken

predominantly up membrane transduction as shown in figure 49. However they only suggest the impact of size on the profile of CPP activity and do not give any quantitative information.

Figure 49: Influence of molecular size and charge on cellular uptake through endocytosis vs. transduction. The mode of uptake is influenced by the charge of the CPP and the size of the cargo molecule attached to it [242].

In our studies the nanoparticles (200 nm) employed were ten folds higher to the quantum dots (20nm), that in a way demonstrates the major role (~80 %) of endocytic pathway (CME) compared to the CPP activity (~29 %). Taken together with the initial studies we could design carriers that are cell penetrating as a consequence of using pARG and employing a dedicated cell penetrating peptide may further increase the uptake and may aid to enhance the intracellular delivery of macromolecules.

5.2.19 Cell penetration - “Mechanism of membrane translocation”

The exact underlying mechanism for the CPPs has not yet been explained. However there are few hypothetical studies that explain them to happen by transient membrane perturbations coupled with formation of membrane structures (reverse micelles) increasing the membrane permeability in the presence of Tat peptide [243], lipophilic ion pair diffusions along the membrane potential [244] and more recently studies where the Tat peptides are able to combine cytoskeleton remodeling activity with membrane translocation. The dense packing of the cationic residues allows it to induce multiplex interaction with the membrane, actin cytoskeleton and cell-surface receptors to facilitate the translocation pathways [245]. However there has been no single mechanism involved in the cell uptake using CPPs and moreover the amount of research that explains the mechanistic pathways are way too less when compared to the studies explain the applicability of these as drug carriers for delivery of a variety of exogenous molecules such as drugs, proteins and oligonucleotides [246]. From an engineering perspective, the

design of next generation particulate carriers should be incorporated with versatile functionalities such as cell penetrating ability for therapeutic applications.

5.2.20 Defoliation pattern of DNA from LbL-HA particles

The aim of this study was to investigate another important aspect of the intracellular behavior of LbL HA nanoparticles; i.e. the effect of layers on controlling the release of the cargo molecules. Towards this goal, we decided to study the release behavior in two different milieus, to demonstrate the sustained effect achieved in the LbL technology.

5.2.20.1 *In vitro* release behavior

The release behavior of DNA was compared among LbL coated HA nanoparticles with the active compound DNA placed in different shells positions, i.e. with 1 layer on top of the DNA and the other with 3 polyelectrolyte layers over the DNA layer.

Figure 50: Cumulative released amount of plasmid DNA from LbL-HA nanoparticles with different shell composition as a function of incubation time. The coating pattern is as follows
 HA/pARG/DXS/pARG/DNA/pARG ($x=1$) and
 HA/pARG/DNA/pARG/DXS/pARG ($x=3$)
 where ($x=n$) denotes the number of polyelectrolyte layers on top of the DNA layer.

As anticipated, the release found to decrease with increasing layer numbers. After ~ 46 hours, about $\sim 7.6\%$ of the DNA has been released from particles ($x=1$) whereas the release of DNA from the particles with three layers on the DNA layer was only $\sim 6\%$ (fig. 50). The *in vitro* release behavior shows that with 2 more additional layers on top of the DNA layers causes a significant reduction in the release of the encapsulated compound. The ideology of the experiment is to mainly emphasize the sustained release pattern as the

experiments here are in simulated condition at 37°C and physiological saline, whereas there are a lot of governing parameters in the intracellular environment.

5.2.20.2 Intracellular DNA release

In order to further elucidate the DNA release patterns, the intracellular release behavior of DNA was studied in HEK 293T cells using pDNA with reporter characteristics that encodes for fluorescent proteins as employed for transfection studies previously. The DNA released from the shells is directly proportional to the amount of fluorescent cells.

Figure 51: Defoliation of pDNA from LbL-HA nanoparticles in HEK293T cells. The successful delivery of pDNA (pDsred) induces the expression of the red fluorescent protein; therefore the number of fluorescent cells is directly related to the amount of DNA released. The number of fluorescent cells is plotted as a function of different amount of nanoparticles coated with two multilayer shell configurations.

The coating pattern is as follows

HA/pARG/DXS/pARG/**DNA**/pARG (x=1) and

HA/pARG/**DNA**/pARG/DXS/pARG (x=3)

Where (x=n) denotes the number of polyelectrolyte layers on top of the DNA layer.

This study offered some insights to the defoliation patterns at the complex intracellular environment from LbL-HA particles through the delivery of nucleic acids that have reporter characteristics like plasmid DNA for the expression of fluorescent proteins. For particles with one layer coated on top of DNA layer the transfection (for e.g. at 20 and 30 μg), the number of transfected cells were ~10 folds higher than the particles with three polyelectrolyte layers on the DNA layer (fig. 51). This shows a time dependent defoliation of the multilayer shells, at the intracellular level. These studies mainly serve to

give a pattern and outlook of layer defoliation, which could be perceived with different dimensions. For instance, the outermost layer could be functionalized with a cell specific ligand for targeted delivery, in that case these carries serve to have dual roles, where the outermost layer can be utilized for cell targeting and the inner multilayer shells for cargo encapsulation. Moreover it can also be explored for added features such as co-delivery of protein and gene by altering the multilayers. The facile packaging of the biomolecules in LbL configuration is of significant advantage compared to the polyplexes and lipoplexes, not only in terms of transfection but also in added functionalities such as targeted delivery, sustained release etc.

5.3 SUMMARY

This chapter explains the fabrication and functionalization of HA (<200nm) nanoparticles with LbL technology for intracellular drug applications. The nano sized colloidal template was chosen as an alternative to the conventional micron sized templates in order to facilitate sophisticated intracellular applications such as gene delivery. The first step involved the identification a suitable polyelectrolyte system for LbL buildup, of which pARG/PLL and DXS were chosen as polycation and polyanion respectively. The multilayer shells offered the possibility of encapsulation of biomolecules based on electrostatic self-assembly. Besides conventional polyelectrolytes as layer materials, model proteins (BSA and lysozyme) and plasmid DNA were employed to replace the polyelectrolyte based on the surface charge, to demonstrate the feasibility of functional nanocarriers. Apart from being a carrier agent, these LbL particles were also investigated for the intracellular delivery of plasmid DNA encoding for reported fluorescent protein as a potential gene carrier. The DNA was coated an anionic layer and to identify an optimal carrier design, 2 shell materials/polycations (pARG and PLL) were used. The cytotoxicity of the carriers tested in HEK293T cells demonstrated that they were biocompatible and did not induce any toxic response in the broad concentration range tested. Qualitative and quantitative data on the cellular uptake and intracellular processing of LbL-HA particles were obtained by flowcytometry and CLSM respectively. The particles coated with pARG as a polycation demonstrated exceptional internalization and transfection efficiency as compared to PLL, and could achieve almost 50% transfection efficiency as compared to LipofectamineTM. Subsequently, we investigated the intracellular trafficking of the system in order to identify the molecular pathways associated with the uptake and intracellular processing. Having demonstrated that dynasore and chlorpromazine was capable of blocking endocytosis of LbL-HA nanoparticles to a major extent (~80%), CME was found to be the predominant uptake pathway. Besides the natural CME endocytic pathway, the LbL-HA carriers also exhibited membrane translocation activity that is attributed to the arginine residues. Finally the defoliation pattern of DNA from the multilayered shells in an *in vitro* and intracellular release model identified the feasibility of LbL-HA particles for sustained release and targeted delivery applications. To summarize, this study has highlighted the significant advantages of using nanoparticles in conjunction with LbL technology to overcome the disadvantages associated with conventional nanoparticulate system for drug delivery applications. This approach presents the potential to encapsulate a wide

