Self-assembled biomimetic architectures : protein-polymer conjugates and lipid-polymer hybrids for biomedical applications

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**SELF-ASSEMBLED BIOMIMETIC
ARCHITECTURES: PROTEIN-POLYMER
CONJUGATES AND LIPID-POLYMER HYBRIDS
FOR BIOMEDICAL APPLICATIONS**

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**INTERDISCIPLINARY GRADUATE SCHOOL
NTU INSTITUTE FOR HEALTH TECHNOLOGIES**

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

2018

DEDICATED
TO
MY BELOVED PARENTS
AND GRANDPARENTS

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

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Date

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(Amit Kumar Khan)

ABSTRACT

Macromolecular self-assembly is the foundation of all natural architecture starting from acellular virus particles to complex cellular constructs. In order to mimic such assemblies, we need to understand the fundamental aspects of self-assembly and the associated parameters governing the self-assembly at the nanometer to micrometer length scales. Self-assembly of single component macromolecular systems based on lipids, polymers and proteins have been studied extensively. However, the self-assembly behavior when they are combined is not well understood. Hence, the objective of this thesis is to design hybrid architectures (i.e., lipid-polymer and protein-polymer) and advance the understanding of their self-assembly behavior. For example, mixing phospholipids and polymers into hybrid self-assembled systems often give rise to bilayer thickness mismatch, which stems from differences in their chemical configurations and size. To overcome this mismatch and to have more energetically favorable system, low-molecular-weight biodegradable polymers and lipids are chosen to self-assemble into vesicles with bilayer thickness similar to that of a natural lipid bilayer membrane. From the experimental results, such hybrid configuration (although containing a high amount of polymer (50%)), possess lipid-like properties. For instance, they display an attenuated response to enzymatic digestion, reminiscent of PEGylated lipid vesicles. Notably, the low-molecular-weight (< 4 kDa) biodegradable polymers alone form micelles. However, when mixed, the presence of phospholipid drives vesicular assembly for this type of hybrid polymer/lipid systems.

For constructing hybrid vesicles, at the nano- and micron-scales, conventional methods such as film rehydration and electroformation methods were employed. To characterize the hybrid vesicles, dynamic light scattering, cryogenic transmission electron microscopy and calcein leakage experiments were performed. The experimental results reveal that such vesicles possess similar properties (size, bilayer thickness, and small molecule encapsulation capability) to vesicular membranes at the nanoscale. Furthermore, at the micro scale (i.e., micron size giant hybrid vesicles), their surface topologies were found

to be homogeneous, independent of cholesterol, suggesting that these polymers possess a different polymer-lipid phase separating behavior as compared to the high molecular weight analogs. While exhibiting a similar bilayer thickness as that of lipid vesicles, functional differences in surface topology were demonstrated using the interfacially sensitive phospholipase A₂. Biodegradable vesicles appear less sensitive to phospholipase digestion, reminiscent of PEGylated vesicles. Varying degrees of sensitivities towards phospholipase digestion were noticed implying that the nanoscale surface topology can further be tuned depending on the nature of the external ligands. This may be useful in designing vesicles for controlled release and supporting integral membrane protein in a semi-synthetic lipid-like environment.

Besides, lipid-polymer self-assembled architecture, another class of giant amphiphile, protein-polymer conjugated assemblies is gaining much attention nowadays. Because of their structural complexity and functional diversity, they are regarded as potentially important candidates over conventional phospholipid and polymer-based assemblies. In Chapter 4, I describe the synthesis and characterization of a new protein-polymer conjugated vesicular architecture, termed as “proteinosome” as an emerging delivery vehicle for biomedical application. It is composed of a milk protein, β lactoglobulin A (β LG A) and poly (ethylene glycol) PEG. β LG A was selectively modified to self-assemble by supercharging via amination or succinylation followed by conjugation with PEG. An equimolar mixture of the oppositely charged protein-polymer conjugates self-assemble into spherical capsules of 80-100 nm in diameter. A major advantage is that the described assembly process is performed entirely in aqueous solution without the need of a template. The self-assembly process is driven by the protein-polymer conjugates’ amphiphilicity and polyelectrolyte nature. Furthermore, the protein-polymer capsules or proteinosomes are reminiscent of viral protein capsids and are capable of encapsulating solutes in their interior. A better understanding of these systems might help employing them in future biomedical applications. The building blocks and the various biomimetic vesicular architectures studied in this thesis are schematically summarized in the illustration below.

Schematic Illustration of Self-Assembled Biomimetic Architecture

Self-Assembled Biomimetic Vesicles based on Lipid-Polymer Hybrids and Protein-Polymer Conjugates.

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LIST OF ABBREVIATIONS

Notations	Descriptions
AFM	Atomic force microscopy
BCP	Block copolymers
β LG A	β -lactoglobulin A
BSA	Bovine serum albumin
CAC	Critical aggregate concentration
CALB	Lipase B from <i>Candida Antarctica</i>
CCMV	Cowpea chlorotic mottle virus
CD	Circular dichroism
Chol	Cholesterol
CTA	Chain transfer agent
DLS	Dynamic light scattering
DMAP	4-dimethylaminopyridine
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
DOPC	1,2-Dioleoyl-sn-glycero-3-phosphocholine
DOXIL	Doxorubicin (DOX) liposomal formulation
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DPPE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
EDA	Diamine dihydrochloride
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
ELP	Elastin-like polypeptide
EPC	Egg phosphatidylcholine
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
GUV	Giant unilamellar vesicles
HIV	Human immunodeficiency virus
HPMA	Hydroxylpropyl methacrylate
HRP	Horseradish peroxidase
ITO	Indium tin oxide
LCST	Lower critical solution temperature
LUV	Large unilamellar vesicles
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time of Flight
Mb	Myoglobin
NMR-1H	Nuclear magnetic resonance spectroscopy (proton)
NP	Nanoparticle
PAA	Polyacrylic acid
PBD	Polybutadiene
PBS	Phosphate buffered saline
PCL	Polycaprolactone

Notations	Descriptions
PDB	Protein data bank
PDI	Polydispersity index
PDMS	Polydimethylsiloxane
pDNA	Plasmid deoxyribonucleic acid
PEE	Polyethyl ethylene
PEG	Polyethylene glycol
PEG-tz	Polyethylene glycol - Tetrazole complex
PEO	Polyethylene oxide
PHPMA	Poly(hydroxylpropyl methacrylate)
PIAA	Polyisocyano dialanine
PIAT	Polyisocyanoalanine(2-thiophene-3-yl-ethyl)amide
PIB	Polyisobutylene
PISA	Polymerization-induced self-assembly
PLA	Poly(lactic acid)
PLA ₂ (sPLA ₂)	Phospholipase A2
PLN	Polymer lipid nanoparticle
PMMA	Poly(methyl methacrylate)
PMOXA	Poly-(2-methyloxazoline)
PNIPAAm	Poly(N-isopropylacrylamide)
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine
PP	Polypropylene
PPO	Polypropylene oxide
PPS	Polypropylene sulfide
PS	Polystyrene
PS-b-PMMA	Polystyrene-b-poly(methyl methacrylate)
RAFT	Reversible addition-fragmentation chain transfer polymerization
Rh-NA (fig 8)	Rhodamine red-X conjugated neutravidin
Rho-PE (Rhod-DPPE)	1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)
RNA	Ribonucleic acid
SLN	Solid lipid nanoparticle
SLS	Static light scattering
ssRNA	Single-stranded ribonucleic acid
SUV	Small unilamellar vesicles
TEM and (Cryo-TEM)	Transmission electron microscopy and (cryogenic-TEM)
TNB	2-nitro-5-thiobenzoate
Tx 100	Triton X-100
UV	Ultraviolet
VLP	Virus-Like particle
W/O/W	Water-oil-water

LIST OF SYMBOLS

Notations	Descriptions
α_0	Optimum head group area
δ	Shape factor (or structure factor)
θ	Scattering angle
λ_{em}	Emission wavelength
λ_{ex}	Excitation wavelength
d	Membrane thickness
D_h	Hydrodynamic diameter
$f_{hydrophilic}$	Hydrophilic weight fraction
F_0	Initial fluorescence of calcein
F_t	Fluorescence of calcein at time interval t
$I_s(q)$	Scattered intensity
κ	Bending Modulus
K_a	Stretching modulus
kDa	Kilo Dalton
kT	Bending Modulus
l_c	Critical hydrocarbon length
M_w	Molecular weight
P_c	Critical packing parameter
q	Scattered vector
R_g	Radius of gyration
R_h	Hydrodynamic radius
R_v	Radius of vesicle
T_c	Crystalline temperature
T_g	Glass transition temperature
T_m	Melting temperature
v	Hydrocarbon tail volume
$wt\%$	Weight %

SYNOPSIS OF THE THESIS

This thesis focuses on two distinctly different self-assembled architectures: 1) hybrid lipid-polymer vesicles composed of a phospholipid and a synthetic, biodegradable amphiphilic block copolymer (BCP) to mimic the lipid-like topology of natural cell membranes. 2) biomimetic virus capsids prepared from protein-polymer conjugated giant amphiphilic building blocks.

The thesis is divided into 5 chapters as summarized below:

Chapter 1 describes the basic introduction of naturally occurring self-assembled architectures such as cells, viruses, and various self-assembled architectures such as a liposome, a polymersome, hybrid lipid-polymer vesicle, protein-based self-assembly, virus assembly and protein-polymer conjugated self-assembly.

Chapter 2 is designated to the current literature on liposome, polymersomes and hybrid vesicle focusing on their physical properties, their fabrication, and applications in biomedicine and biotechnology. This chapter will also include the latest development of self-assembled protein-polymer conjugated assemblies.

Chapter 3 covers the preparation of biodegradable hybrid lipid-polymer vesicles at nano- to microscale by film rehydration process and electroformation respectively. Further, it comprises the thorough structural and morphological characterization using light scattering and Cryo-TEM. The membrane stability, permeability against encapsulated dye and the effect of enzymatic degradation by Phospholipase A2 enzyme will also be discussed in this section.

Chapter 4 presents the design, synthesis, and characterization of a novel protein-polymer conjugated vesicle, named proteinosome which comprises a biocompatible polymer, poly(ethylene glycol) and a milk protein, β -lactoglobulin A (β LG A). This chapter also includes an encapsulation strategy to load DNA, protein, and polysaccharides into proteinosome's interior.

Chapter 5 will give an overall outlook and future perspectives on the novel biofunctional architectures.

CHAPTER 1 INTRODUCTION

1.1 General Introduction of Natural Assemblies

Biomimetics, or Biomimicry, is the concept of mimicking Mother Nature to create smart materials, devices, functions and processes using natural and/or synthetic building blocks. The most basic properties and functions of biological systems are precisely optimized and organized at the nano- and micrometer length scale and they have evolved over long periods of time. The overall aim of biomimicry of such systems is to construct hierarchical assemblies from their first level of organization. Before doing that, it is important to know and to understand the biological system in details. In my thesis, the focus is on cells and viruses.

A cell is the basic structural and functional unit of living organisms. It is classified into two categories - prokaryotic cells [Figure 1.1 C] and eukaryotic cells [Figure 1.1 A-B]. A prokaryotic cell is a unicellular organism, with the absence of a nucleus (containing the genetic materials, DNA or RNA) and other organelles. Genetic material, cytoplasm, and mitochondria all are enclosed by a cell membrane also known as plasma membrane or cytoplasmic membrane. The plasma membrane is the basic boundary wall of a cell, which hold important components inside the cell – separate from the outside environment, and transports molecules and signals selectively in and out of the cell. Common examples of a prokaryotic cell are archaea and bacteria. In contrast, a eukaryotic cell has a well-defined nucleus, endoplasmic reticulum and other organelles with distinct membrane boundary inside the outermost cell membrane. Typical examples are animal cells and plant cells [Figure 1.1 A, B].

Figure 1.1: Structure of natural assemblies: eukaryotic cell (e.g., animal cell (A), plant cell ((B)) and prokaryotic cell (e.g., bacteria) (C). Type of virus: enveloped virus (D) and non-enveloped virus (E). Images are adapted from Encyclopedia Britannica, Inc. (plant cell, animal cell, and bacteria), Bio-foundations.org (cell membrane), Malinovsky et al., [1] (plant cell wall), www.scientistcindy.com (bacteria cell membrane), Borman *et al.*, [2] (virus protein assembly) Buzzle.com (non-enveloped virus) and Anatomy-medicine.com (enveloped virus) respectively.

On the other hand, viruses are infectious agents, another class of natural entities of different shape, size, composition and structural organization [Figure 1.1 D, E]. They possess a unique molecular repertoire designed to infect cells and organisms [3-8]. Structurally, there are two types – enveloped [Figure 1.1 D] and

non-enveloped viruses [Figure 1.1 E]. They are composed of two to three parts, (i) genetic materials, RNA or DNA in the core of the assembly, (ii) a protein capsid that coats the charged genetic materials inside for non-enveloped virus and (iii) outer lipid bilayer or glycoprotein overlay covering the protein capsid for enveloped viruses [3, 4] [Figure 1.1 D]. Viruses are non-living and inactive outside the host cell. They become activated and starts replicating inside the host cell [8-10]. The composition of envelope is almost similar to the composition of host plasma membrane as viruses use host organism's machinery to replicate it membrane [11]; however, slight variation in composition can be observed for few viruses which specifically target certain membrane domain to replicate their envelope (e.g., Human Immunodeficiency Viruses). For example, in HIV-1 and HIV-2 the cholesterol : phospholipids ratio is 2.5 times higher than that of the respective host cell membrane and their membranes are 7.5% and 10.5% more ordered than the host cell membrane, respectively [12]. Interestingly, the physio-chemical properties of each virus differ from each other as even the same family may have different viral capsids and distinctly different properties [4]. Alike cells, virus particles are another example of a hierarchical assembly, where proteins are self-assembled around the nucleus acid core with high precision and symmetry. However, these are much smaller than regular cells. The usual size of viruses is 20-400 nm in diameter, the largest, however, measure about 500 nm in diameter and are about 700–1,000 nm in length [13], whereas cells are significantly larger, from micrometer to a millimeter in diameter and length. Most viruses, animal virus, plant virus, and bacteriophages, infect one or a few specific organisms or specific cell types. In both the cases, molecular self-assembly is the central intuitive element of the cellular and viral construction.

Table 1.1: Structural characteristics of cells, bacteria, and viruses.

Property		Virus	Bacteria	Plant Cells	Animal Cells
Type		Acellular	Prokaryotic cell	Eukaryotic cell	Eukaryotic cell
Size		20-400 nm	300-4000 nm	>1000 nm	>1000 nm
Intracellular characteristics	Genetic materials (DNA/RNA)	Either	Both	Both	Both
	Membrane-bound nucleus	N	N	Y	Y
	Ribosomal protein production ability	N	Y	Y	Y
	Membrane-bound organelles (e.g., Endoplasmic reticulum, Mitochondria, Golgi apparatus)	N	N	Y	Y
Membrane characteristics	Cell wall	N	Y	Y	N
	Major cell wall composition	-	Peptidoglycan	Pectin & cellulose	-
	Cell wall thickness	-	20-80 nm Gram(+), 5-10 nm Gram(-)	< 10 μ m	-

	Plasma membrane	Y (Envelop ed virus only)	Y	Y	Y
	Plasma membrane thickness	3-5 nm	3-5 nm	3-5 nm	3-5 nm
	Major plasma membrane composition	Lipids (phospho lipids and cholesterol), proteins, and carbohydrate groups	Lipids (phospholip ids and cholesterol) , proteins, and carbohydrat e groups	Lipids (phospholip ids and cholesterol) , proteins, and carbohydrat e groups	Lipids (phospholip ids and cholesterol) , proteins, and carbohydrat e groups
	Protein capsid	Y	N	N	N
	Semi-permeable plasma membrane of selective molecules	Y	Y	Y	Y
	Signaling of molecules	Y	Y	Y	Y

Y = Yes or present, N = No or absent, ‘-‘ = Not applicable

From a structural point of view [Table 1.1], these natural architectures, the eukaryotic cell, bacterial cell and virus assemblies are different from one another (e.g., the type of genetic materials present, the presence of membrane-bound organelles, protein production ability, etc.). Yet, they still possess some similar characteristics such as the presence of the plasma membrane or cell membrane in all of these natural architectures, except for non-enveloped virus.

The plasma membrane is an excellent example of biomolecular self-assembly on the nanometer to micrometer length scale. The plasma membranes are mainly composed of lipids (phospholipids and cholesterol), proteins (specific cell membrane receptor proteins, transport proteins: channels, carrier, ion pump proteins, lipoproteins), and polysaccharides or carbohydrate chains (mostly attached to the lipids “glycolipids” and proteins “glycoproteins”). According to the *fluid mosaic model* by Singer and Nicolson (1972) phospholipids are the most important and abundant components of the cell membrane. Functional proteins are also present and constitute ~50% of the whole membrane mass (due to their large molecular weight) [14] [Figure 1.2]. Phospholipids are amphiphilic molecules that self-assemble into bilayers architecture in an aqueous environment with the hydrophilic phosphate head groups orienting towards the aqueous medium and hydrophobic lipid chains residing inside the bilayer membrane. The embedded membrane proteins in the lipid bilayer will be distributed throughout the bilayer and its fluid character allows the membrane components (e.g., phospholipids, cholesterol, proteins) to diffuse laterally in the bilayer plane. Along with lateral diffusion, lipid molecules can undergo flip-flop movement. With the help of enzyme, such as flippases, asymmetrical membrane composition can be maintained [15]. Unlike lipids, proteins cannot flip-flop in the membrane thus, preserve the biofunctionality at right orientation [15]. The lipid bilayer limits water and other molecules (e.g., neurotransmitters, hormones, ions etc.) from permeating across the membrane while proteins transporters allow necessary ions, small molecules, and other signaling molecules to be selectively transferred across the cell membrane.

Figure 1.2: Structure of plasma membrane: composed of lipids (phospholipids and cholesterol), proteins, and carbohydrate groups. It is one of the common feature most natural architecture possess (animal cell, plant cell, bacteria, enveloped virus). Image source Wikipedia.org.

These natural architectures including the simplest unicellular forms of cells are highly complex. Two fundamentally different approaches have been considered to mimic certain aspects of cellular function. A) A minimalistic approach that involves stripping down of the genome to the lowest number of genes to maintain essential properties of the cell [16]; B) A self-assembly approach to engineer cellular life from scratch using basic membrane components as building blocks [17]. Although the two approaches are different, they share the same goal – to construct an autonomous artificial cells. Three basic elements are needed for the construction of a functional an artificial cell using these approaches: 1) information-carrying molecules, RNA or DNA which hold the genetic code, defining the nature and function of the cell, 2) cell membrane, which keeps the intracellular substances secure, and enables communication with the outside world via transport materials, and 3) metabolic systems that generates energy for cell operation, growth, and division [18-20]. Because of this complexity, many studies focus on cell-like structures that exhibit certain key features of living cells such as shape, morphology, surface properties, and a few specific functions. These artificial cells range from basic protocells, a stepping stone to the origin of life (a spherical assembly of lipid or a mixture of lipid and RNA/DNA replicase) to advanced/engineered synthetic cell mimetic vesicles.

1.2 Introduction of Biomimetic Architectures

1.2.1 Liposome

A liposome is an artificial vesicular architecture, generated by self-assembly of simple building blocks that mimics the cellular membrane structure in an aqueous medium. It is made of a phospholipid bilayer, similar to the cell membrane, the closest and most recognized assembly out there that mimics structural and compositional integrity of the cell [Figure 1.3]. This advantageous feature makes the liposome the most studied artificial cell membrane till date. Another advantage of the liposome is its biocompatibility and low immunogenicity which has led to the launch of a few authorized liposomal drug formulations (DOXIL: Doxorubicin liposome formulation, an anti-cancer drug, Caelyx, Daunoxome etc.) into the market. Encapsulation of RNA, DNA, protein, and small biomolecules to enable protein biosynthesis has been carried out to produce functional liposomes [21-24]. Giant unilamellar vesicles (GUVs) also have attracted enormous attention for biochemical reaction and synthesis of a macromolecules because of their large size and easy handling [25-27]. Even more advanced and complex function such as gene expression and production of functional protein have been demonstrated in GUVs [28-30]. Despite all these advantages, liposomes suffer from poor chemical and mechanical stability. Even after 50 years of research, only a few liposomal formulations have reached the market. The major obstacle working with a liposomes or other lipid-based assemblies *in vivo* is the poor mechanical and chemical stability. For example, the high shear rate in the bloodstream through microtubular tiny vessels results in spontaneous leakage, liposome fusion, uncontrolled burst release immediately after the administration, and limited blood circulation time. Moreover, due to faster hydrolysis, prompt oxidation, easily recognized by scavenger cells and lack of customizability limit their use in biomedical applications.

Figure 1.3: Comparison of the cellular architecture vs the bilayer phospholipid assembly: liposome. From a structural point of view both the membrane composed of amphiphilic phospholipid bilayer facing the hydrophilic phosphate group outwards to aqueous medium while hydrophobic lipid chain (hydrophobic tail) inside the bilayer core. Both the cases membrane thickness reported around 3-5 nm. Images are taken from Encyclopedia Britannica, Inc. (animal cell), Bio-foundations.org (cell membrane), Quora.com, and Livlong Alberta Ltd (liposome) respectively.

1.2.2 Polymersome

To overcome the limitations of liposomes, polymersomes have been exploited as the synthetic alternatives to liposomes. Polymersomes are prepared aqueous medium composed by self-assembly of two distinct block copolymers (BCPs), one hydrophilic and other hydrophobic similar to phospholipids [Figure 1.4]. Polymersomes offer advantages over liposomes in terms of mechanical and chemical stability, chemical versatility, easy functionalization, and programmable responsive behavior upon external stimuli such as pH, redox state, temperature, light, small molecules, solvents and others [31-37]. Furthermore, the membrane thickness can be varied by increasing/decreasing the molecular weight of the polymer blocks [38, 39]. This is advantageous in terms of producing entities with customizable permeability (for often used polymersome such as PBD-PEO, PS-PAA, and PMOXA-PDMS-PMOXA (ABA), the typical water permeability

value lies around 1-10 $\mu\text{m/s}$ while typical liposome value is 15-150 $\mu\text{m/s}$ [40, 41]). However, the polymersome permeability largely depends on the chemical composition of the polymer, for example, pluronic ABA tri-block copolymer (PEO₅-PPO₆₈-PEO₅) shows almost no permeability barrier for water while PBD-PEO shows very less permeability [42]. Polymersome membranes are 3-5 nm thick (comparable to the cellular plasma membrane thickness) and up to about 40 nm have been produced [43]. Apart from membrane flexibility and functionalization, polymersomes are capable of encapsulating biomolecules such as protein, enzyme, nucleic acids, and synthetic materials. Regardless of its vast horizon as a potential candidate for the artificial cell, scientists are still struggling to overcome some of the basic issues, such as poor biodegradability and biocompatibility of synthetic copolymers (e.g., polystyrene copolymer, polymethylmethacrylate copolymer, polybutadiene copolymer, etc.) compared to phospholipid which results in adverse immunogenicity. To overcome this issue more biocompatible and biodegradable copolymer have been introduced, such as amphiphilic block co-polypeptides, PEGylated biodegradable hydrophobic blocks, poly (lactic acid)-b-PEG, poly (ϵ -caprolactone)-b-PEG etc. [44-46]. Polymersome membranes formed by biodegradable polymers are usually thicker than those found in common liposomes or the actual cell membranes a feature that may hamper the polymersome in performing certain membrane-associated functions. In general, both the existing systems, liposomes and polymersomes, have their own pros and cons and these are not a perfect mimic for natural architecture. In order to address these issues associated with both liposomes and polymersomes, a lipid-polymer hybrid vesicle has been introduced. The hybrid lipid-polymer vesicles will combine to take advantage of the beneficial aspects of liposomes and polymersomes while minimizing the limitations by supporting each other.

Figure 1.4: The structural difference between lipid bilayer of liposome vs amphiphilic block copolymer membrane for polymersome. Membrane thickness for polymersome was reported ranging from very thin membrane of 5 nm to over 40 nm, which is governed by several factors such as: molecular weight of copolymer, polymer chain length, composition, type of polymer used (e.g., single chain, grafted, branched) etc., while the bilayer thickness of liposome is between 3-5 nm independent of bilayer composition. Images are taken from Quora.com, Livlong Alberta Ltd (liposome), and RSC Publishing Ltd [39] (polymersome), respectively.

1.2.3 Lipid/Polymer Hybrid Vesicles

Numerous studies have demonstrated the superiority of polymer-lipid hybrid systems to their non-hybrid counterparts in providing increased chemical and mechanical stability, improved biocompatibility, modulated bilayer thickness and controlled permeability with variable lipid/polymer composition. Water permeability through liposome is one order magnitude higher compared to common polymersome, which can be tuned by mixing lipid and polymer in a certain ratio. The permeability of H^+/OH^- ions through hybrid lipid/polymer vesicle (PBD₃₇-PEO₂₂/DOPC, 25-50% of polymer) is higher than for pure lipid and pure polymer vesicles and fall in a range of $2 \times 10^{-9} - 5 \times 10^{-9}$ cm/s [47]. Similar permeabilities were observed for other small molecules. However, several

physical and chemical parameters should be taken into account to form a stable hybrid membrane with lipid and synthetic polymer. These are the size of the polymer hydrophilic or hydrophobic polymer segment, the charge of the lipid head group and polymer, hydrophobic domain thickness of the lipid tails and polymer chains, the molar ratio of the interacting components, curvature of vesicles, and the membrane formation process itself [48-55]. The morphology of the membrane depends on the mixing/interpenetration of both phases in each other. The membrane of hybrid vesicles can either be well mixed to give rise a homogeneous membrane, or to a phase separated membrane consisting of polymer-rich and lipid-rich domains which co-exist together to give rise to a textured morphology [Figure 1.5] [56]. To date, the physical and molecular parameter governing the phase separation is not thoroughly understood and more in depth knowledge is required to understand the actual cause and how they can be implemented in future applications. However, a few parameters/conditions play a crucial role for the domain formation in hybrid polymer-lipid vesicles, such as

A) The physical state of lipid (fluid state and gel state): it was observed that a homogeneous phase distribution is favored when the lipid was maintained above its transition temperature ($>T_m$), whereby the lipid exhibits fluid/liquid disordered phase, while a lateral phase separation occurs when lipid used below its transition temperature ($<T_m$), whereby the lipid exhibits solid ordered gel phase [57].

B) Bilayer thickness and hydrophobic chain mismatch between polymer and lipid: the bilayer membrane of most polymersomes is significantly thicker (≥ 8 nm) than that of a conventional liposome or lipid bilayer (~ 5 nm). The excess hydrophobic mismatch causes the hydrophobic group to be exposed to aqueous medium with an energetically unfavorable polymer-lipid interface, which results in domain formation arising from the energy minimization of line tension at domain boundaries [58]. Thermodynamically, the line tension tends to favor domain coalescence to minimize the boundary length, which leads to a big single domain formation and the gained free energy dissipates through lateral phase separation and budding formation [58, 59].

C) Lipid fraction in hybrid vesicle: it was observed that above a critical lipid fraction, lipid tends to phase separate [51, 60].

D) Another interesting parameter is chemical compatibility of lipid and polymer in the hybrid vesicles. According to the recent report by O. Sandre *et al*, [50] it has been suggested that the solubility of hydrocarbon moieties of lipids in the hydrophobic polymer blocks is governed by the chemical compatibility between two hydrophobic segments. Close chemical compatibility results in better solubility of both segments which leads to the more stable bilayer membrane.

E) The architecture of the copolymer: it has been observed that mixing of same molecular weight copolymer, PDMS₂₆-g-(PEG₁₂)₂ (one tri-block copolymer and another grafted copolymer) with lipid POPC exhibits different phase separation behavior at different lipid weight %. The grafted copolymer /POPC exhibit a visible phase separation at 22 weight % of lipid (50/50 mol %) and above whereas, tri-block copolymer exhibits at only 16 weight % of POPC [58].

Figure 1.5: Hybrid vesicles prepared by combining lipids and BCPs into blended membranes forming either a homogeneous phase vesicle or a phase separated textured vesicle.

Given the above observations, it's essential to gain an in-depth knowledge of insight molecular and macroscopic parameters to apply such tools for future biomedical applications. Upon understanding the phase separation, membrane structure can be tuned either to homogeneous mixing phase or phase separated domain to perfectly exploit the potential of such systems to validate the future applications.

1.2.4 Peptide-based Self-assembly

Apart from the synthetic BCPs, polypeptides have been used as the hydrophilic and/or the hydrophobic blocks to prepare polymersomes [61-63]. Such polymersomes are particularly interesting because of their diverse biofunctionality, biocompatibility, and biodegradability. Various amphiphilic α -

helical polypeptides have been reported to self-assemble into vesicle in the aqueous medium named peptosome [62-65] such as charged block polypeptide, poly(L-lysine)-*b*-poly(L-leucine) (PLL-P(Leu)) designed to form vesicle by charge-charge repulsion (same charge) from the PLL blocks [61, 66]. Another class of vesicles designed from polypeptides of oppositely charged building blocks named polyion complex vesicle, PICsomes (or nano-PICsome) [67-73]. Mixing of a PEGylated cationic polypeptide (poly(ethylene glycol)-*b*-poly([2-aminoalkyl]- α,β -aspartamide)) with PEGylated anionic polypeptide (poly(ethylene glycol)-*b*-poly(α,β -aspartate)) results in vesicle formation in the aqueous buffer.

1.2.5 Protein Self-assembly

Beside lipid, polymer, and polypeptide self-assembly, protein self-assembly has drawn a tremendous attention over past decades. Decades of research on protein self-assembly and protein-small molecule (e.g., ions) self-assembly have revealed insight of several natural assemblies, which play a huge role in animal and plant body. A few example of these natural assemblies are collagen fibers (a structural protein assembly, one of the most abundant protein complex in our body, which constitutes 25-35% of whole body protein, is associated with various connective tissues and help to form corneas, cartilage, bones tissue, blood vessels etc.), amyloid fibers (a fibril type protein assembly associated with several diseases in human body, e.g., Alzheimer's disease), β -clamp (a protein self-assembled architecture that plays an important role in DNA replication. This type of protein assembly is also observed in bacterial DNA replication and in ferritin assemblies (a universal intracellular iron cage, which stores iron and releases in a controlled manner), etc. [Figure 1.6]. These protein architectures are a consequence of precise self-assembly at molecular level from protein building blocks [74, 75]. The mechanism of protein-assembly depends on the protein structure, its interaction with other protein (such as hydrophobic interaction, H-bonding, π - π and van der Waals interactions) and the net thermodynamic binding energies of the assembly [76-78]. In addition, environmental factors such as the pH, temperature, ionic strength, and choice of

solvent, also play an important role for the stability of the constituting proteins and their assemblies [79]. However, the understanding of the programmable self-assembly of proteins is still inadequate in comparison to polymer, lipid and DNA self-assembly. Nonetheless, this field holds great potential due to the structural and functional diversity of proteins. [80]. Recent development in chemical and biological technologies help to design new self-assembled structure governed by external stimuli include metal ions, molecular template, symmetric fusion based assembly etc. Using these techniques various protein nanostructures have been constructed, ranging from nanofibers, multiscale layers, ring-like structures and hollow tubes to spherically shaped cages [74, 81-83].

Figure 1.6: Natural Protein Assemblies with Diverse Structures for Symmetry-Dependent Biological Functions: Collagen (PDB: 3B0S); Amyloids (PDB: 2MXU); β -clamp (PDB: 2POL); Cowpea Chlorotic Mottle Virus (CCMV) (PDB code: 1CWP) [82]; Ferritin (PDB: 1BFR) [83].