range of therapeutic molecules such as proteins, DNA, siRNA without compromising the biological activity. In addition, the intracellular trafficking studies provide novel insights on the internalization mechanism of LbL-HA particles that are useful for the design and development of more sophisticated delivery systems for gene therapy. Taken together, these studies cover the entire spectrum of factors to be considered in the design of a particulate carriers for intracellular application ranging from encapsulation of bioactive molecules (proteins and nucleic acids), cytotoxicity analysis, cellular uptake, intracellular delivery (transfection efficiency), and in depth intracellular trafficking studies. In addition the studies provide new ventures for using a strategy involving the endogenous cellular signaling and uptake pathway for intracellular delivery of macromolecules.

Chapter 6: Surface functionalization of LbL multilayer shells with RBC membranes towards passive targeting

OVERVIEW

This chapter reports the fabrication and functionalization of membrane vesicles derived from human red blood cells towards targeted drug delivery applications. The vesicles were prepared from erythrocyte membranes with two different membrane orientations namely inside-out and right-side-out. Inside-out vesicles (IO) are those with inverted membrane orientation, whereas the right-side-out (RO) with the same orientation as compared to the parent RBC membranes. The vesicle orientation was confirmed by the presence of surface ligands phosphatidylserine (PS) and sialic acid with flow cytometer using ligand specific fluorescent markers. In a normal RBC, the membrane constituents such as phosphatidylcholine (PC), N-acetylneuraminic acid (sialic acid) are located in the outer monolayer whereas sphingomyelin (SM) and phosphatidylserine (PS). Hence detecting the ligand orientation was used to monitor differences in the membrane asymmetry. Subsequently, the stability of the vesicles in physiological conditions was analyzed. Following the fabrication of vesicles, they were investigated for phagocytic uptake with monocytes; the key players in immune system towards MPS clearance. Qualitative and quantitative information on the internalization of vesicles by monocytes were obtained using confocal microscope and flowcytometry. Finally the vesicles were introduced as a shell material on silica particles (5 μm) coated with PAH/PSS polyelectrolyte pairs. Following the vesicle coating, the membrane orientation of the vesicle on the particles was monitored by flowcytometry. These studies demonstrate the feasibility to surface functionalize the LbL particles with RBC membrane derived vesicles in a controlled fashion that may serve towards the design of passive targeted drug carriers.

6.1 MATERIALS AND METHODS

6.1.1 Materials

Poly (allylamine hydrochloride) (PAH; ~58,000), poly (sodium 4 styrene-sulfonate) (PSS; MW ~70 kDa), 5-([4,6-Dichlorotriazin-2-yl]amino)fluorescein hydrochloride (DTAF) were purchased from Sigma aldrich. Silica particles, (4.99 ± 0.22) μm in diameter, 5% stock solution, were purchased from GmbH microparticles (Germany). Recombinant human Annexin-FITC, Cell mask OrangeTM (cell membrane stain), Wheat Germ Agglutinin (WGA)-Alexa Fluor 488 conjugate was purchased from Invitrogen. Buffers used for all the experiment is phosphate buffered saline (PBS) buffer. Phosphate buffered saline solution (PBS) containing 137mM sodium chloride; 2.7mM potassium chloride and 10mM phosphate buffer was prepared from a 10x PBS stock solution. PBS with different osmolality was prepared from 10x PBS stock solution by adjusting the water volume.

6.1.2 Preparation of inside-out and right-side-out vesicles

Blood was collected directly into EDTA coated vacutainer (BD) tubes and centrifuged at 1000g for 10 minutes at 4°C to remove the plasma. The cells were resuspended in isotonic PBS (300 mOsm/kg) pH 7.4 to an approximate hematocrit of 50%. 1 ml of the cell suspension of the above 50% cell suspension was added to 10ml of hypotonic PBS (20 mOsm/kg) at 4°C. The tubes are allowed to stand for 5 minutes prior to centrifuging at 20000g for 10 minutes at 4°C. An index of the progress of hemolysis process is the development of deep red color from the hemoglobin released in the soluble portion. A total of 4-5 washes are necessary before the membranes are colorless. The ghost membranes are resuspended in hypotonic PBS and then transferred to respective vesiculation buffers. To initiate vesiculation 1 ml of ghost pellets were resuspended in 10ml of 0.5mM Na₂HPO₄ for inside-out vesicles and 0.5mM MgSO₄ in 0.5mM Na₂HPO₄ for right side out vesicles. The membranes were incubated in ice for 60 minutes and were pelleted by centrifugation at 20000g for 10 minutes at 4°C. The membranes were resuspended to 1 ml in the respective buffers and passed 3-5 times through no.27 gauge needle with a 1ml syringe to complete vesiculation. The vesicles were quantified in terms of protein content by UV spectrophotometry.

6.1.3 Characterization of RBC derived vesicles

6.1.3.1 Sialic acid exposure

Alexafluor 488 conjugated WGA was used for the detection of sialic acid exposed on the outer leaflet of the vesicles. 0.5mg of vesicles were treated with 100 μ l of 50 μ g/ml WGA solution and incubated for 30 minutes in the dark at room temperature. The vesicles were washed thrice with the respective vesiculation buffers at 20000g for 10 minutes at 4°C to remove the unbound molecules and analyzed by flow cytometer. A total of 10,000 events were acquired for analysis.

6.1.3.2 Phosphatidylserine (PS) exposure

FITC-conjugated Annexin V was used as a probe for the detection of phosphatidylserine on the outer membrane of the vesicles. 0.5mg of vesicles were treated with 5 μ l of FITC-annexin V and incubated for 30 minutes in the dark at room temperature. The vesicles were washed thrice with the respective vesiculation buffers at 20000g for 10 minutes at 4°C to remove the unbound molecules and analyzed by flow cytometer. A total of 10,000 events were acquired for analysis.

6.1.4 Internalization of vesicles by THP-1 Monocytes

The phagocytic uptake profile of vesicles by monocytes was studied using FACS Calibur (Beckton Dickinson). THP-1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS. For cellular internalization studies, cells were transferred to 12 well plates (250K cells/well) in optimem. DTAF (5-[4,6-Dichlorotriazin-2-yl] amino) fluorescein hydrochloride], that binds to the N-amino groups of proteins was used to label the RBC derived vesicles. The labeled vesicles were then treated with THP-1 cells and incubated for 4h at 37°C. Following incubation the cells were removed and washed twice with Dulbecco's PBS and analyzed by FACS. 10 μ l of trypan blue (0.4%) was added to 300 μ l of cell suspension in order to quench the fluorescent signal from the vesicles adsorbed on the outer surface of the cell. FACS calibur is equipped with an argon laser emitting at 488nm. The fluorescence intensity of the FITC from the microcapsules is detected in the FL-1 channel that uses a 530/30nm band pass filter. The data (10⁴ events) were acquired for forward scatter intensity, side scatter intensity and for fluorescence intensity (FL-1) in logarithmic mode. WinMDI software was used to analyze the results.

6.1.5 Surface functionalization of Silica particles with RBC derived vesicles

Silica particles were coated with polyelectrolytes as described before. Polyelectrolytes (PAH/PSS) were dissolved in 0.1 M NaCl solutions at a concentration of 2 mgml⁻¹. The particles were alternatively incubated with PE solutions for 10 min under constant shaking. Three washing steps in 0.1 M NaCl were used to remove unbound PE from the supernatant. Following PE coating, the vesicles (right-side-out/inside-out) were coated as the outermost layer. The coating schemes for SiO₂ particles were as follows:

The particles were then treated with WGA solution of varying concentrations (6.25- 50 µg/ml) of WGA-Alexafluor solutions to monitor the sialic acid exposure after membrane coating. The particles were incubated with the solutions for 30 minutes followed by subsequent washing steps in 1x PBS to remove the unbound fluorescent molecules. The particles were then analyzed for sialic acid exposure by flow cytometer. The fluorescence intensity of the alexafluor from the micro particles is detected in the FL-1 channel that uses a 530/30nm band pass filter. The data (10⁴ events) were acquired for forward scatter intensity, side scatter intensity and for fluorescence intensity (FL-1) in logarithmic mode. WinMDI software was used to analyze the results.

6.2 RESULTS AND DISCUSSION

The chapter deals with the fabrication of a coating material based on the natural biomembrane of red blood cells. The first step involves the preparation of vesicles from RBC ghost membranes and characterization by biochemical steps. The successive steps include the study of phagocytic uptake by monocytes to identify the membrane dependent uptake effects. Finally the vesicles are introduced as a multilayer shell material on LbL coated silica particles (5 μ m) and the membrane orientation of the vesicles following coating were investigated.

6.2.1 Preparation of RBC derived vesicles

The erythrocytes (RBC) were first subjected to hypotonic lysis, where the hemoglobin is removed and the so formed cells referred to as Erythrocyte Ghost (EG) (fig. 52a). When the cells are placed in a hypotonic medium, the RBCs swell and change their shape from a biconcave disk to a perfect sphere. The ghost membranes were then converted into vesicles with two different orientations by disruption in alkaline buffer of low ionic strength. The vesicles generation from the ghost membranes is based on the budding of the parent membrane into ghost interiors leading to the accumulation of small vesicles within parent ghosts, which are subsequently released from the interior by homogenization steps, that by passing through 27-gauge needle several times [247].

6.2.2 Vesiculation mechanism

Inside-out vesicles are those with inverted membrane orientation as compared to the parent membranes, whereas the right-side-out with the same orientation as of the parent ghost membranes. The lysed erythrocytes ghosts are placed in vesiculating medium (0.5mM Na₂HPO₄) to yield vesicles with inside-out orientation (fig. 52d) whereas the addition of 0.5mM MgSO₄ to the solution retains them in the parent membrane orientation/right-side-out (fig. 52e). The presence of Mg²⁺ ions in the RO buffer are known to activate the Mg²⁺ ATPase that restores the phospholipid asymmetry in the native orientation and prevents membrane flipping [248].

Figure 52: Schematic illustration for the preparation of inside-out and right-side-out vesicles from human red blood cells. (a) Human red blood cells are subjected to hypotonic lysis by treatment with hypotonic solutions (20mOsm/kg PBS) that yields hemoglobin free RBC membranes, termed as RBC ghosts. The ghost membranes are then incubated with vesiculating solutions for the formation of (b) inside-out and (c) right-side-out vesicles respectively. The ghost membrane are supposed to (d) undergo endocytosis to yield inside-out vesicles with a inverted membrane orientation whereas in (e) right-side-out, the membrane undergo exocytosis to form vesicles with the native orientation as compared to that of the parent RBC ghost membranes.

6.2.3 Cytoskeleton of RBC membrane

RBC membrane is basically composed of proteins, lipids (phospholipids and cholesterol) and carbohydrates (sugars, hexosamines and sialic acids). It plays many roles in regulating surface deformability, flexibility, adhesion as well as immune recognition. Aging RBCs differentiate themselves through changes in the membrane after they live in blood circulation for 100 to 120 days. It is believed that these changes lead to selective recognition of macrophages and removal of aging or damaged RBCs from circulation.

6.2.4 “Lipid bilayer” architecture

The predominant phospholipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM) and phosphatidylserine (PS). The asymmetry of the membrane lipid bilayer is a special feature of the RBCs. Choline containing phospholipids, phosphatidylcholine (PC) and sphingomyelin (SM) are predominantly localized in the outer monolayer of the plasma membrane whereas the aminophospholipids comprising phosphatidylethanolamine (PE) and phosphatidylserine (PS) are enriched in the cytoplasmic leaflet of the membrane.

This arrangement appears to depend on the activity of proteins called flippase and floppase (fig. 53). The asymmetry is under control of a flippase, an inward-directed pump specific for PS and PE that is responsible to translocate them to the inner monolayer, and an outward- directed pump referred to as floppase that catalyzes the translocation of phospholipid to the outer monolayer, and a lipid scramblase that catalyzes unspecific, bidirectional redistribution of phospholipids across the bilayer [249].

Figure 53: Membrane transporter found in various eukaryotic cells. Flippases catalyze the transport of lipids toward the cytoplasm and require ATP, floppases catalyze the ATP-dependent transport of lipids away from the cytoplasm, and “scramblases” catalyze the bi-directional, non-energy-dependent transport of lipids [249].