1.2.6 Virus Particles Self-assembly

The understanding of the hybrid vesicle self-assembly and protein self-assembly process will also serve as a guide for viral assembly mimicking. However, complete mimicking of the virus can only be possible by mimicking the viral capsid, before more complex protein-protein self-assembly and protein-nucleic acid self-assembly can take place. Viruses and virus capsid are highly ordered, advanced biological self-assembled architectures capable of performing multiple complex functions during their infectious cycle. The understanding of the molecular self-assembly of viral capsid aids in the pursuit of biomedical strategies in fighting against viral infections [3, 4, 8]. Moreover, a better understanding of protein - protein and protein – nucleic acid assembly mechanism opens up for alternative use in (i) design novel anti-viral drugs that could inhibit viral growth by affecting the capsid assembly; (ii) design viral capsid without the infectious gene to suit specific application such as, vaccine generation, drug and gene delivery, cancer treatment, fight against infectious bacteria, imaging, nanobiosensing and more; (iii) creation of artificial virus particles or mimicking of the viruses with same/other protein to suit specific application; (iv) optimize the conditions for artificial viruses or virus like particles (VLPs) to achieve the efficient in vitro self-assembly; (v) design other protein-based, non-biological or hybrid self-assembling nanoparticles, nanomaterials and nanodevices [Figure 1.7] [3, 4].

Figure 1.7: Applications of VLPs in biomedicine and other technology [84].

1.2.7 Protein-Polymer Conjugated Self-assembly

Nowadays, protein-polymer conjugates are gaining increasing attention in nanobiotechnology because of their programmable and complementary role from each component into the composite self-assembly [84-87]. Protein provides a high degree of structural complexity, multifarious reaction cascade and specific molecular recognition that cannot be achieved by synthetic materials [88-92]. Thus, to use an existing or marginally modified protein to fulfil the gap of synthetic materials is the key governing factor for protein-polymer conjugates. Recent years, more advanced and sophisticated polymer has been reported with excellent thermal and mechanical stability, end-less chemical functionalities/diversity, altered solubility, superior stimuli responsiveness by pH, temperature, redox, light etc. [31-37]. Nonetheless, these advancements are fairly simple as compared to protein's functional diversity. Still, in some cases this polymer conjugate plays a significant role in protein solubility, stability, and functions as the first protein-polymer conjugate has been reported in the 1970s as a functional PEG (polyethylene glycol), to increase protein solubility. PEGylation further showed that it increases protein's stability in water, prolong blood circulation and reduce immunogenicity. Nowadays, researchers have begun to use greater structural and chemically diverse polymer to achieve higher order hierarchical self-assembly [34]. These protein assemblies combine the

beneficiary effects of both protein and polymer which in the stand for useful applications such as catalysis, biosensing, drug delivery and other biomedical applications [93].

In order to form spontaneous hierarchical protein-polymer self-assembly, molecular interaction parameters play a gigantic role. Molecular parameters involve van der Waals interaction, hydrophobic interaction, H-bonding, π - π interaction etc. Two major approaches have been taken into count for successful protein-polymer conjugations: A) Physisorption of protein to polymer moieties e.g. via π -, hydrophobic interaction, H-bonding, π - π interaction); and B) chemical attachment (covalent or non-covalent bonding) of protein to the functional polymer. In both cases, the final assembly behavior is governed by the associated interaction parameters.

Over the past decades, several strategies have been adopted to design the protein-polymer conjugated nanostructure such as: A) a non-lithographic block copolymer template assisted patterning for protein array on pre-formed polymer nanostructure by hydrophobic interaction [94]. IgG protein was patterned on top of a polystyrene-b-poly(methyl methacrylate) (PS-b- PMMA) coated silicon wafer for improved biofunctionality [94]. B) Nolte's group demonstrated that polystyrene (PS), covalently linked to horseradish peroxidase (HRP) or myoglobin (Mb) enzymes in solution, generated a vesicular aggregate [95-97]. C) Olsen and co-workers investigated the mechanism of protein-polymer self-assembly in concentrated aqueous solution as well as the resulting solid-state nanostructures using a thermoresponsive polymer poly(N-isopropylacrylamide) (PNIPAM) (which exhibits hydrophilic to hydrophobic transition above lower critical solution temperature) with globular protein (mCherry, GFP, and Mb) [98-100]. Their findings suggest that protein structure, the protein shape, solubility, surface charge, and are largely responsible for the self-assembly behavior of these protein-polymer assemblies [100].

In recent years, a fairly simple and general approach has been proposed by S. Mann and co-workers to overcome the protein structure and shape dependency in self-assembly [101]. A protein-polymer vesicle termed proteinosome was assembled at the oil/water interface. Amine ($-\text{NH}_2$) groups on

Bovine Serum Albumin (BSA) was linked with a mercapto thiazoline-activated PNIPAAm polymer produced a giant amphiphile that self-assembled into proteinosome of micrometer size (20-50 μm) [Figure 1.8]. The construct was stabilized by post-synthetic cross-linking of carbodiimide-activated carboxylic acid residues (aspartic and glutamic acid) of protein using 1,6-hexanediamine.

Figure 1.8: General procedure for preparation of proteinosomes. (a) preparation of protein–polymer nano-conjugates by coupling of mercapto thiazoline-activated PNIPAAm polymer with primary amine groups of cationized BSA-NH₂. (b) Preparation of proteinosome by protein-polymer conjugates at oil/water interface was stabilized further by cross-linking using 1,6-hexanediamine [101].

The latest advancement in the protein self-assembly and approaches of protein-polymer self-assembly addresses a few earlier challenges of biomimicry of protocell design and holds a huge potential for a future toolkit for the advance biomedical application. Olson demonstrated the effect of protein structure, shape, solubility and charge variation on self-assembly [100]. S. Mann has addressed the issue of protein dependency by adopting a general approach of protein-polymer self-assembly in oil/water emulsion [101]. The proteinosome prepared in emulsion process minimize the role of protein's structure and shape dependency by considering protein as a hydrophilic segment and polymer as a

hydrophobic segment in assembly formation. However, various aspects remain untouched such as, (i) using protein as hydrophobic domain, as most reports deemed protein as hydrophilic component and polymer as hydrophobic. However, the opposite fact has not been investigated with hydrophobic water-insoluble protein. (ii) Formation of proteinosome in a completely aqueous environment similar other biological assembly needs to be developed.

Table 1.2 provides a comparison of a few critical parameters for commonly used vesicular systems.

Table 1.2: Comparison of common biomimetic vesicular systems

Properties	Liposome	Polymersome	Polymer-lipid hybrid	Protein/peptide based assemblies
Component	Lipid and phospholipid (natural or synthetic)	Polymer (natural or synthetic)	Polymer, lipid and phospholipid (natural or synthetic)	Protein, peptide and polymer (natural or synthetic)
Size (nm)	30 nm to >20 μm	30 nm to >20 μm	30 nm to >20 μm	30 nm to ~200 μm
Morphology	Vesicular	Vesicular	Vesicular or tubular	Vesicular
Membrane thickness	3-5 nm [43]	5-40 nm [43]	5-40 nm	~ 10-20 nm [101]
Biocompatibility	Yes	Yes (mostly for biodegradable polymers only)	Yes (mostly blends with biodegradable polymers only)	Yes
Biodegradability	Yes	Yes (mostly for biodegradable	Yes (mostly blends with biodegradable	Yes

		polymers only, not for non- biodegradable polymer)	polymers only)	
Stability	Less stable	More stable	Stable	Stable
Chemical versatility	Poor	Very good	Very good	Very good

1.3 Motivation

Inspired by nature and natural assemblies to design sophisticated materials, not only because of the precise and beautiful organization, but also because of the huge potential of such assemblies in various applications. Biomimicry gives us the opportunities to extract the best features from natural assemblies, and likewise allow us to merge the best properties from several systems into one assembly. Synthesis of novel materials by merging properties from various assemblies is relatively common nowadays. Other advantages of biomimetic assemblies are customizability and stimuli responsiveness. Virus assemblies are being used decades to generate a vaccine against the same virus after removal of infectious genetic materials. However, these assemblies are extremely specific for certain types of viruses. Instead, recent development with polymersome, virus-like particles or advanced proteinosomes can be used as a multipurpose carrier and a toolbox for multi-target vaccines generation, drug delivery, cancer prevention, fight against bacterial infection, nanobiosensing, imaging to nano-reactor for controlled chemical synthesis. Furthermore, the state-of-the-art progression of programmed self-assembly behavior by external stimuli, such as pH, temperature, ionic strength, light, UV irradiation etc., make these assemblies more versatile and adequate. Currently, biomimetic assemblies are well advanced and able to perform multi-tasking beyond their natural architectures. Even though, these natural assemblies are superior over the synthetic ones in certain areas, especially in complexity, adaptability, and self-healing ability that only

natural assemblies can achieve. Thus, there is a continuous need for improvement and advancement in future.

1.4 Research objective

- a) Both liposomes and polymersomes have practical limitations, such as stability and biocompatibility. The hybrid polymer-lipid assembly has been introduced to address these limitations. Even though hybrid vesicles are not up to the mark to natural cell membranes because of a substantial mismatch in physio-chemical properties between lipid and polymer building blocks, e.g. in terms of chemical compatibility, membrane disparity, and molecular weight dissimilarity, they still constitute an attractive model system. Thus, serious efforts are required to develop hybrid vesicles for demanding practical applications. In addition, in-depth characterization and understanding the system is also necessary to compare their properties with natural cell membranes and effectively use these vesicles for future.
- b) Protein-polymer mediated self-assembly has been reported by several research group, however, each system has their own limitations, such as, the current approach by S. Mann, that requires an oil/water emulsion technique to create this assembly which limits its use in many oil sensitive bioprocesses. Thus, a serious urge is to develop a method that mimics viral protein capsid in a fully aqueous medium. If successful it can serve as a broad-ranging tool for viral mimicry independent of the virus of choice.
- c) A popular approach to design a vaccine against viruses, a genetic material free artificial viral capsid constructs from the same viral proteins, which are prepared by genetic engineering using *E. Coli* and/or other foreign organism: mostly know as virus-like particles (VLPs). Use of a VLP in the vaccination is one of a growing demand. In spite of genetic engineering approach, a fairly easy and cost-efficient alternative could be a synthetic vesicle such as polymersome, lipid/polymer hybrid vesicle or proteinosome embedded with spike proteins from the virus of interest.

Beside vaccine generation, these biomimetic vesicles serve as an on-demand toolkit for various biomedical (e.g., targeted drug/gene delivery, biosensing, imaging etc.) and non-biomedical disciplines (e.g., gas separation, optoelectronic devices, catalysis etc.).

1.5 Aim of the project

The aim of the project is

- a) To design a stable, robust, and homogeneous hybrid polymer-lipid vesicle to mimic the cellular membrane and lipid viral envelope by keeping the membrane thickness as close to actual cellular membrane thickness. Moreover, to use these hybrids as future drug delivery carriers. The stable and robust membrane will ensure extended circulation times in the complex media for drug delivery application.
- b) Design and synthesis of stable protein-polymer conjugated nanovesicles to mimic viral capsid from entirely aqueous medium eliminating the oil from self-assembly process. Furthermore, to apply these nanovesicles as a future gene delivery/drug delivery system, and potentially also as a multipurpose vaccine generator.

1.6 Hypothesis

I hypothesize that,

- a) Low molecular weight polymers (< 4 kDa) contribute to improved chemical and mechanical stability of model hybrid vesicles by reducing the polymer-lipid mismatch. Such hybrid constructs potentially offer a platform for therapeutic applications in demanding environments.
- b) The combined effect of electrostatic interaction, hydrophobic interaction and protein-polymer amphiphilicity enables self-assembly of two oppositely charged and PEGylated proteins in aqueous solution into nanosized vesicular architectures without using a template or oil/water emulsions.

The evolved nano-vesicles, the lipid-polymer hybrid, and protein-polymer conjugate will keep a step ahead toward the imminent biomimetic architecture and will unleash the possibility of future nanomedicine applications including, targeted drug delivery, nanobiosensing, imaging, next-generation multi-targeted vaccine generation with appropriate surface receptor-ligand binding and more.

CHAPTER 2 LITERATURE REVIEW

2.1 Introduction of Self-assembly

Self-assembly is a process where the components of a disordered system organize themselves spontaneously and reversibly into a higher order structure or patterns without the help of an external stimulus. The process of self-assembly follows the law of thermodynamics where the assembly attain a lower Gibbs free energy of the system compared to the un-assembled components. Lowering of Gibbs free energy drives the formation of relatively defect-free self-assembled structures. In general, self-assembly of macromolecules are determined by the local weak interaction forces (e.g., Van der Waals force, the capillary force, π - π interaction, hydrophobic interaction, and hydrogen bond) in contrast to the stronger covalent, ionic, or metallic bonds.

2.2 Self-assembly of phospholipid: Liposome

Liposome is the simplest form of artificial vesicle that mimic the spherical shape of the cellular structure. It is composed of natural amphiphilic phospholipid and cholesterol, which self-assembles into bilayer morphology in an aqueous medium. In aqueous medium, the bilayer structure is fully governed by thermodynamics principle and generally referred to as lamellar structure [102]. The size of the vesicle can vary from 30 nanometer to tens of micrometers depending on the preparation process. The liposomal properties vary with the composition of the lipid, head group charge and size, and method of preparation.

2.2.1 Characteristics of lipid membranes

2.2.1.1 *Lipid structure*

The type of phospholipid used generally determines the formation of a ‘rigid’ or ‘fluid’ membrane. Saturated phospholipids generally form more stable and dense bilayer compared to unsaturated phospholipids. The presence of one or more double bonds in the unsaturated lipid tail disrupts the van der Waals forces resulting in a loose packing of the phospholipids. This property largely

affects the permeability of the membrane as fluid membrane is more permeable to water and small molecules [Figure 2.1(A)] [103-106].

Figure 2.1: (A) Saturated lipid vs unsaturated lipid packing in liposome membrane. (B) Different phases of lipid below and above phase transition temperature.

2.2.1.2 Thermodynamic state

Lipid membrane exists mainly in two states, i) solid-ordered state (or gel state) below its transition temperature (T_m) and ii) liquid-disordered state (or liquid crystalline state or fluid state) above its T_m [Figure 2.1(B)]. The lipids in a solid-ordered bilayer are tightly packed due to straightening of the acyl chains, resulting in a thicker bilayer membrane and reduced area occupied by the head group. The opposite is observed for liquid disordered phase lipid, characterized by a thinner bilayer and disordered packing. For instance, for DPPC membrane in solid ordered phase, average area per lipid is 0.48 nm^2 and bilayer thickness is 4.79 nm , whereas the fluid phase exhibits the an average area per lipid of 0.63 nm^2 and a thickness of 3.92 nm [107, 108]. Lipid membrane at fluid phase exhibit more flexibility, swelling and more lateral diffusion [109, 110].

2.2.1.3 Molecular dynamics

Lipid membrane in a liquid phase is more dynamic compared to a gel phase. In liquid phase membrane, lipid molecules are allowed to move freely or exchange their lateral position in bilayer, measured by lateral diffusion coefficient. The lipid molecules also traverse from one leaflet to another by the so-called flip-flop movement, measured by the transverse diffusion coefficient.

2.2.1.4 Permeability

Permeability is a parameter that indicates the rate at which molecules cross the membrane. Major factors that govern the lipid membrane permeability are, i) membrane thickness, ii) packing density, and iii) fluidity of the membrane. The typical value for water permeability across the liposome membrane is 15-150 $\mu\text{m/s}$ [41].

2.2.1.5 Mechanical properties

The mechanical strength of liposome is typically characterized by stretching modulus, bending modulus (κ) and the lysis tension. The bending modulus (κ) and the lysis tension depend on membrane thickness, as lipid with lower thickness severely suffers from mechanical stability which limits its applications.

2.3 Self-Assembly & Fabrication of Polymersomes from Amphiphilic Block Copolymers

In the past decade, self-assembled polymers have attracted tremendous attention due to their vast possibility in various disciplines. Numerous self-assembled structures have been fabricated, e.g., polymer micelles, cross-linked polymer nanoparticles, dendrimers, a polymersome, worm-like, tubular, rod-like polymer assemblies and multicompartament capsules [111-117]. Among these self-assembled architectures polymeric vesicles i.e., polymersomes, have attracted ample interest for their analogous structure to cellular compartments [17], mimicked also by liposomes. Polymersomes have extensively used in different disciplines ranging from chemical to material science, pharmaceutical, medical to biotechnological sectors. They are used for applications such as nanoreactors, drug or gene delivery vehicles, biosensing, nanomedicine, vaccine generation, drug screening, drug discovery and catalysis [118, 119]. In analogy to liposomes, Polymersomes are self-assembled vesicles composed of amphiphilic BCPs in an aqueous medium. This section presents a review on the physio-chemical properties of polymersomes made of amphiphilic BCPs; such as diblock copolymers [113, 120], triblock copolymers [120, 121], grafted polymers [122], dendritic polymers [123, 124] and their formations.