The asymmetrical distribution of phospholipid is crucial for RBC especially in the interaction between the cell and its environment. The membrane surface area asymmetry is considered to be a major driving force for vesicle formation and that transient asymmetries in lipid concentration contributes to the formation of endocytic/exocytic vesicles [250].

6.2.5 Characterization of RBC derived vesicles

The vesicles derived from the RBC membrane were confirmed by biochemical test procedures and some marked changes that can be observed visually.

Figure 54: Erythrocyte derived inside-out and right-side-out vesicles. The parent ghost membranes prepared from RBCs are white in colour after hemolysis with exact resemblance to the right-side-out vesicles. The inside-vesicles appear to be slightly red in colour, which is attributed to the inward bound hemoglobin of the parent ghost membrane that is visible after membrane flipping.

The ghost membranes prepared were creamy white and morphologically intact. Figure 54 shows the micrograph of the vesicles derived from the ghosts following the incubation of the parent membranes in the respective vesiculation buffers. The ghost membranes initially were creamy white and resemble as such as the right-side-out vesicles. It could be observed that the inside-out vesicles are bright red in color compared to the right-side-out vesicles. The sudden appearance of the red color after the inside out vesiculation procedure is mainly attributed to the inward bound hemoglobin, which was not removable during the ghost preparation. This observation serves as qualitative information to give an idea of the membrane orientation during their preparation and they are further supported by biochemical tests.

A schematic illustration of the membrane architecture of RBC membrane and their derived vesicles are demonstrated in figure 55.

Figure 55: Membrane orientation of the RBC derived vesicles compared to the native RBCs. In native RBC membranes, the outer leaflet is rich in sialic acid whereas the phosphatidylserine is present on the cytoplasmic face. During inside-out vesiculation, the inward bound phosphatidylserine (PS) extends to the outer leaflet and sialic acid vice versa, whereas right-side-out-vesicles retain the native membrane orientation as the RBC membranes.

6.2.5.1 Drawbacks of age-old methods to study membrane orientation

The conventional method to study membrane orientation of vesicles involved membrane sidedness assays based on biochemical tests. The most common and well-established assay utilized the sialic acid content to differentiate the inside out and right out vesicle preparation. The vesicles were first exposed to sialidase; enzyme specific to cleave the surface exposed sialic acids. Following sialidase treatment the sialic acids removed were then quantified using the conventional thiobarbituric acid assay to detect sialic acid [179, 251, 252]. The disadvantage associated with this method is the two-step procedure involved, i.e. enzymatic treatment to cleave the sialic acid and the next exhaustive time consuming procedure involved in the assay for sialic acid detection. The

absence of the other methods can also be attributed to the period of research, i.e. the vesicles originated as early as 1970. One more reason was due to minimal scope of these vesicles used in different research areas. For the first time we studied the membrane orientation of the vesicles using a flow cytometer with a one step procedure. The membrane orientation of the vesicles were studied using ligand specific fluorescent markers that has significantly reduced the time consuming detection step and the possibility to study the orientation of different surface ligands.

6.2.5.2 Biochemical characterization of RBC vesicles- FACS

The orientation (right-side-out or inside-out) of membrane vesicles was determined by two independent biochemical tests:

- 1) Assessment of the amount of sialic acid exposed on the surface of the vesicles,
- 2) Percentage of phosphatidylserine (PS) exposure by FITC-annexinV binding assay.

6.2.5.2.1 Sialic acid exposure

WGA binding assay was performed to detect the sialic acid exposed on the outer surface. Sialic acid has been reported as a standard marker for the characterization of the inside-out and right-side-out vesicles prepared from erythrocytes [252]. Sialic acid is a member of family of acetylated or glycosylated derivatives of neuraminic acid, is a component of many glycoprotein and glycolipids. In a normal erythrocyte membrane the sialic acids are exposed on the extracellular surface of erythrocytes (fig.55) [253]. A prominent member of the cell-surface sugar; the N-acetylneuraminic acids (sialic acids) are nine-carbon monosaccharides, with a negative charge are most commonly found at the non-reducing terminal positions of N-and O-glycan. Wheat germ agglutinin (WGA) is a carbohydrate-binding protein of approximately 36 kDa that selectively recognizes sialic acid and N-acetylglucosaminyl sugar residues found on the plasma membrane [254].

Figure 56: Detection of sialic acid exposed on the surface of the RBC derived vesicles. Alexafluor488 labeled WGA was used to bind to the sialic acid present on the outer surface of the vesicles and the fluorescence shift obtained is proportional to the amount of sialic acid present.

The control vesicles that are not treated with WGA are shown in black histogram. As shown in fig. 56, the red histogram and the blue histogram represents the inside-out and right-side-out vesicles treated with WGA. The g.mean values are directly proportional to the amount of WGA bound to the vesicles. For the same concentration of WGA used, the g.mean value for inside out was about 86.48 ± 3.51 a.u. whereas right-side-out vesicle had a value of 422.42 ± 4.5 a.u. The reduced amount of sialic acid ($\sim 5x$) exposed on the outer leaflet of inside out vesicles confirms the membrane flipping induced by vesiculation.

6.2.5.2.2 Phosphatidylserine exposure

As an additional method to determine the orientation of the prepared vesicles, we studied the phosphatidylserine exposure on the membrane-derived vesicles. The study of PS externalization in membrane vesicles has been reported for the first time in our studies. In a normal erythrocyte, the phosphatidylserine (PS) is present on the inner membrane layer [255]. The phosphatidylserine externalization of RBC vesicles prepared from the parent ghost membranes was measured with the FITC-annexin V binding assay. Annexin V is a human placental anticoagulant protein of molecular weight 35kDa that binds to the membrane and lipid bilayers containing phosphatidylserine. Annexin V binding to cell surfaces as a result of transmembrane movement is often used as a diagnostic indicator of apoptosis

Figure 57: Detection of phosphatidylserine exposed on the outer surface of RBC derived vesicles. The distribution of the fluorescence intensity in dependence of forward scattering (FSC) from single vesicles in arbitrary units (events distribution) is shown. The positional shift in the fluorescence intensity is directly related to the amount of PS exposed.

FITC-annexin V was used a fluorescent marker to detect the amount of PS bound to the outer surface of the vesicles. As observed from the graph (fig. 57), the fluorescence intensity of the inside out vesicles is higher than the right-side-out vesicles, which is directly proportional to the amount of phosphatidylserine present at the outer surface. Inside-out vesicles had a PS exposure of about $68.56 \pm 1.59\%$ whereas right side out had $34.95 \pm 1.52\%$. The amount of PS exposed in inside-out was almost $\sim 2x$ higher as compared to the right-side-out, which confirms the inverted orientation of the inside-out vesicles.

These studies clearly demonstrate the membrane asymmetry of the prepared vesicles and the results have been confirmed by testing the orientation of two important markers in the plasma membrane of the erythrocytes.

6.2.6 Stability of vesicles at physiological conditions

The stability of vesicles is an important parameter that has often been overlooked by researchers in this field. The vesicle preparation procedures were carried out at hypotonic conditions (vesiculation medium), that was of low ionic strength and hence we investigated the membrane stability and orientation of the membrane after subjecting them to physiological saline (isotonic PBS).

Figure 58: Membrane orientation of vesicles exposed to isotonic conditions. Sialic acid exposure on vesicles exposed to physiological conditions. The G.mean (fluorescence intensity) of the vesicles (inside-out and right-side-out) storage vesicles is plotted versus two types of buffer conditions with one being the native vesiculation buffer (V.B) and PBS (1X) that represents the physiological saline. The higher fluorescence intensity of the inside-out vesicles in PBS compared to the right-side-out vesicles suggests that there is a rapid reorientation of the membrane lipid groups as compared to the vesicles prepared in respective vesiculation buffers

It was interesting to find out that there was a drastic difference in the membrane orientation after subjecting them to physiological conditions. The vesicles stored in the vesiculation buffer (V.B) retained the same orientation as it was observed during the preparation procedure. The IO vesicles in V.B had a g.mean of 41.27 ± 2.19 a.u. (fig. 58a) whereas for RO vesicles in V.B it was found to be 192.9 ± 7.01 a.u (fig. 58b). In stark contrast, the IO vesicles subjected to isotonic condition exhibited higher amount of sialic acid compared to RO vesicles. The IO in PBS had a g.mean value of 387.7 ± 7.65 (fig. 58c) much higher than that of its counterpart RO that had a value of 275.6 ± 3.08 a.u. (fig. 58d) , i.e. approximately 1.4x lower than that of IO vesicles.

The phenomenon exhibited here is demonstrated for the first time using RBC derived vesicles, and it is considered as a natural inherent ability of RBC membrane that remains beyond human engineering. Owing to the plasticity of the lateral organization of the membrane into domains, known for precise alignment of the lipids and protein species, a given external stimulus can be expected to induce the membrane deformations in a controlled fashion. Such an external stimulus (vesiculation buffer) guided the orientation of the vesicles in a controlled manner to derive them as RO and IO, however once returned to isotonic conditions, the lipid membranes undergo rapid rearrangements

in the complex membrane that alters the native configuration. More specifically, the stimulation of the cytoskeletal membrane leads to a redistribution of lipids and proteins associated with the cell membrane, leading to changes in micro domain organization, cytoskeletal reorganization and membrane orientation.

6.2.7 Mechanism of vesiculation

Vesicle generation involves a crucial step of membrane invagination, which is the bending of a comparatively flat plasma membrane to generate high curvature necessary for vesicle generation. The curvature generation is achieved by the interplay between proteins and lipids or by leaflet specific changes in the lipid composition of the membrane. The proteins can be an integral part of the membrane as such or through the recruitment of specific curve generating proteins that are recruited to specific endocytic pathways such as clathrin [256]. It has been explained that the micro vesicle formation by bending of the plasma membrane occurs in two ways. In the case of exocytosis (inside-out vesicles formation), it requires the translocation of the lipids from the outer leaflet to the inner surface whereas the native configuration is retained in endocytosis (right-side out vesicle formation) [257]. This is in agreement with our experimental results as examined by sialic acid and phosphatidylserine exposure (fig. 55).

The aim of the current research is directed to identify the role of membrane orientation towards MPS clearance and application of the obtained knowledge towards novel carrier design. The membrane of the red blood cell plays many roles that aid in regulating their surface deformability, flexibility, adhesion to other cells and immune recognition. Active translocation of phospholipids across the plasma membrane has been demonstrated both from the inner to the outer leaflet and vice versa. The loss of membrane phospholipid asymmetry is known to play an important role in signaling mechanism. Hence in our next study we investigated the membrane orientation as a factor that determines the phagocytic uptake by the MPS system and their supporting evidences

6.2.8 Role of membrane orientation

In the search for a injectable, biocompatible and long-circulating material for multilayer coating, evaluation of their interaction with monocytes; the key players in immune systems towards MPS clearance represents a good preliminary test predictive of the *in vivo* fate of the LbL coated carriers in the circulation. Quantitative confirmation of vesicles uptake by THP-1 cells were visualized by CLSM.

Figure 59: Confocal Laser Scanning Microscopy (CLSM) image of THP-1 cells to demonstrate cellular uptake of RBC derived vesicles. The image (c) corresponds to the overlap of (a) red fluorescent signal from stained membrane (Cellmask™) and (b) green fluorescence emitted from the DTAF labeled vesicles respectively.

The fact that both the cellular membrane and the RBC vesicles are seen in the same confocal plane indicates that the vesicles are effectively taken up by cells, and not merely sticking to the cell surface by electrostatic interactions. The CLSM images shows that both the vesicles (IO and RO) were associated in the cell cytoplasm after incubation, which confirms the phagocytic uptake by the cells (fig. 59). This interaction was further corroborated by the quantitative analysis of cells by flowcytometry.

Figure 60: Cellular uptake profile of fluorescent labeled vesicles by THP-1 monocytes. The G.mean (fluorescence intensity) of the cells following internalization of vesicles is plotted against concentration of 2 types of RBC derived vesicles. The high fluorescent intensity of the cells treated with right-side-out vesicle compared to inside-out-vesicles illustrates the higher phagocytic uptake of right-side out vesicles by monocytes.

The simultaneous recording of fluorescence emitted from labeled vesicles and forward scattering intensity allows quantification of the vesicles taken up by the cells. An

unequivocal distinction between internalized and surface adherent vesicles was made possible by trypan blue quenching. The plasma membranes of living cells are impermeable to trypan blue and therefore, only the fluorescence of those vesicles that are attached to the cell surface from the bulk will be quenched. Figure 60 demonstrates the cellular internalization profile of THP-1 cells treated with IO and RO vesicles. By examining the effect of vesicle concentration on the efficiency of uptake, a difference can be observed between inside-out and right-side-out vesicles. The right-side-out vesicles had higher cellular uptake as compared to inside-out vesicles. Moreover the index of uptake was higher for RO vesicles with increasing concentration compared to IO vesicles. The mechanism behind the increased uptake and the difference in the profile observed is directly attributed to the membrane configuration of the vesicles. In particular, the high exposure of sialic acid on the inside out vesicles serves to evade the uptake by monocytes compared to their counterpart, right-side-out vesicles. A detailed review on the role of sialic acid towards RBC senescence is explained in detail in section 5.3.9.