2.3.1 Characteristics of block copolymer membranes

The size of the polymersome can vary widely from few nanometers to tens of micrometers in diameter (e.g., 10 nm to ~100 μm) depending on their chemical constitution, size, shape, and molecular weight of the polymer blocks. Method of preparation also plays a significant role in size distribution [125]. In comparison to liposomes, polymersomes exhibit negligible exchange of block copolymer between vesicles, low critical aggregate concentration (CAC), and enhanced mechanical stability [111, 126].

2.3.1.1 *Hydrophobic weight fraction*

Similar to lipids, the geometry of the polymersomes is governed by the hydrophilic and hydrophobic weight fraction (denoted as f). In order to form a vesicle geometry, hydrophilic fraction, f of the block copolymer need to be within 25-40 % of the total weight fraction. Hydrophilic fraction above 40 % leads to wormlike or spherical micelles whereas, f below 25 % gives rise reverse micelles [38, 39, 120, 127].

2.3.1.2 *Membrane thickness*

The bilayer membrane thickness of polymersomes is determined by the relative length, size and molecular weight of their hydrophobic and hydrophilic chains [38, 39]. Thus, high molecular weight polymers yield thicker membrane with reduced permeability and a greater mechanical stability [38, 113, 120, 121]. In addition, the chemical versatility of polymers allows polymersomes to be engineered easily to improve their mechanical stability, to introduce target functionality or to endow stimuli responsiveness [125, 128]. Few examples of commonly used amphiphilic block copolymers (BCPs) that assemble into polymer vesicles are shown in Table 2.1.

Table 2.1: The chemical structures of common examples of amphiphilic block copolymers (BCPs) that assemble into polymersomes.

Chemical Structure	Abbreviation
	PEG-PDMS
	PEG-PLA
	PEG-PCL
	PEE-PEO
	PBD-PEO
	PS-PAA
	PS-PIAA
	PS-PIAT
	PEO-PDMS-PEO

PDMS = polydimethylsiloxane, PEG = polyethylene glycol, PLA = polylactic acid, PCL = polycaprolactone, PEE = polyethyl ethylene, PEO = polyethylene oxide, PS = polystyrene, PBD = polybutadiene, PAA = polyacrylic acid, PIAA = polyisocyanodialanine, PIAT = polyisocyanooalanine(2-thiophene-3-yl-ethyl)amide, PMOXA = poly-(2-methyloxazoline), PPS = polypropylene sulfide, PPO = polypropylene oxide.

2.3.1.3 Type of block copolymer architecture

The amphiphilic block can be of an AB or of ABA/ABC triblock copolymers where the B block is a hydrophobic block and A or C being the hydrophilic block respectively. A or C presents at the outer surface and in the inner surface of the vesicles while the B block stays the center of the bilayer membrane. A major challenge of polymersomes formation is to find a method that ensures a controlled membrane formation. As polymeric assemblies (e.g., micelles, vesicles) are generally very stable and unlikely to exchange polymer chain due to higher kinetic barrier once formed, it is important to obtain target the correct self-assembly structure directly.

2.3.2 Biodegradability of polymersome

Biodegradability is a term used to describe the ability to degrade a material either by a biological or chemical approach over time inside or outside the body. In biological prospective, biodegradability means breaking down the materials into the smaller parts by enzymatic degradation or by ecological means (by bacteria, fungus etc.). In case of polymer materials degradation is mostly by hydrolysis or by oxidative cleavage of labile chemical bonds. In this thesis the

biodegradable term will be used mostly on chemical perspective as most biodegradable polymersomes are easy hydrolyzable (e.g., polycaprolactone (PCL), polylactic acid (PLA) etc. [128-132]).

The essential characteristic of a biodegradable polymersome is the ability to cleave at least one or more block of a di (or tri) block copolymer. Up until now, plentiful cleavable linker-based polymersomes have been explored (e.g., acetal [133, 134], ortho ester [135], imine [136], and hydrazone [137]. The reports suggests that more hydrophobic block has been targeted as biodegradable along with hydrophilic polymer (e.g., polyethylene glycol, PEG) to produce biodegradable block copolymer and the resulting polymersome degrade through the degradation of the hydrophobic block [131, 132].

2.3.3 Basic physical properties of liposome and polymersomes: A comparison

The basic physical properties of polymer membranes are shown in [Table 2.2] Compared to lipid membranes, polymer vesicles possess higher mechanical toughness and lower permeability that can be modulated via the molar mass of the copolymer used. Moreover, they present more versatile chemical functionality compared to liposomes.

Table 2.2: Physical and mechanical properties of liposomes and polymersomes and scaling with membrane thickness (d) and polymer molar mass (M_w) (Adapted from [139]).

Property	Liposomes	Polymersomes	Scaling
Bending modulus (kT)	11 – 30 [138]	40 - 460 [139, 140]	$\sim d^2$ [140]
Stretching modulus (mN.m ⁻¹)	250 ± 2 [138]	80 – 100 [141]	$\sim d^0$ [141]
Lysis strain (%)	5 [141]	20 – 50 [141]	$\sim d^{0.6}$ for $M_w < M_e$ [141]

Membrane surface shear viscosity (mN.ms ⁻¹)	10 ⁻⁵ [142]	15.10 ⁻³ [139]	-
Water permeability (μm.s ⁻¹)	15 – 150 [41]	0.7 – 10 [41]	~d ⁻¹ [126]
Lateral diffusion coefficient (μm ² .s ⁻¹)	3.8 [143]	0.12 – 0.024 [143]	~M _w ⁻¹ (Rouse scaling for M _w < M _e) ~M _w ²⁻³ (bulk reptation for M _w > M _e)

2.3.4 Preparation method of vesicles

Several methods for preparation of polymersomes are available:

1. Film rehydration [Figure 2.2]: A thin film of polymer is first prepared by dissolving the polymer in an organic solvent followed by evaporation of the solvent. Next step is to rehydrate the thin film by aqueous solvent, allowing polymer films to swell and form the final closed lamellar structure. This process is widely used to prepare liposomes from a phospholipid. This approach usually results in more polydisperse and multilamellar polymersomes, which require further processing to reduce the polydispersity [144-149].

Figure 2.2: Preparation of vesicles by thin film rehydration [152].

2. Direct hydration/Co-solvent method: Block copolymer is dissolved in a very small amount of water-miscible organic solvent followed by the addition of the polymer solution into an aqueous solvent under vigorous stirring. This is a “bottom-up” approach called direct hydration or co-solvent method. This technique generates polydisperse but more unilamellar vesicles [39, 41, 125, 144, 150, 151].
3. Electroformation: Electroformation usually favors giant unilamellar polymersomes (in the range of 1-100 μm) formation. This protocol work as similarly as thin film rehydration in presence of AC current at varying frequency and voltage. A thin film of polymer is dried on a conductive ITO (indium tin oxide) coated glass plate followed by addition of aqueous solvent [Figure 2.3 (b)] [40, 152, 153].

Figure 2.3: (a) Vesicle sizes achieved by different kinds of film rehydration, controlled and uncontrolled and also using electroporation [156]. (b) Electroformation itself is shown on the left where dome-like structures evolve with PEG-PDEA [155].

4. Water-oil-water (W/O/W) double emulsion: A novel approach was recently reported to prepare monodisperse polymersomes (in the μm range) by the use of water-oil-water (W/O/W) double emulsion method [41, 150, 151]. The polymersome prepared by this method exhibit uniform polymer vesicles and excellent encapsulation efficiency [41, 150, 151].
5. Lecommandoux *et al.* developed a multi-step emulsion/extrusion method where first water is added to a solution of the polymer in an organic solvent (i.e., toluene). Thus, a water-in-oil emulsion is formed that is stabilized by the amphiphilic polymer at the water-organic interface [115, 154]. The size of the polymersomes depends on the size of the droplet formed during the emulsion process, making large or giant polymersomes of several micrometers. The final step of a multi-step emulsion process is the pre-formed water-oil-emulsion, extruded into an aqueous solution with higher density than the organic solvent (sucrose solution). Once the water droplets of the emulsion reach the sucrose-solution/oil interface, the polymer self-assembled to bilayer in the interface of the oil/sucrose solution stabilized the water-oil-water emulsion, which gives rise the actual polymersome [Figure 2.4] [154, 155]. This process also presented a better technique to form polymersome-in-polymersome by encapsulating small polymersome in the very first of the water-in-oil emulsion [155] [156].

Figure 2.4: Polymersome prepared using water-oil-water double emulsion method. Polymersome-in-polymersome is also prepared by this approach. The green large polymersome encapsulating small polymersomes (red) [158].

6. Weitz and Battaglia *et al.* presented a microfluidics-based technique to prepare one step water-oil-water emulsion with very high encapsulation efficiency [Figure 2.5] [157, 158]. Moreover, this process can prepare multicompartmental polymersomes [156-158].

Figure 2.5: (a) Schematic illustration showing the formation of multi compartmentalized polymersomes containing several inner polymersomes using a microfluidic device. (b) Optical microscope image showing double emulsion formation by injection of polymersomes into the innermost droplets of double emulsion drops [158].

7. Polymerization-induced self-assembly (PISA): This approach is recently developed by Armes *et al.* which describes the *in situ* polymersome formation during polymerization [159]. Polymersome formation takes place instantaneously after the formation of the hydrophobic block from the aqueous solution [Figure 2.6] [159, 160]. The polymerization of water-soluble hydroxylpropyl methacrylate (HPMA) leads to a water-insoluble polymer PHPMA using a macroinitiator. The process of polymerization follows the reversible addition fragmentation chain transfer polymerization (RAFT) without a metal catalyst starting from a water-soluble prepolymer as chain transfer agent (macro- CTA) [159-161].

Figure 2.6: PMPC-PPHMA vesicles are synthesized using PISA. With an increasing block length of the building block copolymer, the self-assembly structures change from spheres (micelles) to worms to vesicles [163].

2.4 Lipid polymer Hybrid

In recent years, nanomedicine has become extremely popular among scientists and clinicians for its versatile treatment modality for various diseases, especially for cancer therapies [162, 163]. Numerous nanoparticles have been developed thereafter, although liposomes remain the most widely used nanocarrier system in the clinic [163, 164]. Besides liposomes, other nanocarriers are also exhibiting potential applications over liposomes such as polymeric nanoparticle, solid lipid NP (SLN) [165], polymeric micelles, more recently polymer-lipid hybrid NP (PLN) [166] and inorganic materials based nanomaterial (e.g., metals or metal oxides) [167], where many of these nanoparticle systems have been extensively studied in pre-clinical settings. A major hurdle faced by these nanocarriers is to overcome bio-barriers. In order to address these challenges, polymer-lipid hybrid systems are now being designed to merge the beneficial features of both polymeric and lipid-based nanocarriers. Numerous studies demonstrated the superiority of polymer-lipid hybrid systems to their non-hybrid counterparts in providing improved drug encapsulation, modulated drug release, and improved cellular uptake, which encourage their applications in the delivery of chemotherapeutics, proteins, peptides, and vaccines.

The structure of the lipid-polymer hybrid nanoparticles is classified into several categories like monolithic polymer-lipid hybrid matrix systems, nano/micro type hybrid, polymerized liposomes, core-shell nanoparticles (includes polymer-core lipid shell and lipid-core polymer-shell), layer-by-layer coated lipid-polymer hybrid nanoparticles and polymer-lipid hybrid bilayer vesicles. However, only polymer-lipid hybrid bilayer vesicles will be described briefly in this section because of its great potential and structural similarity with the natural cell membrane.

2.4.1 Lipid-polymer hybrid vesicles

Vesicles can be individually fabricated from naturally occurring lipid or synthetic block copolymer molecules via self-assembly in aqueous solutions; the blending of both vesicle-forming amphiphiles leads to the formation of hybrid membranes. Their final stabilities and lateral morphologies are strongly determined by the molar composition, size, and charge properties of the interacting components as well as by the lipid chain melting temperature. Upon merging the best properties of lipo- and polymersome membranes, hybrid lipid/polymer vesicles represent a new scaffold for medical applications combining, e.g., combining the biocompatibility of liposomes with the high thermal and mechanical stability and functional variability of polymersomes within a single vesicle type.

2.4.1.1 Effect of lipid-polymer lateral phase separation

One of the earlier attempt to prepare hybrid vesicle by Ruyscharet *et al.*, demonstrated that lipid Egg phosphatidylcholine (EPC) or 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) spontaneously produced homogeneous phase vesicles even with 2-3 times higher molecular weight ABA triblock copolymer, poly(2-methyloxazoline) – block - poly(dimethylsiloxane) – block - poly(2-methyloxazoline) (PMOXA-PDMS-PMOXA) [168]. Later Binder and Vanderlick demonstrated the formation of stable inhomogeneous hybrid vesicles of clear phase separation of lipid and block copolymer domains [169, 170]. Binder showed that hybrid vesicles exhibited a phase separation behavior at certain mol % of the block copolymer. DPPC (1,2-dipalmitoyl-sn-glycero-3-

phosphocholine) blended with polyisobutylene (PIB), at 20-28 mol % of PIB, generated vesicles with distinct domains whereas other mol % of PIB below 20 mol % or above 30 mol % displayed a homogeneous mixing phase throughout the entire membrane.

Vanderlick et. al [169] demonstrated that at 30 mol% of PBD-b-PEO (polybutadiene-b-polyethylene oxide) with POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine), the membrane undergo phase separation over time from a homogeneous mixing phase [Figure 2.7]. These studies suggest that the type of polymer and lipid-polymer ratio hugely influence the phase behavior of the hybrid vesicle.

Figure 2.7: Confocal microscopy images of rhodamine red-X conjugated neutravidin (Rh-NA) (red) bound to biotinyl PBD-b-PEO (30 mol %)/POPC hybrid vesicles labeled with BODIPY-DHPE (green) with different incubation times: (a-f) single image sections through the vesicle equator (superposition of red and green channels) and (a'- f') 3D reconstruction of vesicle hemispheres. Arrows indicate large Rh-NA-rich domains with irregular curvatures that deviate from the native spherical curvature of the vesicle. The scale bar represents 10 μ m [169].

2.4.1.2 Effect polymer molecular weight on membrane thickness

The thickness of the membrane is a crucial parameter to form a stable vesicle. The bilayer membrane of most polymersomes is significantly thicker (≥ 8 nm) than that of a conventional liposome or lipid bilayer (≤ 5 nm). Mixing of lipid and polymer results in a significant membrane thickness mismatch and this is one of the major factors governing the morphology and stability of the giant

hybrid vesicles [171]. To overcome the membrane mismatch and minimize the membrane tension between lipid domain and polymer domain T. P. Tuyen Dao *et. al.*, [49] blended a thinner bilayer polymersome forming block copolymer, poly-(dimethylsiloxane) grafted with two poly (ethylene oxide) moieties ($M_w = 3000$), PDMS-g-(PEO)₂ with DPPC or POPC at a molar ratio of 50/50. The thickness of polymer bilayer was ≈ 5 nm, close lipid bilayer thickness of 3-5 nm. The author demonstrated that the newly formed sub-micrometer (50-100 nm) sized hybrid large unilamellar vesicles (LUVs) forms a more stable nano-sized domain compared to GUVs (≥ 1000 nm) of the same composition [49, 51]. These results suggest that phase segregation is more common in macroscale hybrid vesicles. Nevertheless, the lateral phase segregation also has been observed at the nanoscale even for individual liposomes and polymersomes [49, 57, 60, 172, 173], which undoubtedly influence the membrane properties and selected transmembrane processes. These types of heterogeneous phase segregations also have been observed for lipid-lipid mixtures [57].

2.4.1.3 *Effect of chemical compatibility between lipid and polymer*

Another interesting parameter is chemical compatibility of lipid and polymer in the hybrid vesicles. According to the recent report by O. Sandre *et al.*, [50] it has been suggested that the solubility of hydrocarbon moieties of lipids in the hydrophobic polymer blocks is governed by the chemical compatibility between two hydrophobic segments. Close chemical compatibility results in better solubility of both segments which leads to the more stable bilayer membrane.

2.4.1.4 *Effect of lipid-polymer composition*

Besides the bilayer thickness, chemical compatibility and lateral phase separation, the composition of lipid/polymer plays an essential role in vesicle formation, permeability, and stability. It was observed that for certain hybrid vesicles compositions such as, below 50 mol % of PDMS-g-PEO in mixture with POPC [51], above 60 mol % of PIB-b-PEO in mixture with DPPC, [170] and 0–65 mol % of PBD-b-PEO in mixture with POPC, [169] did not result in the formation of stable hybrid vesicles. X. Hu *et al.*, [174] showed that the effect of

composition on the stability and permeability of hybrid vesicles. A hybrid vesicle made from DOPC and ABA triblock copolymer (PMOXA-PDMS-PMOXA) resulted in a massive increase in membrane stability (against vesicle rupture and fusion) compared to pure DOPC and DOPC liposomes with increasing amount of ABA block copolymer. It was observed that water permeability also increased by increasing copolymer incorporation into the membrane [174]. In contrary, S. Lim *et al.* [175], reported that an increase in copolymer (PBD_{1.2k}-b-PEO_{0.6k}) content in PBD-b-PEO/POPC hybrid vesicles delayed the release of encapsulated cargo compared to release from POPC liposomes, because of a less permeable membrane [Figure 2.8]. However, the encapsulation efficiency of water-soluble entities was drastically decreased by increasing polymer content. A recent report N. Pippa *et al.* [176] demonstrated an improved encapsulation efficiency of an amphiphilic drug Indomethacin and release rate by using a poly(ϵ -caprolactone)-b-poly(ethylene glycol), (PCL_{2.5k}-b-PEO_{5k}), (~30 % PCL, hydrophobic contain) and DPPC mixture.

Figure 2.8: Illustration of hybrid POPC/PBD-PEO vesicles with controlled permeability [178].

S. Lim *et al.* [177], demonstrated that fine-tuning of the chemical composition of lipid/polymer not only provides a more stable membrane, it also plays a significant role in morphology and structure of the assemblies. Optical turbidity [Figure 2.9 (b)], high-resolution Cryo-TEM [Figure 2.10], and

tomography showed the formation of hybrid polymer/lipid tubular morphology that co-existed with vesicles in the aqueous phase for diverse compositions from 15–85 mol % of polymer component [Figure 2.9 (a)].

Figure 2.9: Turbidity of vesicle preparations. (a) Optical photographs of the spontaneously formed vesicle suspension, prior to extrusion, showing the difference in turbidity depending on vesicle composition. Vesicles were rehydrated with PBS (top) or labeled with 0.5 % Rhod- DPPE (Bottom). (b) Turbidity (absorbance at 630 nm) as a function of PBD-PEO molar fraction for the pre-extruded 5 mg mL⁻¹ PBD-PEO/POPC hybrid vesicles sample [177].

A 50:50 molar mixture of a fluid-phase phospholipid (POPC) and an amphiphilic block copolymer (PBD_{1.2k}-b-PEO_{0.6k}) they spontaneously self-assembled into an exclusively tubular morphology of diameter ~15 nm; length >1 mm; core ~2.0 nm; membrane thickness ~5–6 nm [Figure 2.10 d,e,f]. Such spontaneous stabilization of cylindrical vesicles, unaided by extraneous forces, requires a significant spontaneous bilayer curvature, which in turn necessitates a strongly asymmetric membrane composition. Compositional asymmetry produced in aqueous mixtures was suggested that a significant proportion of the polymer component accumulated in the outer expanded leaflet whereas the lipid component spontaneously segregated into the compressed inner leaflet most plausibly due to the difference in lateral steric pressures the two components experience. These results further suggested that self-assembly in microscale and nanoscale does not behave in similar fashion. Doping of amphiphilic polymer into spherical liposome does not necessarily give the same property in a hybrid vesicle at the nanoscale. It contradicts the conventional impression of

extrapolating studies of the hybrid lipid-polymer giant vesicle ($\sim$ tens of micrometers in diameter) to nanovesicle that hybrid vesicle will combine the properties of both spherical liposome and polymersome.

Figure 2.10: Polymorphism of aqueous suspensions of lipid-polymer mixtures. Cryo-EM images of nanostructures formed from thin film rehydration of phospholipid POPC, block copolymer PBD-PEO, and POPC/PBD-PEO mixtures. (a–c) POPC; (d–f) POPC/PBD-PEO (50: 50); (g–i) PBD-PEO. Scale bars as indicated are 100 and 200 nanometers. Arrows (middle panel) indicate line-scan regions (below). Insets. Photographs showing the difference in macroscopic turbidity of vesicle suspensions, depending on vesicle composition. Vesicles formed by rehydration using PBS (top, unlabeled; bottom, labeled with 0.5 % Rhod-DPPE) [177].

These opposing findings of permeability, variable encapsulation efficiency and other miscellaneous behavior of hybrid vesicle lead to the assumption that

the composition of lipid/polymer, chemical nature of hydrophobic domain, the molecular weight of the polymer used, the thickness of the membrane dramatically influences the final membrane properties. Several studies have already been conducted to improve the membrane property as close to the natural membrane by changing different variables. In recent times biodegradable and biocompatible polymer-lipid hybrid nanoparticles are being popular, in particular, poly(DL-lactic-co-glycolic acid (PLGA) because of its excellent bioproperty as well as an FDA approved polymer for drug delivery and biomedical devices application [178-181]. Apart from PLGA, other biodegradable/biocompatible polymers such as PCL (or PCL-b-PEG) [176, 182], PLA (or PLA-b-PEG) [183-185], poly (N-isopropylacrylamide) (PNIPAm), polyisobutylene (PIB) [170] and chitosan [186] based hybrid nanoparticles are getting enough attention in nanobiotechnological applications. Nevertheless, only a few reports are available on the biodegradable polymer-lipid vesicular system and there is always room for further enhancement.

2.5 Protein-Polymer Conjugates

Nowadays, protein-polymer conjugates are gaining increasing attention because of their controllable and programmable assembly characteristics. These assemblies are mostly categorized into two classes: (i) Proteins are being decorated on top of pre-formed polymers nanostructure by specific interacting moieties present on the polymer surfaces. (ii) Protein and the polymer were initially linked together to form a protein-polymer conjugate, which is self-assembled into an ordered macromolecular architecture using a bottom-up approach. The second category of protein-polymer conjugates is further classified into two sub-groups depending on the nature of conjugation. These are (i) protein and polymer linked via covalent chemical reaction, and (ii) protein and polymers are linked by non-covalent binding [97, 187, 188], such as the weak interaction forces (van der Waals interaction, hydrophobic interaction, H-bonding, π - π interaction) or by molecular recognition (receptor-ligand interaction). In most cases, the hydrophobic polymer linked with hydrophilic protein, create these giant amphiphiles for spontaneous self-assembly.

2.5.1 Non-covalent linked protein-polymer self-assembly

2.5.1.1 Temperature and pH induced self-assembly

Nolte *et al.* utilized AFM to demonstrate the formation of a protein-polymer monolayer at the air/water interface by non-covalent protein-polymer conjugation of biotinylated polystyrene with streptavidin [189]. Hoffman, Stayton, and co-workers designed a stimulus responsive protein-polymer conjugate controlled by temperature [190] and pH [188]. The authors presented a temperature responsive biotinylated polymer based on poly(*N*-isopropylacrylamide) (PNIPAAm) non-covalently conjugated with streptavidin to produce nanoparticles of controlled size (~250-900 nm) by varying polymer molecular weight, concentration, and rate of heating. PNIPAAm is hydrophilic below the lower critical solution temperature (LCST) and remains in solution, but transforms into a hydrophobic polymer above LCST resulting in a defined nanostructure stabilized by streptavidin [Figure 2.11(A)] [190]. The temperature responsiveness and pH-dependent behaviours were also observed for PNIPAAm-*b*-PAA (PAA= poly(acrylic acid)) blocks [188].

Figure 2.11: (A) Schematic representation of the temperature-dependent formation of PNIPAAm-streptavidin smart nanoparticles [193]. (B) Synthesis of covalently linked CALB-PS giant amphiphiles that self-assemble into micellar rods in the water [97].

2.5.2 Covalent linked protein-polymer self-assembly

Covalently linked protein-polymer conjugates rely on stable bond formation rather weak interactions. Side chain amino acids such as lysine, aspartic acid, glutamic acid, cysteine, tyrosine, and tryptophan are usually

targeted for bioconjugation with the functional polymer. However, cysteine is preferred for site-specific modification over others because of its relatively low abundance on the protein surface and high reactivity with maleimide groups [191]. Nolte and co-workers [96] conjugated a protein, lipase B from *Candida Antarctica* (CALB) with maleimide functionalized hydrophobic polymer, polystyrene, using thiol-maleimide chemistry. This giant amphiphile self-assembled into micrometer long and 20-30 nm thick fibers in aqueous medium [Figure 2.11(B)].

2.5.2.1 Effect of protein head group on self-assembly: structure, shape, and size

Olsen and co-workers investigated the mechanism of protein-polymer self-assembly in concentrated aqueous solution as well as the resulting solid-state nanostructures. Covalently linked red fluorescence mCherry–PNIPAAm has been used as a model bio hybrid copolymer [98]. At 40 °C and above (above LCST) the thermo-responsive polymer PNIPAAm turns hydrophobic and only the protein part is dissolved in water, whereas, at room temperature PNIPAAm is hydrophilic and both protein and polymer dissolve in water. Influence of this transition mCherry–PNIPAAm self-assembled into a heterogeneous nanodomain structure at 40 °C and hexagonal perforated lamellar phase at room temperature [Figure 2.12(A)] [98]. Analogous nanostructure was observed for similar β -barrel-shaped proteins, green fluorescent protein (GFP)–PNIPAAm bioconjugates [Figure 2.12(B)] [99]. However, a variation of the protein “head” introduced some differences: functional Mb–PNIPAAm copolymers self-aggregate into weakly ordered lamellar domains at a concentration of 50 wt % and above [100]. In comparison with previous mCherry–PNIPAAm and GFP–PNIPAAm conjugates, Mb–PNIPAAm conjugates are poorly ordered and desolvation occurs with mCherry–PNIPAAm, which generates a wide two-phase region. GFP–PNIPAAm, on the other hand, forms a homogeneous disordered micellar solution suggesting that the protein structure, the protein shape, solubility, and charge are largely responsible for the self-assembly behavior of these protein–polymer conjugates.

Figure 2.12: Two possible pathways toward self-assembly of mCherry–PNIPAAm bioconjugates. Room-temperature water provides a non-selective solvent, whereas at 40 °C water acts as a protein-selective solvent [98]. (B) mCherry–PNIPAM and GFP–PNIPAAm conjugates exhibit similar self-assembly behavior such as forming the lamellar phase [99].

2.5.2.2 Template supported self-assembly: water/oil emulsion based assembly

S. Mann and co-workers, [101], immersed the protein-polymer conjugate to a water/oil emulsion, where protein act a hydrophilic segment and thermo-responsive PNIPAAm polymer a hydrophobic domain in the self-assembly process, to minimize the protein structure and shape dependency in self-assembly. A protein-polymer vesicle produced from oil/water interface termed as proteinosome. Amine ($-\text{NH}_2$) groups on Bovine Serum Albumin (BSA) linked with a mercapto thiazoline-activated PNIPAAm polymer produced a giant amphiphile that self-assembled into proteinosome of micrometer size (20-50 μm) [Figure 2.13]. The construct was stabilized by post-synthetic cross-linking of carbodiimide-activated carboxylic acid residues (aspartic and glutamic acid) of protein using 1,6-hexanediamine. The proteinosome prepared by this process also was employed to other proteins such as myoglobin- NH_2 /PNIPAAm or hemoglobin- NH_2 /PNIPAAm [101]. Furthermore, the proteinosome can

encapsulate hydrophilic moieties such as protein, DNA, small molecules, inorganic nanoparticles and other functional components like ribosomes [101, 192]

Figure 2.13: Functional protein-polymer protocell or proteinosome capable of encapsulate molecules and perform enzyme-mediated cascade reactions [195].

2.5.2.3 Dual responsive self-assembly

Cornelissen and co-workers [193] constructed a dual responsive viral protein assembly using thermally responsive elastin-like polypeptide (ELP) and the CCMV protein. Because of polypeptide nature of ELP, it does not fall into the synthetic polymer and can be integrated from the genetic approach. ELP switch its property from completely water-soluble to total hydrophobic state with an increase in temperature.

The latest advancement in the protein self-assembly and approaches of protein-polymer self-assembly addressed few earlier challenges of biomimicry and protocell design. Olson demonstrated the effect of protein structure, shape,

solubility and charge variation in self-assembly [100]. S. Mann has addressed the issue of protein dependency by adopting a general approach of protein-polymer self-assembly in oil/water emulsion [101]. The proteinosome prepared in emulsion process minimize the role of protein's structure and shape dependency by considering protein as a hydrophilic segment and polymer as a hydrophobic segment in assembly formation. However, various aspects remain untouched such as, (i) using protein as hydrophobic domain, as most reports deemed protein as hydrophilic component and polymer as hydrophobic. However, the opposite fact has not been investigated with hydrophobic water-insoluble protein. (ii) Formation of proteinosome in a completely aqueous environment similar other biological assembly needs to be developed.

CHAPTER 3 HYBRID LIPID/POLYMER VESICLES: MIMIC CELL MEMBRANE AND VIRAL ENVELOPE

3.1 ABSTRACT

Hybrid polymer-lipid vesicles are classically known to sustain high degrees of hydrophobic mismatch. Here, I have used low-molecular-weight (1.2-3.45 kDa) biodegradable polymers to construct such hybrid vesicles, at the nano- and micron-scales, by conventional film rehydration and electroformation methods, respectively. By cryogenic transmission electron microscopy, calcein leakage assay and dynamic light scattering, the hybrid vesicles display attractive physical properties (size, bilayer thickness and small molecule encapsulation capability) as well as a semipermeable vesicular membrane boundary. Importantly, I found that the polymers themselves did not self-assemble into vesicles/polymersomes. Thus, they could not be used as a vehicle for encapsulation of hydrophilic molecules. Furthermore, using giant hybrid vesicles, their surface topologies were found to be homogeneous, independent of cholesterol, suggesting that these biodegradable polymers display unique polymer-lipid phase behavior. While exhibiting a similar bilayer thickness as that of lipid vesicles, differences in surface topology were demonstrated using the interfacially sensitive enzyme, phospholipase A₂. Biodegradable vesicles appear less sensitive to phospholipase digestion, which is reminiscent of PEGylated vesicles. Varying degrees of sensitivities towards phospholipase digestion were noticed upon modification with polymers implying that the nano-scale surface topology can be chemically tuned. My results reveal a role of phospholipids in complementing low-molecular-weight polymers for spontaneous vesicular self-assembly, generating protective polymer-lipid surface topology. This may be useful in designing vesicles for controlled release and supporting integral membrane protein in a semi-synthetic lipid-like environment.

3.2 SIGNIFICANCE STATEMENTS

Hybrid self-assembled systems of synthetic polymer and natural phospholipid often give rise to bilayer thickness mismatch, which stems from

differences in their chemical configurations. To construct a more energetically favorable system, low-molecular-weight biodegradable polymers are designed to self-assemble into vesicles with bilayer thickness similar to that of a natural lipid bilayer membrane. Though containing high amount of polymer (50%), these hybrid vesicles possess a lipid-like topology. They also possess an attenuated response to enzymatic digestion, reminiscent of PEGylated vesicles. Importantly, the presence of phospholipid is shown to drive the vesicular assembly of this type of hybrid polymer-lipid vesicles. Thus, this may find important applications in areas such as membrane protein reconstitution and controlled release.

3.3 INTRODUCTION

Nature has developed a self-assembled lipid barrier that encloses a crowded pool of proteinaceous constituents in a compartment that allows selective exchange of information between the intracellular milieu and the extracellular environment [194, 195]. The topology of such barriers exhibits various shapes and sizes (i.e. nano- and micron-sized vesicles) and is controlled by an integrated set of factors [196, 197] including acyl chains of different lengths, degrees of saturation, polar head charges, chemical structures and constituent protein entities that regulates the information exchange and membrane structure integrity [198, 199]. Bearing structural homology to lipids, *artificial* block copolymers (BCPs) have emerged as alternatives, with enhanced stability and highly tunable interfacial properties [200, 201], such as thickness and chemical composition.