6.2.9 Vesicles as shell material

Following the initial studies of material fabrication and characterization, we introduced the vesicles in the multilayer shells as a surface coating. For this purpose, silica particles were LbL coated with PAH/PSS multilayers. The last layer was coated with PAH (cationic layer) to facilitate the deposition of the vesicles on the outer surface of the particles. The coating of vesicles was confirmed by fluorescent microscopy using labeled vesicles and zeta-potential measurements to confirm the assembly. We further investigated whether it was possible to control the membrane orientation of the vesicles after coating, or if both the vesicles exhibited a uniform orientation following LbL coating.

Figure 61: Membrane orientation of red blood cell derived vesicles coated on LbL coated SiO₂ particles. The G.mean (fluorescence intensity) of the particles coated with vesicles (inside-out and right-side-out) as function of concentration of alexa fluor labeled WGA (marker for sialic acid). The particle concentration was kept as constant whereas the concentration of WGA solution was varied to detect the variation in sialic acid exposure. The coating pattern of particles is as follows

Particles coated with inside-out vesicles had higher sialic acid exposure as compared to right-side out vesicles. This is well in agreement with the membrane orientation of the vesicles as such, which demonstrates that the membrane orientation of the vesicles is retained after introducing them in the multilayer shell configuration (fig. 61). A broad concentration range (6.25 – 50 μg/ml) of WGA was used to neglect any undesired saturation effects and at all the concentration range tested the amount of sialic acid present on IO vesicles coated particles were ~3.5x times higher compared to the RO vesicle coated particles. These results suggest that the vesicles are adsorbed on the surface of the particle; the reason by which the free vesicles and the membrane bound vesicle exhibit similar membrane orientation. The outcome of LbL carriers with two membrane orientation does not only signify the identification of long circulating carriers, but also opens up the opportunity to tailor the life time as a function of membrane orientation without any undesired side effects as compared to the synthetic PEG system.

After summarizing the different aspects of the current research work on the fabrication of RBC membrane vesicles, internalization by phagocytic cells and their association with LbL coated particles, we now consider the available knowledge with the current research in the design of next generation carriers. The following part of the thesis

details the ideology from vesiculation to membrane orientation and their applicability in targeted delivery systems.

6.2.10 RBC senescence

A complete understanding of why RBCs have a definite life span after which they are cleared by the monocyte-macrophage system is still not available. Due to absence of intracellular organelles and nucleus in RBCs; they are unable to synthesize any proteins or signaling components unlike the other cells of the human system. Hence it is evident that the changes in the membrane protein or phospholipids are probably involved in the RBC senescence.

6.2.10.1 Regulators of RBC life span- Sialic acid

One widely accepted mechanism towards RBC clearance involves senescent antigen of the band-3 protein. The degradation of hemoglobin and crosslinking of band-3 generates a senescent antigen recognized by IgG antibodies, that mediates the clearance of aged RBCs by the MPS system [258, 259]. In addition, a difference in the glycosylation patterns exists between the old and young RBCs, which functions as a signaling molecule. Older RBCs exhibit decreased sialylation pattern as compared to young RBCs [260, 261]. Aminoff *et al* demonstrated that the presence of sialic acid on the cell surface is crucial for the survival of nonnucleated mammalian erythrocytes. Furthermore desialylated RBCs were rapidly cleared *in vivo* [262, 263] and bind macrophages *in vitro* [264]. In addition, evidences suggest that desialylated RBCs may be cleared by asialoglycoprotein receptor mediated phagocytosis (the lectins that recognize D-galactose exposed by desialylation) [260] and/or FcγR mediated phagocytosis because of the increased autoantibody binding to senescent RBCs [265]. Apart from erythrocytes, a similar mechanism was found to exist in lymphocytes. The cleavage of sialic acids by neuraminidase from the lymphocytes enhanced the phagocytic uptake, suggesting the importance of the glycocalyx in immune clearance [266].

6.2.10.2 PS- a trivial factor in RBC aging

Although PS is an important factor for removal of apoptotic cells, some controversial phenomenon exists in red blood cells. It is important to note that older RBCs obtained by combined centrifugation method do not expose PS more often than younger RBCs, i.e. on approximately 0.2% of the cells [267]. Moreover in another study, annexin binding to human RBC populations with a life span of 100 to 120 days amounted

only to 0.1 to 0.8% of the cells with exposed PS [268], which is significantly low. It is generally accepted that PS exposure on RBC and their direct recognition by macrophages add redundancy to the pre-existed clearance mechanisms and may represent a leftover from cellular evolution which generates more problem than it solves in RBC senescence [269].

The erythrocyte life span is not regulated by PS exposure of the aged cells, which was recently reported by a specialized *in vivo* assay, double *in vivo* biotinylation (DIB) for tracking age related changes in circulating erythrocytes. The changes in PS expression on erythrocytes were monitored as a function of age. There was no significant difference in the PS expression between young and old erythrocytes, invalidating the role of PS externalization in the destruction of old erythrocytes from circulation. This may also give rise to an intriguing question of whether the old erythrocyte is rapidly removed by macrophages following PS expression, and hence undetectable in the circulation population. To test this hypothesis, the PS expression in circulating erythrocytes were tested in macrophage depleted mice; no significant difference in PS expression was observed among different populations [270].

The above-mentioned discussion is devoted to explain the rationale behind choosing sialic acid as a marker in our studies. The expression of sialic acid on the carriers and their recognition by phagocytes are inevitably considered to be a decisive down regulating factor for phagocytic uptake [271].

6.2.10.3 Concluding remarks on RBC senescence

Research aimed at understanding these complex biological processes, has yielded a variety of mechanism to be involved in RBC senescence. Till date, the exact mechanism of RBC senescence or the signaling pathway and molecular participants has not yet been established. In particular, studies have identified the contribution of different antigens such as band-3, PS, sialic acid, CD-47 etc. to contribute the MPS clearance are well accepted [272]. It is highly important at this juncture to mention that none of the above observations towards the senescent antigen has been completely accepted or refuted/abolished, but which in our opinion deserves much significance to study further was selected based on our system. The decrease of sialic acid content could well be correlated with the physiological mechanism of erythrocyte ageing and senescence and be responsible for the removal of oldest RBCs from circulation.

6.3.11 “Sialic acid ” – in a different dimension

In another approach, polysialic acids have been used to prolong the circulation lifetime of nanoparticles. Similar to the PEGylated systems polysialylation of proteins is currently actively studied by the pharmaceutical industry to enhance their circulation half-life [273]. Surface modification of liposomes by gangliosides GM1 and GM3 were shown to possess an increased blood circulation time compared to control liposomes that were rapidly cleared, the effect of which this was attributed to their sialic acid moieties . Very recently in 2010, Bondioli et al demonstrated surface decorated sialic acid functionalized PLGA nanoparticle and found a significant difference between the sialic acid functionalized particles and uncoated particles in the cellular uptake by monocytes [274]. The key idea behind this discussion is to explain the applicability of sialic acid for long circulation in a different dimension and apart from sialic acid there are many other components in the RBC membrane whose functionality have not yet been clearly delineated.