Mixing liposomal and polymeric materials to fabricate hybrid polymer-lipid vesicles integrates individual strengths of the self-assembling lipid and polymer entities and the distinct features of these systems have been extensively studied [48, 50]. In past decades, liposomes have been widely exploited in the pharmaceutical and cosmetic industries as carriers, due to their excellent biocompatibility, albeit low stability and shelf life [202, 203]. Polymersomes, on the other hand, show high mechanical and chemical stability (tunable membrane permeability and longer shelf life) and biofunctionality [204], but low biocompatibility, and therefore have not been as widely applied as the lipid

counterpart. A mixed system allows one to obtain a vehicle that is robust (mechanically stable with low permeability and long shelf-life) [205], chemically versatile (possibility to be tuned and functionalized) and biocompatible. Indeed, this has been explored in recent years using different di- and tri-block copolymers, in combination with fluid- or gel-phase lipids [48, 206, 207].

In (hybrid) assemblies, BCPs and lipids display two distinct lateral membrane topologies where homogeneously mixed and segregated phases co-exist. Domain formation is controlled by the interplay of multiple parameters [208], including, but not limited to, lipid phase, thickness mismatch [209, 210], lipid/polymer ratio, cholesterol [211], and protein-lipid crosslinking [169]. Given the high compositional degrees of freedom of polymer chemical architectures and structural adaptation to specific geometries and dynamic requirement of the bilayer, domain formation is not completely understood and predictable. Nonetheless, several domain-promoting conditions have been identified including existence of lipid gel phase below the gel-liquid-crystalline transition temperature (T_m), increased hydrophobic thickness mismatch and the presence of cholesterol. Compositions promoting the formation of de-mixed lipid-rich and polymer-rich phases are demonstrated by PEG₁₃-b-PBD₂₂:DPPC and PEG₁₃-b-PBD₂₂:POPC:Chol (1:1:1), at room temperature [211]. These domains are likely to persist in both micron- and nano-sized vesicles, at a comparable domain size-to-vesicle size ratio [212]. Such polymer-lipid hybrid topology has found applications in enzymatic sensing [211] and in directed protein insertion [206, 213].

Several recent findings have hinted on the physical properties that control the appearance of de-mixed phases [50, 208, 209]. By comparing vesicle surface topology for different composition, the stability of de-mixed phases appears to be dependent on the competition between line tension that arises from the hydrophobic mismatch between the polymer and lipid, and the bending energy cost of the curved phase separated domains. Polymers that contribute to greater hydrophobic mismatch are more likely to give rise to a transient phase separated topology, which is then followed by a homogenous surface (as a consequence of vesicle budding and separation) [208]. Consistent with this, polymers that show

a molecular weights $> 3800 \text{ g mol}^{-1}$ form vesicles with homogenous surface [211, 214-217]. In consideration of these factors, designing and synthesizing hybrid vesicles of comparable thickness may be energetically more favorable without compromising the stability of polymer vesicles that are usually attributed to increased thickness at the expense of permeability and lateral mobility. Hence, low-molecular-weight (M_w) biodegradable polymers ($1250\text{-}3450 \text{ g mol}^{-1}$) are selected to have minimal thickness mismatch in the hybrid bilayer in contrast to higher M_w BCPs ($>3800 \text{ g/mol}$) used often for hybrid vesicles assemblies in most other studies [211, 214-217]. These low M_w biodegradable polymers are relatively unexplored, whereas high M_w polymers ($>9500 \text{ g mol}^{-1}$) previously have been used in the form of polymeric micelles for encapsulation of hydrophobic molecules [218, 219]. I hypothesize that these low-molecular-weight and biodegradable polymers will self-assemble with lipids in a more energetically favorable configuration thereby offering a better mimicking of the topology of a lipid membrane. I have produced hybrid vesicles composed of a fluid phase lipid, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and a biodegradable BCP (PEG₁₂-b-PCL₆, PEG₁₂-b-PCL₉, PEG₁₆-b-PLA₃₈) at equimolar composition, at the nano- and micro- length scales. PEG₁₃-b-PBD₂₂, one of the most studied BCP is used as a comparison. These hybrid vesicles were characterized by cryogenic-transmission electron microscopy (cryo-TEM), dynamic light scattering (DLS), fluorescence microscopy and calcein leakage assay for topology and morphology. Furthermore, to assess the bio-functional characteristics of such hybrid vesicles, they are subjected to an interfacial-dependent phospholipase digestion assay.

In this work, I present the following results: **First**, the hybrid vesicles exhibit a bilayer thickness close to that of a lipid vesicle, ensuring that minimal mismatch is achieved, allowing for further meaningful characterization. **Second**, phospholipid actively mediates vesicle formation of the BCPs, forming vesicles readily both at the nano- and micron length scale – a role not previously emphasized. These polymers do not form vesicles in the absence of phospholipid, reminiscent of phospholipids deviating from a critical packing parameter, $P_c = v/a_0 l_c$, of 0.5-1.0, [220], even though the polymers possess hydrophilic fraction

of $0.2 < f_{\text{hydrophilic}} < 0.45$. The PEG₁₃-*b*-PBD₂₂ with hydrophilic fraction of $0.2 < f_{\text{hydrophilic}} < 0.45$ is known to form vesicles. **Third**, the ability to form stable homogeneous small- (SUV) and giant- vesicles (GUV), of which the membrane tensions, which are proportional to $1/R_v^2$ where R_v is the vesicle radius, differ by about four orders of magnitude, supporting the notion that the polymer and lipid are mutually compatible. **Forth**, the appearance of phase-separated topology does not follow a simple correlation with known domain-forming conditions [211]. Rather, it is likely dependent on the specific chemical nature of the polymer [204, 221].

Finally, I show that the nano-sized hybrid vesicles exhibit varying sensitivity to an interfacial-dependent phospholipase, demonstrated by a calcein leakage assay and DLS, suggesting that the surface topology of hybrid vesicles may be further tuned by using these polymers at different stoichiometry. At micron-scale, when the curvature of the lipid membrane decreases, these hybrid vesicles appear unperturbed. Since the interfacial interaction mechanism of PLA₂ is well understood, this observation suggests that the nuance difference in the nanointerface on the hybrid vesicle surface plays a role in determining the binding and accessibility for external ligands.

3.4 MATERIALS AND METHODS

3.4.1 MATERIALS

PEG₁₂-*b*-PCL₆, PEG₁₂-*b*-PCL₉, PEG₁₆-*b*-PLA₃₈ and PEG₁₃-*b*-PBD₂₂ were purchased from Polymer Sources Inc. Canada. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhod-DPPE) and cholesterol were from Avanti Polar Lipids, USA. Phospholipase A₂ (P9279) and Naphtho[2,3- α]pyrene (380946) was from Sigma-Aldrich, Singapore. Triton X-100 was from BioRad, Singapore. Calcein from TCI Chemical, Singapore. Phosphate-buffered saline (10X) solution was purchased from Vivantis Technologies, Malaysia. All other chemicals were purchased from Sigma-Aldrich unless stated.

3.4.2 METHODS

3.4.2.1 *Preparation of small unilamellar vesicles (SUVs)*

SUVs were prepared by film rehydration followed by extrusion. In a 25-ml round-bottom flask, 0.5 equivalent of POPC and 0.5 equivalent of polymer were dissolved in CHCl_3 and mixed for 10 min (for a POPC liposome, one equivalent (5 μM) of POPC in CHCl_3 was taken). Then, CHCl_3 was removed slowly using a rotary evaporator to form a thin film of polymer/lipid mixture. The thin film was further dried under vacuum for another 5-6 hr. 1 ml phosphate-buffered saline (PBS), pH 7.4 was added to the dried polymer/lipid thin film to make a 5 mM POPC/polymer suspension. For calcein-encapsulated SUVs, 1 ml of 30 mM calcein in PBS was used. The solution was stirred at 50 °C overnight and the resultant solution was extruded by passing through a 100-nm membrane filter (Avanti Polar Lipid Inc., USA) 21 times using a 1 ml a mini-extruder (Avanti Polar Lipid Inc., USA) to get monodispersed SUVs. Unencapsulated calcein was removed by overnight dialysis using 300 kDa regenerated cellulose membrane against PBS.

3.4.2.2 *Preparation of giant unilamellar vesicles (GUVs)*

GUVs were prepared by the electroformation method [222]. Briefly, 20 μL of a 1 mg/mL POPC or polymer/POPC mixture at 1:1 molar ratio (or 1:1:1 for polymer/POPC/cholesterol blend) containing either 1% of Rhod-DPPE (or 0.5% Rhod-DPPE and 0.5-1.0% Naphthopyrene) in CHCl_3 was spread and dried on two Indium tin oxide (ITO)-coated glass slide (Nanion Technologies GmbH, Munich, Germany) using a glass syringe to form a thin polymer/POPC film was formed by evaporating the CHCl_3 . The film was further dried for another 1 hour under vacuum. Subsequently, an “O” ring was placed on a ITO-coated glass slide. 250 μL 300 mM sucrose solutions was added to the “O” ring enclosed area, followed by a second ITO-coated glass slide. A current was applied at 3 V, 5 Hz, for 120 min at 50 °C and the formed GUVs were collected and used immediately or stored at 4 °C for up to a week.

3.4.2.3 *Calcein leakage assay*

Dialyzed calcein encapsulated polymer/lipid hybrid SUVs were 100-fold diluted with PBS before leakage experiment. 100 μ l of the diluted calcein encapsulated samples were added to a 96-well flat transparent plate (Corning Incorporated, Corning, USA). Fluorescence was measured at 525 nm (excitation at 485 nm) using a fluorescence plate reader (Infinite 200 Pro, Tecan, Salzburg, Austria). 5 μ l of 1% Triton X-100 was added for total lysis of the vesicles. % Leakage was calculated based on the following equation: $100 \times (F_t - F_0)/(F_T - F_0)$, where F_0 is the initial fluorescence of calcein, F_t is the fluorescence of calcein at time interval t and F_T represents total fluorescence, *i.e.*, complete release of calcein upon addition of 0.05% Triton X-100.

3.4.2.4 *Phospholipase A2 enzymatic assay*

For phospholipase A2 (sPLA₂) assay, 50 μ l of dialyzed calcein-encapsulating SUVs (1:50 diluted in PBS) were mixed with 50 μ l of PBS containing 1 mM of CaCl₂ and different concentrations of sPLA₂ (10-400 nM). The release of calcein from the vesicles, as result of the addition of sPLA₂ was monitored for 60 min (about 30-second interval) at room temperature using a fluorescence plate reader (Infinite 200, Tecan, Salzburg, Austria; λ_{ex} = 480 nm, λ_{em} = 520 nm). The dialyzed vesicles were used within a week. The percentage of calcein release over time is calculated as above. For GUVs, 5-10 μ l of the GUV solution was added to a prediluted 100 nM sPLA₂ solution (in 300 mM glucose for osmotic equilibrium), supplemented with 200 nM CaCl₂.

3.4.3 CHARACTERIZATION TECHNIQUES

3.4.3.1 *Dynamic light scattering (DLS)*

DLS was performed on Malvern Zetasizer Nano ZS (Malvern, United Kingdom). 100 μ L of the purified-filtered sample was placed in a micro cuvette (Eppendorf® UVette, Sigma-Aldrich, Singapore) and an average of 30 runs (10s per run) was collected using the 173° detector. Data was plotted in Microsoft Excel (Microsoft, Washington, USA)

3.4.3.2 Cryogenic-transmission electron microscopy (Cryo-TEM)

For Cryo-TEM, 4 μ l of sample containing polymer/lipid hybrid vesicles were adsorbed onto a holey carbon-coated grid (Quantifoil, Germany). The grid was afterwards blotted with Whatman filter paper (GE Healthcare Bio-Sciences, MA, USA) for 2 seconds and then was vitrified into the liquid ethane at -178°C using a Vitrobot (FEI company, Netherlands). Frozen grids were transferred into a Titan Krios microscope (80-300 kV, FEG, LN2-cooled stage for 12 grids and imaged using Ultrascan 4000 (4k CCD), Tridiem GIF (Ultrascan 1000, 2k CCD)) (FEI company, Netherlands).

3.4.3.3 Wide-field deconvolution microscopy

A DeltaVision microscope (Applied Precision, Inc., Washington, USA), fitted with a PLAPON 60XO/1.42 NA oil-immersion objective from Olympus and TRITC and FITC Semrock filters (New York, USA), was used for imaging GUVs in real time. Samples were imaged in a 96-well uncoated μ -plate with a glass bottom (ibidi, Planegg / Martinsried, Germany). Briefly, 5-10 μ l of sucrose-encapsulating GUVs were added into 200 μ l of osmotically balanced 300 mM glucose solution. The sucrose-loaded GUVs settled to the bottom of the well within 1-2 minutes and were imaged using TRITC and/or FITC filter sets.

3.5 RESULTS AND DISCUSSION

3.5.1 Selection of block co-polymers (BCPs)

The propensity of block co-polymers to self-assemble into vesicles is mainly defined by the fraction ratio of the hydrophilic block, $f_{hydrophilic}$ [204, 223]. In this study, I compared four polymers with varying Mw (1.2-3.45 kDa) and $f_{hydrophilic}$ (0.2-0.44). In comparison with other studies, which are based on high Mw polymers (>3.5 kDa), I have chosen low Mw biodegradable BCPs (PEG₁₂-b-PCL₆, PEG₁₂-b-PCL₉, PEG₁₆-b-PLA₃₈) and a commonly used PEG₁₃-b-PBD₂₂ for comparison, abbreviated as PCL_{0.7}, PCL_{1.1}, PLA_{2.75} and PBD_{1.2}, respectively (Table 1). Based on theoretical understanding [224], the PCL BCPs, which have Mw of 1.25 and 1.65 kDa (for PCL_{0.7} and PCL_{1.1}, respectively), are predicted to form vesicle ($0.25 > f_{hydrophilic} < 0.45$) [204]. PLA_{2.75} has the highest Mw but the

lowest $f_{hydrophilic}$, at the theoretical limit for vesicle assembly. The selection of the low M_w BCPs, in general, approximating the lipid-like $f_{hydrophilic}$ (≈ 0.4 , except for PLA_{2.75}), rationalizes the design of hybrid polymer-lipid vesicles that preserve lipid-like properties, such as higher fluidity and lipid membrane thickness. Even though flexible, polydisperse polymers with bilayer thicknesses approximately twice that of the transmembrane segments of integral proteins have been shown to accommodate membrane proteins, I examine in this study if lipid-like hybrid bilayer may present as a feasible alternative.

3.5.2 Formation of hybrid POPC/BCP small unilamellar vesicles

BCPs or POPC/BCP mixtures at 50 mol % BCP were employed to prepare small unilamellar vesicles (SUVs) using the film rehydration method. The SUVs were then morphologically characterized by using cryo-TEM and DLS. POPC/BCPs mixtures readily formed unilamellar vesicles once rehydrated and extruded in phosphate-buffered saline (PBS) (<1 wt %). The membrane showed a homogeneous curvature and bilayer thickness for all the biodegradable polymers, as shown by the contrast as well as the Fresnel interference fringes on the inner and outer membrane edges, given by the hydrophobic core of the membrane (Figure 3.1A). The measured bilayer thicknesses of the hybrid vesicles (6.5 ± 0.5 nm, 6.7 ± 0.5 nm and 5.7 ± 0.5 nm for POPC/PCL_{0.7}, POPC/PLA_{2.75} and POPC/PBD_{1.2}, respectively) were comparable to that of POPC vesicles (5.3 ± 0.5 nm). For POPC/PBD_{1.2}, in addition to the classic lamellar structure, cylindrical tubes with a diameter of 14 nm were also observed, in agreement with previous studies on such formation [225]. The size distribution estimations of the hybrid vesicles by dynamic light scattering (DLS) show unimodal intensity-weighted peaks (> 92 % area intensity) with PDI values ranging between 0.2-0.4 for all compositions. The hydrodynamic diameter, (D_h), of POPC/PCL_{0.7} (138 nm) closely matches that of POPC (135 nm), while POPC/PCL_{1.1} (114 nm) and POPC/PLA_{2.75} (123 nm) [123] were slightly smaller. POPC/PBD_{1.2} were larger (165 nm), likely due to an ensemble averaging effect with the existence of elongated cylindrical tubes [225] (Figure 3.1A).

Figure 3.1: Formation of small- and giant- hybrid POPC/BCPs vesicles. (A). Cryo-TEM images of extruded small hybrid vesicles. **(B).** Intensity-weighted size distribution of extruded small hybrid vesicles, showing a largely unimodal distribution. **(C).** Fluorescence microscopy images of giant hybrid POPC/BCPs vesicles. The vesicles are doped with 1 mol % of Rhod-DPPE. Dense Rhod-DPPE accumulation is observed for PCL-containing vesicles. POPC/PBD_{1.2} and POPC/PLA_{2.75} giant vesicles show a homogeneous surface. **(D).** Encapsulation efficiency of small hybrid vesicles. Vesicles were extruded in 50 mM calcein and dialyzed extensively. Total leakage is induced by adding 0.05 % Triton X-100.

In addition, a small amount of larger aggregates was also present in the samples (< 2-8 %), indicated by the larger z-average values for all samples, with the exception of POPC/PCL_{0.7}, which shows a smaller z-average of 130 nm. In contrast, BCPs alone did not yield any vesicle.

I further determined whether the hybrid vesicles maintained a closed shell by preparing vesicles in buffer containing calcein at self-quenching concentration (30 mM) [226, 227]. For hybrid vesicles, the total molar mass of POPC and polymer was kept constant to that of the POPC preparation so that comparable amount of vesicles was prepared for each samples. Total encapsulated calcein was determined by vesicle lysis with 0.05 % Triton X-100, after removal of unencapsulated calcein by dialysis [Figure 3.1 (D)]. Encapsulation efficiency was compared (normalized) to POPC after background subtraction. The two hybrid POPC/PCL SUVs showed about 2-fold increase in calcein encapsulation while POPC/PLA_{2.75} and POPC/PBD_{1.2} showed similar amount of calcein encapsulation. As expected, PBD_{1.2} a significantly higher calcein encapsulation (4-fold). Importantly, all biodegradable polymers did not form vesicular structures capable of encapsulation, indicating that POPC mediates vesicle formation of such polymers.

Corroborating with the cryo-TEM images, the results show that populations of hybrid SUVs with similar structural properties were formed. These results are important because low M_w polymers are not known to microphase separate. In this study, I show that lipids can mediate the microphase separation of these low M_w polymers, likely due to the highly compatible physicochemical properties of the lipid and polymers.

3.5.3 Formation of hybrid POPC/BCP giant unilamellar vesicles

To visualize the surface topology and morphology of the hybrid vesicles, I prepared giant unilamellar vesicles (GUVs) by the electroformation method [222]. Polymer-lipid mixtures in chloroform were dried on ITO-coated glass slides and electroformed in 300 mM sucrose solution. The vesicles were doped with 0.5mol% of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl), Rhod-DPPE, and 0.5-1.0 mol % of naphthopyrene,

known to partition into liquid disordered and liquid ordered lamellar phases, respectively [228].

POPC/PBD_{1.2} and POPC/PLA_{2.75} formed GUVs with homogeneous surface topology comparable to GUVs consisting of POPC alone, indicating that no micron-sized polymer-lipid phase separation occurred [Figure 3.1(C)]. For POPC/PCL_{0.7}, a somewhat faceted topology with areas of condensed dye aggregates and dye-excluded regions were observed. Based on the liquid-disordered phase preferential of Rhod-DPPE, this suggests that micro-sized phase separation has occurred, albeit with a morphology distinct from the typical phase separation on GUV topology [229], whereby homogeneous patches of dye-enriched and dye-excluded regions are seen. The dye appeared to segregate into membrane defects, as has been observed on faceted polyhedron vesicles [230]. Studies using Brownian dynamics [231] suggests that the liquid-ordered facets are connected by highly curved fluid regions in the vesicles, which could result in accumulation of Rhod-DPPE. For POPC/PCL_{1.1}, both homogeneous and faceted topologies were observed, likely a consequence of the longer PCL block, which may complement the acyl chains of POPC in a slightly different geometry. The faceted topologies for POPC/PCLs may suggest that the interfacial tension of these vesicles is distinct from the other polymers. Consistent with the inability of the polymers to form assemblies of nano-sized polymersomes, I did not observe any micron-scale vesicles when electroformation was performed, except for PBD_{1.2} (<10 μm GUVs). The failure to form vesicular structure may be constraint by the much smaller polymer size used, as larger size polymers of the same polymer types are able to form vesicular structures [176, 232-234].

3.5.4 Phase separation of hybrid POPC/BCP giant unilamellar vesicles

Mapping a phase-separated topology is useful to better control the functionality of reconstituted protein in the hybrid bilayer [213, 235]. With the exception of the PCL_{0.7} polymers, the hybrid vesicles have shown a homogenous surface. To understand the formation of polymer-rich and lipid-rich domains, I have incorporated cholesterol into each of the polymer-lipid composition at

equimolar ratio. Cholesterol previously has been shown induce polymer-lipid phase separation by condensing fluid phase lipid [211, 221]. To further elucidate if the topology of such low M_w BCP/POPC vesicles could be induced to form a phase separated topology, I prepared hybrid POPC/BCP/Chol at 1:1:1 mol %, doped with 0.5-1 % of Rhod-DPPE and 1-1.5 % of Naphthopyrene. As described above, Rhod-DPPE partitions into fluid disordered lipid phase, whereas Naphthopyrene partitions into polymer-rich phase.

Figure 3.2: Hybrid giant POPC/BCPs vesicles. Hybrid giant vesicles consisting of POPC/BCP/Chol at 1:1:1 mol %, doped with 0.5-1 % of Rhod-DPPE and 1-1.5 % of Naphthopyrene are produced by electroformation method. 3D projections are shown, except for POPC/PCL_{1.1}/Chol, for which an optical section is shown.

A phase separated surface topology was observed for POPC/PBD_{1.2}/Chol, where Rhod-DPPE and Naphthopyrene partitioned into mutually exclusive regions [Figure 3.2]. A spherical polymer phase was surrounded by an interconnected lipid phase, consistent with minimization of line tension for a de-

mixed fluid phase membrane [211, 236]. The biodegradable hybrid GUVs show a homogenous surface, which points toward statistical mixing between the lipid and the polymer. For POPC/PCLs/Chol, Rhod-DPPE-dense or excluded regions in hybrid vesicles without cholesterol were replaced by a homogeneous one, suggesting that the nano-sized defects that may have contributed to dye accumulation [Figure 3.1(C)] have vanished in the presence of cholesterol. This is consistent with the fluidizing effect of cholesterol observed in other studies [211, 230]. A similar homogeneous surface was observed for POPC/PLA_{1,1}/Chol and other POPC/BCP/Chol compositions [Figure 3.3]. Although a lipid chain-ordering effect of cholesterol [221] is known and has been experimentally demonstrated [211] and repeated in this study, I could not find evidence for chain-ordering in the case for low M_w BCPs at the stoichiometry used in this study. This is perhaps not surprising under conditions where thickness mismatch is kept low, resulting in a reduced pre-existing lateral line tension [229]. Hence, the result implies that conditions promoting phase separation may involve different physicochemical interactions between the low M_w polymers and phospholipid, not necessarily limiting to thickness mismatch and cholesterol.

Figure 3.3: Hybrid giant PCL_{1.1}/POPC/Chol vesicles. Hybrid giant vesicles consisting of PCL_{1.1}/POPC/Chol at different mol % depicted in the figures as P:L:C, doped with 0.5-1 % of Rhod-DPPE and 1-1.5 % of Naphthopyrene are produced by electroformation method.

3.5.5 Enzymatic activity on hybrid POPC/BCP small unilamellar vesicles

To further investigate the topological properties of these hybrid polymer/lipid vesicles, I examined the effects of phospholipase on membrane structural integrity. The underlying motivation to select phospholipase is to address the following questions: Does the homogeneous vesicle surface influence the enzyme accessibility? How does the change in membrane curvature of such

polymer/lipid assembly influence the bioactive topology? Could I draw direct parallel between observations on small- and giant- vesicles?

I have used Ca^{2+} -dependent, surface-active, secreted phospholipase A_2 (sPLA₂) from bee venom (*Apis mellifera*). Briefly, sPLA₂, which belongs to a superfamily of lipase, catalyzes the hydrolysis of ester bond at the *sn*-2 position of phospholipids, producing free fatty acids and lysophospholipids and it has been extensively characterized on its properties on membrane for the resultant reaction products [237-240]. The initial interfacial binding of sPLA₂ with the membrane is mediated by a defined binding surface (*i*-face) on sPLA₂ [241-243]. To begin, I examined the sensitivity of the vesicles to sPLA₂ by using calcein leakage as a measure of membrane disruption. Vesicles encapsulating calcein were prepared as described above (more detail in Methods section). A range of sPLA₂ concentrations was tested, from 10-100 nM. This range of sPLA₂ concentrations would allow a theoretical surface coverage of 1-10 %, which translates to sPLA₂: vesicle ratios of 25:1 to 250:1. As expected, POPC is most susceptible to sPLA₂ digestion (15, 55 and 100 % leakage at 10, 50 and 100 nM sPLA₂, respectively) [Figure 3.4]. POPC/PCL_{0.7} and POPC/PCL_{1.1} show a much lower leakage and appear to be more resistant to higher concentrations of sPLA₂ as 10, 35 and 50 % leakage was observed at sPLA₂ concentrations of 10, 50 and 100 nM, respectively. It is to be noted that POPC/PCL_{1.1} consistently show a marginally higher resistance (about 5 % less leakage) to the lower concentration (10 nM) of the enzyme, as compared to POPC/PCL_{0.7}. In comparison, POPC/PLA_{2.75} was more resistant to higher concentration of sPLA₂ at 100 nM. Surprisingly, they showed a similar digestion profile to POPC, and even more susceptible to lower concentrations of sPLA₂ (10 and 50 nM). At much higher concentrations of sPLA₂ (200-400 nM), all vesicles containing biodegradable polymers showed complete leakage (data not shown). As a negative control, PBD_{1.2} vesicles remained intact (about 5 % leakage at all sPLA₂ concentrations). Similarly, POPC/PBD_{1.2} was also resistant to enzymatic digestion, consistent with previous findings [225].

Figure 3.4: Calcein leakage assay for hybrid small unilamellar vesicles (SUVs). Enzymatic digestion of the hybrid SUVs is performed at 10 (top), 50 (middle) and 100 nM (bottom) of sPLA₂. % Leakage is calculated based on values obtained after 0.05 % Triton X-100 addition and normalized to the amount of POPC. Representative plots from two independent experiments are shown.

Complementary to measuring membrane permeability, I further examined size changes of the vesicles after enzymatic digestion. DLS measurement on SUVs incubated with 100 nM sPLA₂ for 1 hour were undertaken. From the enzyme kinetics [Figure 3.4], sPLA₂ reaction has completed as no further leakage was observed, indicating a steady membrane state was reached. Membrane restructuring, if any, could then be measured. POPC, POPC/PLA₂.75 and POPC/PCLs SUVs, which have shown leakage in

response to sPLA₂, exhibited an increase (at least 2-fold) in the hydrodynamic diameter (D_h) [Figure 3.5], consistent with the reported increase in vesicle size after sPLA₂ hydrolysis [244, 245].

Figure 3.5: Dynamic light scattering measurement of hybrid small vesicles. The samples before and after 1-hour sPLA₂ incubation were measured. Black lines are DLS data measured before sPLA₂ digestion and red lines are after 1 hour sPLA₂ digestion.

In comparison, POPC/PBD_{1.2} did not show observable size increase. Surprisingly, PBD_{1.2} vesicles, which did not respond to sPLA₂, showed an increase in size. As sPLA₂ binding does not necessarily lead to hydrolysis, this apparent mismatch may be reconciled by the known consequence of surface area increase on the bilayer when external ligands are bound [246]. The overall increase in size for hybrid vesicles is counterintuitive as digestion of lipid by PLA₂ is expected to cause vesicle shrinkage. This could be explained by vesicle opening and re-assembly [244, 245] after the emergent of hydrolyzed products –

lysophospholipids and fatty acids, which are known to destabilize membrane, either created *in situ* in a preformed lamellar phase or added externally [237, 238, 240]. The comparable enzymatic activity on the hybrid vesicles demonstrates that the vesicles retain a dynamic, lipid-like surface topology likely interspersed with polymeric substances.

3.5.6 Enzymatic activity on hybrid POPC/BCP giant unilamellar vesicles

To further understand the membrane effect as a consequence of sPLA₂ interaction, hybrid GUVs, with or without cholesterol (at 1:1 or 1:1:1 molar ratios, respectively), were prepared. At micron-scale, GUVs allow us to readily visualize morphological changes of membrane. I first characterized and benchmark sPLA₂ effects on GUVs consisting of POPC. Consistent, qualitatively similar response was observed. The main morphological change was vesicle size shrinkage [Figure 3.6A (i-vi)]. In some instances, formation of membrane tubules was observed [Figure 3.6A (iv-v)]. Ultimately, the digested vesicles shrank to a dense lipid aggregate or appeared as a bilayer on the glass substrate, indicating that the phospholipids were hydrolyzed, producing lysophospholipids and free acids, which resulted in instability in the vesicle membrane [Figure 3.6A (vi)]. The second reproducible morphology that emerged involved a series of complex, spontaneous expulsion of interior vesicles [247] [Figure 3.6A (ii-iii) and B (i-v)].

Figure 3.6: Effects of sPLA₂ on giant hybrid vesicles. 100 nM sPLA₂ is added to the vesicles and morphological changes are monitored with time. [A (i-x) and B (i-v) are different time frames]. Two typical morphological change of POPC giant vesicles when incubated with sPLA₂. (C) No change is observed for phase separated POPC/PBD_{1.2}/Chol giant vesicles. (D) Membrane flickering on POPC/PBD_{1.2} giant vesicles. (E) No change is observed for other hybrid vesicle. A representative image after 30-min incubation with sPLA₂ is shown. In this case, POPC/PCL_{0.7}/Chol is shown.

I subsequently investigated if interfacial enzymatic activity of sPLA₂ (100 nM) can cause qualitatively similar morphological changes on hybrid GUVs, with or without cholesterol. In comparison to the observations on the hybrid SUVs, where only partial limited enzymatic activity was observed for calcein leakage and hydrodynamic size increase, their corresponding GUVs did not show drastic morphological changes, such as that demonstrated by POPC GUVs when added to a sPLA₂ solution [Figure 3.6C]. The response of POPC/PBD_{1,2} GUVs to sPLA₂ was qualitatively different from the hybrid biodegradable GUVs, showing transient membrane flickering. Surprisingly, POPC/PBD_{1,2}/Chol GUVs, which showed a phase-separated topology, did not show size shrinkage nor membrane tubulation from the lipid-rich phase. This may suggest that PBD_{1,2} might still be interspersed in the POPC-rich phase and perturb the binding of sPLA₂ although the majority of the PBD_{1,2} were segregated from the POPC-rich region.

To understand the contrasting effect of sPLA₂ on POPC GUVs and on GUVs containing biodegradable polymers, a unifying biophysical mechanistic understanding of sPLA₂ needs to be laid out. Based on a series of theoretical and experimental works [242, 247-250], briefly, when sPLA₂ hydrolyzes POPC into the two membrane destabilizing compounds, a lysophospholipid and a free fatty acid, their conical shapes perturb the thermodynamic equilibrium of POPC bilayer [237, 239, 246]. As a consequence, the nucleation energy barrier lowers and is more easily overcome. In this case, the energy barrier is overcome when an object from the vesicle interior comes in proximity to the inner monolayer [247] [Figure 3.6A (ii)]. From the time-lapse images, I also observed that during the expulsion of inner vesicle, the outer vesicle remained spherical and tense, driven by both membrane tension and line energy (due to the exposure of hydrophobic tail to solvent) [247] [Figure 3.6A (iii)]. Not all parent vesicles remained tense during the expulsion of inner vesicle. In some instances, the parent vesicle could become flaccid [Figure 3.6B (iv)], likely due to convection of solute and solvent through nano-scale width surrounding the daughter vesicle, effectively lowering volume-to-area ratio of the parent vesicle.