Hence in our studies we chose sialic acid as the dominant factor to regulate the phagocytic uptake, though there may be other components involved that requires a thorough investigation following initial carrier design. Our approach in this pilot study was directed to investigate whether the membrane orientation of RBC membrane had an influence on the phagocytic uptake and if there was one, whether it could be translated to particulate carriers for passive targeting application. The mechanism underlying the erythrocyte lifespan is apparently important for tuning the circulation lifetime of biomimetic carriers.

Numerous studies have reported the use of lipids in the surface modification of carriers, even in the LbL technology. However the aim of this study was to utilize a natural biomembrane as a surface coating and to tailor the carriers with well defined membrane orientation as of the native vesicles. The electrostatic interaction between the glycocalyx of the vesicles and the cationic outer layer PAH provides a coating of vesicles on the surface. The present study may serve to enhance the circulation life time of nanoparticles coated with RBC membrane by membrane extrusion [183]. The precise tailoring of the membrane orientation could also be used to control the circulation lifetime with our proposed carrier system. The IO vesicle coated particles with high exposure of sialic acid are hypothesized to have higher circulation half-life times as compared to RO vesicle coated particles. Despite the generous amount of experimental evidence about the

MPS clearance, we are still far from understanding the complex interplay that remains elusive. Clearly advent of such biomimetic carriers for passive targeting application will be of utmost importance in therapeutic applications. On the whole, initial interpretation of the results suggests that a careful control of the membrane orientation can serve to control the lifetime of the carriers.

6.4 SUMMARY

This chapter reports the fabrication and functionalization of membrane vesicles derived from human red blood cells towards targeted drug delivery applications. The aim of the study was to identify a novel material for the surface functionalization of LbL carriers for long-term circulation. For particle engineering, we have taken a lead from nature i.e. exploring membrane orientation of red blood cells that plays the major determinant role in circulation to evade macrophage recognition. Towards this, we sought to prepare membrane vesicles from red blood cells with two different membrane orientations namely inside-out and right-side out vesicles. Inside-out vesicles (IO) are those with inverted membrane orientation, whereas the right-side-out (RO) with the same orientation as compared to the parent RBC membranes. The lipid asymmetry of the vesicles (IO and RO) was compared to that of the parent membrane for surface orientation of ligands such as phosphatidylserine (PS) and sialic acid. The assay procedure reported here for detecting the surface orientation was based on flowcytometry, accomplished by the advent of ligand specific fluorescent markers, that follows a simple one-step procedure rather than the conventional time-consuming biochemical tests. In a normal RBC, the membrane constituents such as phosphatidylcholine (PC), N-acetylneuraminic acid (sialic acid) are located in the outer monolayer whereas sphingomyelin (SM) and phosphatidylserine (PS) are localized to the inner leaflet. Hence the surface exposure of the ligands serves to identify the membrane asymmetry of the prepared IO and RO vesicles. Annexin V-FITC and alexafluor-WGA were used as labeled markers that are highly specific for PS and sialic acid respectively. The RO vesicles had 5x higher exposure of sialic acid compared to IO membranes. On the other hand, the IO vesicles exhibited 2x higher PS exposure compared to RO vesicles, which confirmed the membrane variation between two vesicles. Subsequently, the stability of the vesicles in physiological conditions was analyzed. Subjecting them to isotonic condition induced rapid cytoskeletal reorganization changes in the lipid bilayers; as a result of which IO vesicles had higher sialic acid exposure compared to RO, in stark contrast to the vesicles in their native buffers. Following the fabrication of vesicles, they were investigated for phagocytic uptake with monocytes; the key players in immune system towards MPS clearance. Qualitative and quantitative information on the internalization of vesicles by monocytes were obtained using confocal microscope and flowcytometry. The RO vesicles were found to have higher phagocytic uptake compared to IO vesicles. The difference observed is directly attributed to the higher exposure of sialic acid present on

the IO vesicles, which are known to play a dominant role in the senescence of red blood cells. Further we introduced the vesicles as a shell material on the LbL coated silica particles (5 μm) coated with PAH/PSS polyelectrolyte pairs, to test the feasibility of surface coating in a controlled fashion. As desired, the membrane orientation of the vesicles was retained following coating on the LbL particles. The IO vesicles had higher amounts of sialic acid exposed compared to RO coated particles similar to that of the native vesicles. Lipid vesicles were prepared from RBCs in with two different membrane orientations that exhibited differential response to monocytes are key players in the immune clearance. These studies clearly demonstrate the feasibility to surface functionalize the LbL particles with RBC membrane derived vesicles in a controlled fashion that may serve towards the design of passive targeted drug carriers. Taken together these studies suggest the importance of novel design strategies that combine the advantages of both colloidal and biological systems. In light of the recent approaches made towards using RBC-nanoparticulate systems, this work is envisaged as a valuable study to increase the therapeutic efficiency of the current systems.

Chapter 7: Conclusion And Future Recommendations

Inevitably future advances in the delivery of therapeutic molecules require more specific exploitation of sophisticated designed drug carriers for potential applications. As described in detail in the previous sections, a wide variety of methods have been proposed for the design of carriers for delivery applications. LbL technology has come a long way to become the successful partner to drug that alone display a poor therapeutic index. Many studies have already shown the versatility of the system that has resulted in the design of multifunctional drug carriers that displays innate properties such as high payload efficiency, controlled release, feasibility of active and passive targeting. Therefore research in layer-by-layer systems may contribute to the effective development of smart devices to combat various life threatening diseases in the future that requires highly sophisticated drug delivery carriers.

Novelty and Scientific contributions:

- Polyelectrolyte microcapsules based on a dissolvable core, showed successful intracellular cathepsin B inhibition in vitro for tumor therapy.
- RBC carriers functionalized with LbL technology were successful in controlling the release of encapsulated bio active molecules while retaining their bioactivity.
- HA nanoparticles (<200nm) functionalized with LbL technique was efficient in gene delivery compared to the conventional micron sized systems; in comparison to lipofectamine, the HA-LBL system offers the potential of sustained gene delivery.
- Intracellular trafficking pathways associated with LbL-HA nanoparticles elucidated the bottlenecks associated with the carrier system that is valuable information in the future design of drug carriers.
- The concept of erythrocyte derived vesicles as a biomimetic shell material was explored and shown to be feasible towards targeted drug delivery applications.
- - RBC vesicles were characterized by a novel method based on flowcytometry and their phagocytic competence were studied as a function RBC membrane configuration.

- These studies serve to demonstrate for the first time, the usefulness of RBCs to act as drug/protein encapsulants that can show significant enhancements in passive targeting.

This concluding chapter explains the unique features and novelty of the LbL carriers designed for specific applications; further suggesting the potential future applications using these carriers. Based on the existing knowledge of the design of multilayer shells as a functional scaffold, they have opened up new directions for further research in the design of next generation particulate technology.

- Chapter 2 dealt with the design of LbL carriers for exogenous protein delivery applications. The current work is the first successful report to induce tumor cell death by cystatin delivery using a drug carrier.

The main objective was to design a subtle carrier for the intracellular delivery of cystatin to inhibit cathepsin B, which plays a major role in aggravation of tumor pathology. Previous studies were carried out using well-established carriers systems such as PLGA, chitosan [199, 275]. However they failed due to the apparent cytotoxicity of the carriers. In our study, the cystatin-loaded microcapsules were successful in inducing cell death following cathepsin inhibition whereas the empty capsules were completely inert to the tumor cells. These studies have not only identified a subtle carrier but have also identified a therapeutic target in tumor therapy that is recently gaining widespread attention. As a further extension, the carriers also have the possibility of surface modification to target the specific tumor cells. The tumor cells are differentiated from the normal cells by the presence of surface antigens that could be utilized for targeted delivery. Therefore, surface functionalization with moieties that are recognizable by the tumor cells adds more functionality to the carriers for targeted applications. The carriers have by far demonstrated the basic attributes of drug carriers such as high encapsulation and loading amounts, biocompatible, biodegradable, intracellular uptake and delivery. Hence adding the functionality of targeted delivery makes them versatile carriers that are desired in various therapeutic applications.

- Chapter 3 reports the surface modification of RBC ghost carriers with LbL coated polyelectrolytes towards controlled release applications.

RBC carriers have been in existence for the past 30 years and reported for encapsulation of wide array of biomolecules in different applications. One major

drawback faced was the rapid leakage of the drug from the carrier. The only method that exists till date is crosslinking the membranes with glutaraldehyde. However crosslinking only served to slow the release of the encapsulated molecules and do not control the release. Towards this, LbL modification was envisaged to control the release of the encapsulated biomolecules. As expected we were able to control and tune the release of the molecules as a function of multilayers thickness. Besides controlled release we also demonstrated that the carriers fabrication does not induce any modification to the cargo molecules that completely retained their biological activity. Having demonstrated the design of LbL carriers based on erythrocyte ghosts we now propose the design of the future carriers. These two aspects of the design i.e. the ghost carriers and LbL –ghost carriers are demonstrated first and the interplay is drawn towards the end.

“Erythrocyte carriers in a different dimension”: After years of preliminary research with red blood cells as carriers, the application has been translated now to new heights reaching advanced clinical trials. This was made possible by the development of “red blood cell loader”, a device that can automatically load the red blood cell in a highly repetitive fashion. With as little as 50 ml of blood sample, therapeutic drugs can be encapsulated in the erythrocytes within 2 hours, in a way similar to blood transfusion [276]. This approach has widened the prospects for erythrocyte based drug delivery and with successful pre-clinical trails involving drugs such as dexamethasone, advanced clinical trials are going on [277].

“Universal blood via LbL modification”: In a different approach, LbL surface modification was utilized as tool to mask the surface antigens of RBC, towards the development of universal blood. The LbL coating was successful in masking the surface antigens of RBC that was compatible with the RBCs of other blood groups. Moreover the LbL modification did not induce any undesired alteration to the template RBCs and were functional as tested by the O₂ transfer capacity [278].

The above reported two individual studies can be unified under roof with the concept of “universal drug loaded RBC carriers”. Specifically, in future drug loaded erythrocyte carriers can be coated with polyelectrolyte multilayers that serve not only to control the release but also as a universal blood with O₂ transfer abilities.

- Chapter 4 explains the fabrication and functionalization of HA nanoparticles (<200nm) for intracellular drug delivery applications. Nano

sized templates were employed as an alternative to the conventional micron sized templates that are beneficial for intracellular applications.

The LbL surface modification was used to functionalize the nanoparticles in a simple way, by encapsulation of bioactive molecules (proteins and nucleic acids) in the multilayer shells. The transfection efficiency was enhanced by several folds using LbL-HA nanoparticles compared to the previous studies that employed micron sized templates; mainly attributed to the nano size and precise engineering of the multilayer shells. Besides in-depth mechanistic investigations on cellular uptake pathways for the LbL carriers are reported for the first time that provides a framework for the design of future carriers aimed at intracellular targeted delivery. In particular, the clathrin mediated endocytosis (CME) pathway was found to be the predominant portal for cellular entry. However, such mode of entry is not desired as the carriers are prone to degradation by the acidic compartments of endolysosomes, whereas caveolar mediated pathway is relatively inert and is highly preferred. Towards this, the future design is proposed to use templates in the size range of (>500nm) that can utilize the caveolar pathway and avoid endosomal degradation. The hypothesis that nano size templates have higher edge over the micron size particles for intracellular drug delivery applications was thus proven.

- Chapter 5 reports the identification of a novel material based on red blood cells towards the design on biomimetic carriers. This is the first study reported till date to employ membrane-derived vesicles as a surface modification material that combines the advantage of both colloidal system and natural biological system.

For particle engineering, we have taken a lead from nature i.e. exploring membrane orientation of red blood cells that plays the major determinant role in circulation to evade macrophage recognition. Besides using vesicles with two different membrane orientations, the experimental observations were substantiated by mechanistic investigation to identify the governing factor. As further extension, the surface modification can easily be translated to drug loaded LbL carriers for various applications. There are many therapeutic applications that require its presence in circulation for prolonged time periods. Moreover till date, long circulating particulate systems have not received much attention for their ability to act as sustained release systems for controlled release of drugs as the present system for passive targeting is not yet well established. In this context, the LbL coating was already shown to control the release and addition of the vesicle coating would aid in increasing the plasma half-life. The potential advantages of

the proposed carrier system includes optimal levels of therapeutic agent in the blood for extended time intervals, inert to the MPS systems and absence of any adverse immune reaction as compared to the synthetic methods.

To summarize, application of LbL assembled carriers opens immense/multifarious opportunities in producing drug delivery systems with a vast array of required properties for broad spectrum of therapeutics applications. The future research on nanomedicine requires integration between physicochemistry, biology and materials science, which shows exciting prospects for pharmaceutical scientists working in different field towards a unified goal of improvement in human healthcare. This domain of research will inevitably be one of the most exciting arenas in the multidisciplinary field of nanomedicine, driven by polyelectrolyte multilayers with new combinations of properties. The studies reported in this thesis may contribute to the rational design of novel strategies to overcome the barriers in design of particulate carriers for drug delivery applications.

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