The reduction in activity of sPLA₂ on hybrid SUVs and GUVs can be understood based on several factors. As the sPLA₂ activity on phospholipid is dependent on the availability of a binding surface of around 1500 Å² (equivalent to about 30 POPCs) [243, 251], existence of nanoscale mixed topology of lipid and polymer [212] likely perturb its binding through height fluctuations at the monomer scale [201, 252], masking the accessibility of the enzyme to the *sn*-2 position of phospholipids. This increased stability towards enzymatic digestion is reminiscent of PEGylated-lipid, but with the advantage of forming vesicular structure at high polymer fraction, i.e. 50 mol % in the current study as compared to a upper limit of 10mol% for PEGylated lipid before transforming into a micellar form [253]. The difference may also due to a high mechanical stability towards the presence of conically shapes molecules (lysophospholipid and fatty acids) as membrane tension tends to be higher for a highly curved surface of the small vesicles [136, 254].

3.6 CONCLUSION

I have shown by mixing lipid and biodegradable polymers at 1:1 molar ratio, that nano- and micron-scale hybrid polymer/lipid vesicles can be prepared by the extrusion and electroformation methods, respectively. The low *M_w* polymers used in this study were shown not to form closed lamellar structures on their own. However, they improved the chemical and mechanical stability while self-assembled with POPC. Thus, emphasizing the importance of POPC to facilitate the assembly. The key findings from this study, as hypothesized, was that the low *M_w* polymer reduced the membrane mismatch and provided a robust hybrid bilayer with a thickness close to that of the natural lipid cell membrane, around 5 nm. The hybrid vesicles were shown to encapsulate hydrophilic small molecule dye, confirming the vesicular assembly with a stable semipermeable boundary. The surface topology of these vesicles is lipid-like but with a higher resistance to enzymatic digestion as compared to POPC vesicles. While I have shown here with POPC, other phospholipid types should be in principle expand this phospholipid-assisted hybrid vesicle forming strategy and contribute to designing functional vesicles for future applications.

CHAPTER 4 CONTROLLED SUPRAMOLECULAR SELF-ASSEMBLY OF “SUPERCHARGED B- LACTOGLOBULIN A – PEG CONJUGATES” INTO NANOCAPSULES.

4.1 ABSTRACT

I describe the synthesis and characterization of a new protein-polymer conjugate composed of β lactoglobulin A (β LG A) and poly (ethylene glycol) PEG. β LG A was selectively modified to self-assemble by supercharging via amination or succinylation followed by conjugation with PEG. An equimolar mixture of the oppositely charged protein-polymer conjugates self-assemble into spherical capsules of 80-100 nm in diameter. The self-assembly is driven by the protein-polymer conjugate's amphiphilicity and polyelectrolyte nature. The protein-polymer capsules or proteinosomes are reminiscent of viral protein capsids, and are capable of encapsulating solutes in their interior. I envisage this approach to be applicable to other globular proteins.

4.2 INTRODUCTION

Viruses are the epitome of protein self-assembly and are unparalleled in their ability to form highly symmetric protein capsids. Manipulating proteins to self-assemble into virus-like particles is therefore a critical task in the overall process of developing novel delivery vehicles with unique therapeutic properties [255-258]. One approach is to create giant amphiphiles with a protein that serves as the inert hydrophobic part of the amphiphilic building block [96, 101, 189, 192, 259, 260]. Despite numerous studies reported on self-assembled structures using protein-polymer conjugates, they suffer from drawbacks that include harsh conditions during their synthesis or involve non-aqueous steps to mediate self-assembly. Recent approaches in designing multi-component protein nanocapsules with defined architecture, including the use of computational methods, have helped overcome some of the drawbacks [261-266].

With the self-assembly of viral subunits in mind, I have together with previous students in the group developed a synthetic strategy to produce a protein-polymer conjugate that spontaneously self-assembles into nano-sized capsules in aqueous environment. The protein moiety itself (β LG A) plays an active role in the self-assembly process. It is an ubiquitous component of milk that displays well known self-assembly and aggregation characteristics under denaturing conditions (low pH and elevated temperature) [267, 268].

Figure 4.1: Synthesis and self-assembly of supercharged β LG A–PEG conjugates into nanocapsules. The supercharged protein-polymer conjugates are synthesized in four steps (illustrated in two parts). Top panel: (1) Functionalization of PEG3300 with tetrazole. Bottom panel: (2) Allylation of the native protein. (3) Succinylation or amination of the allylated protein; and (4) PEGylation with PEG-tz. Mixing the two oppositely supercharged protein-polymer conjugates at equimolar ratio leads to their self-assembly into nanocapsules (center) capable of encapsulating solutes.

To control self-assembly and allow the process to occur at ambient temperature and neutral pH, the protein was “super-charged” through the introduction of negative and positive charges, while a PEG-chain was introduced to impart amphiphilic character to the conjugate. PEGylation has the added advantage of restricting the conformational freedom of the protein and thereby limiting its denaturation [269]. Interestingly, while solutions of either charged conjugates were stable, mixing the two oppositely charged conjugates led to rapid self-assembly, yielding nano-sized capsules. All this happened in an aqueous environment, without the need for any harsh conditions or elaborate steps to initiate self-assembly.

4.3 MATERIALS AND METHODS

4.3.1 MATERIALS

4-Formylbenzoic acid, Benzenesulfonyl hydrazide, 4-Dimethylaminopyridine (DMAP) and Succinic anhydride were purchased from TCI Chemical (Singapore). PEG3300 was purchased as lyophilized powder from Polymer Source (Quebec, Canada). The plasmid, pCMVTnT was purchased from Promega (Singapore). Xho1, Not1, and NE Buffer 3.1 were purchased from New England Biolab (Singapore). β Lactoglobulin A (β LG A), and all other chemicals were purchased from Sigma-Aldrich (Singapore), unless otherwise stated.

4.3.2 METHODS

Supercharged protein-polymer conjugates of β LG A were constructed in four steps [Figure 4.1]: (1) Functionalization of PEG₃₃₀₀ with tetrazole (PEG-tz) (2) Modification of β LG A with a double bond for conjugation with PEG-tz (3) supercharging the β LG A-Allyl by introducing excess positive or excess negative charges, and (4) conjugation with PEG-tz to achieve the final protein-polymer conjugates, β LG A-PEG (+) and β LG A-PEG (-).

4.3.2.1 Preparation of 4-(2-phenyl-2H-tetrazol-5-yl)benzoic acid (Tetrazole; tz)

Step 1

Step 2

Figure 4.2: Preparation of Tetrazole

Tetrazole was prepared using a method previously described by S. Ito, *et al.*, (1976) [270] and W. Song, *et al.*, (2008) [271]. Briefly, to a 50 mL solution of 5 mmol 4-formylbenzoic acid in ethanol, 5 mmol of benzenesulfonyl hydrazide was added, and the reaction was stirred for 30 min. 100 mL water was added to the above solution which resulted in a white precipitate that was collected in a funnel [Figure 4.2, step 1]. The white precipitate was dissolved in 30 mL pyridine to give solution A. In parallel [Figure 4.2, step 2, a solution of 5 mmol NaNO_2 in 2 mL water was added dropwise to a pre-chilled solution of 5 mmol aniline that was previously dissolved in a mixture of 8 mL ethanol-water (1:1) and 1.3 mL concentrated HCl to give solution B. Solution B was added slowly to solution A while being cooled on an ice-salt bath (-15 to $-20\text{ }^\circ\text{C}$), and the reaction mixture was then extracted with ethyl acetate three times. Upon the addition of 250 mL 3N HCl to the combined organic layers, precipitates formed which were collected and dried overnight. The dried crude product was purified by flash chromatography using ethyl acetate/hexane mixture. The final compound was characterized by $^1\text{H-NMR}$ and MALDI.

4.3.2.2 Preparation of Tetrazole-functionalized Poly(ethylene glycol), PEG₃₃₀₀-Tetrazole

A solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) ($75.75\text{ }\mu\text{mol}$, 2.5 equivalent) in 0.5 mL of chloroform was

added dropwise to a mixture of PEG₃₃₀₀ (30.3 μmol , 1 equivalent) and tetrazole (tz) (60.6 μmol , 2 equivalent), and 4-Dimethylaminopyridine (DMAP) (6.06 μmol , 0.2 equivalent) in 1 mL of chloroform at -15 to -20 °C on an ice-salt bath. After complete addition, the reaction mixture was stirred for 20 hr at room temperature. Then, the reaction mixture was extracted with 0.1 M HCl twice, followed by the addition of saturated NaCl. The organic layer was dried with magnesium sulfate and reduced to 1 mL by evaporating excess chloroform in a rotary evaporator under high vacuum. The orange colour liquid was then purified by ether precipitation by adding 10 mL diethyl ether. The precipitate was washed three times with 10% chloroform/ether solution and dried under high vacuum overnight. The crystalline solid was then dissolved in milliQ water and centrifuged at high speed for 10 min to remove undissolved material. The filtrate was freeze-dried overnight to yield 91.3 mg of a white powder.

4.3.2.3 Preparation of $\beta\text{LG A-Allyl}$

Allyl-functionalized $\beta\text{LG A}$ was prepared using a method previously described by J. M. Chalker *et al.* (2009) [272]. Briefly, 2 mL of 3 mg/mL $\beta\text{LG A}$ in 5 mM PBS, pH 7.4 was mixed with 300 μL of allyl chloride (3.68 mM) in DMF. The mixture was incubated under agitation at 37 °C for 2 hr, followed by removal of the small molecules using a PD-10 column (GE healthcare, Singapore), eluting with 5 mM PBS, pH 7.4. An Ellman's test was performed to determine the yield of the reaction. The product was freeze-dried overnight to yield a white powder that was stored at -20 °C until further use.

4.3.2.4 Ellman's Test to determine loss of thiol groups.

The Ellman's reagent, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) was used to determine the amount or concentration of free thiol groups in proteins. The Ellman's test is based on the fast and sensitive reaction of DTNB with thiol to give the mixed disulfide and 2-nitro-5-thiobenzoate (TNB^-), which ionizes to TNB^{2-} dianion in water at both neutral and alkaline pH. This TNB^{2-} dianion is yellow in color and can be quantified easily by its absorbance at 412 nm using UV/Vis spectrophotometer with molar absorption coefficient, ϵ_{412} of 14,150 $\text{M}^{-1}\text{cm}^{-1}$ for dilute buffer.

4.3.2.5 Preparation of β LG A-Allyl (+)

360 μ L of 0.1 M of ethylene diamine dihydrochloride (EDA), pH 4.7 (36 μ mol, 7.5 equiv.) was added to 3 mL of 2 mg/mL β LG-A-Allyl in 0.1 M MES, pH 4.7, followed by addition of EDC (36.0 μ mol, 7.5 equiv.). The reaction mixture was incubated at room temperature for 2 hr, followed by removal of the low mass components using a PD-10 column, eluted with milliQ water. The collected fractions were freeze-dried overnight to yield a white powder. MALDI-TOF and zeta potential were used for characterizing the final product (see details below).

4.3.2.6 Preparation of β LG A-Allyl (-)

Briefly, succinic anhydride (48 μ mol, 10 equiv.) was added to 3 mL of 2 mg/mL β LG A-Allyl in 0.1 M PBS, pH 8.6 with shaking while maintaining the pH between 8 - 9. The reaction was incubated at room temperature for 2 hr, followed by desalting on a PD-10 column, eluted with milliQ water. The collected fractions were freeze-dried overnight to yield a light yellow powder. MALDI-TOF was used for characterizing the final product (see details below).

4.3.2.7 Preparation of Positively Charged Protein-Polymer Conjugates, PEG- β LG A(+) and PEG- β LG A(-) using Photoclick Chemistry

PEG- β LG A(+) and PEG- β LG A(-) were prepared using a procedure reported by W. Siti *et al.* (2015), but with slight modifications [4]. Briefly, 4 mL of β LG A-Allyl (+) or β LG A-Allyl (-) (0.3 μ mol, 1 equiv.) in milliQ water was mixed with 0.5 mL of mPEG₃₃₀₀-Tetrazole (1.5 μ mol, 5.0 equiv.) in milliQ, into a quartz cuvette (10 mm pathlength). Under stirring, the mixture was irradiated at 302 nm with a handheld UV-lamp at a distance of 5 cm from the sample for 10 min, resulting in a pale-yellow solution. The resulting solution was purified using a SEC column (BIO SEC 3, Agilent, Singapore), eluted with 50 mM MES, pH 4.7 and 50 mM PBS, pH 8.0 for PEG- β LG A(+) and PEG- β LG A(-) respectively. 500 μ L fractions were collected and PEGylated fractions were determined by MALDI-TOF. The resulting PEG- β LG (+) or PEG- β LG (-) was desalted using PD-10 desalting column, eluted with MilliQ water and freeze-dried overnight yielding a white/ pale yellow powder. The final product was

further characterized by fluorescence measurements using a TECAN, Infinite M200 Pro (Singapore) with the excitation set at 475 nm and emission recorded at E_m 495 nm, and SDS-PAGE.

Figure 4.3: Preparation of Positively Charged Protein-Polymer Conjugates.

4.3.2.8 Preparation of proteinosomes

0.5 mL of 2 mg/mL PEG- β LG (+) and 0.5 mL of 2 mg/mL PEG- β LG (–) in milliQ water were mixed and incubated at room temperature overnight. Next day, the samples were filtered through a 0.2 μ m RC membrane syringe filter followed by 100 kDa viva spin centrifuge filter (GE Healthcare, Singapore). The final product was characterized by DLS and TEM (see details below).

4.3.2.9 Encapsulation of pDNA

50 μ L of 1 mg/mL pCMVTnT pDNA in MilliQ water was mixed with 50 μ L 2 mg/mL PEG- β LG (–) followed by addition of 50 μ L PEG- β LG (+). The mixture was incubated overnight at room temperature to complete the encapsulation. The encapsulated samples were run on a 0.8% agarose gel by mixing 10 μ L pDNA encapsulated polymersomes with 5 μ L 6x Sample loading buffer (50 % Glycerol with $\sim$ 0.05 mg bromophenol blue in sterile H₂O). 10 μ L 1x Syber Safe DNA intercalated dye was used to stain the gel. For nuclease digested samples, 1 μ L pDNA (pCMVTnT) were incubated with 2 μ L mixture of Xho1 and Not1 in NE buffer 3.1 buffer for 30 min at 37 °C. The reaction was quenched by increasing the temperature to 75 °C for 10 min, before loading the samples in the agarose gel.

4.3.2.10 Encapsulation of GFP and FITC labelled dextran

Green Fluorescence Protein (GFP) (Recombinant *Aequorea victoria* GFP, Mol. Wt. 28.7 kDa, purchased from Sino Biological Inc, Beijing, China) was added either during or after the vesicle formation step. Briefly, GFP encapsulated proteinosomes were prepared by mixing 5 μ L of 3.48 μ M GFP with 20 μ L of 1.5 mg/mL β LGPEG(+) and 20 μ L of 1.5 mg/mL β LGPEG(-) followed by incubation for 2 hr at room temperature. For control samples, 5 μ L of 3.48 μ M GFP was added after incubating the empty proteinosomes for 2hr. The final concentration of GFP in both samples was 0.38 μ M. The above samples were purified through a 100 kDa vivaspin centrifuge filter (GE Healthcare, Singapore) (speed: 1500 rpm for 10 min) followed by three washes with MiliQ water to remove un-encapsulated GFP from the sample. After washing, fluorescence intensity of the samples was collected by exciting them at 480 nm and collecting their emission at 520 nm. A similar approach was employed for encapsulating Fluorescein isothiocyanate (FITC) labelled Dextran (3-5 kDa) (purchased from Sigma- Aldrich, Singapore). The final concentration of the FITC-dextran was 5.55 μ M. DLS measurements were carried out on all the above samples to confirm the size of the assembly.

4.3.3 CHARACTERISATION TECHNIQUES

4.3.3.1 Dynamic Light Scattering (DLS) and Zeta Potential Measurement

DLS and Zeta Potential measurements were carried out in a Brookhaven Zetapals (Holtsville, NY, USA) or in a Zetasizer Nano ZS (Malvern, Worcestershire, UK.). Purified samples were filtered with 0.2 μ m RC syringe filter before collecting their scattering profile. 100 μ L of the purified-filtered sample was placed in a micro cuvette (Eppendorf® UVette, Sigma-Aldrich, Singapore) and average of 10 run (1 min per run) was collected angle of at 90°. For zeta potential measurements, 600 μ L of the purified-filtered sample was placed in a Folded capillary cells (Malvern) were used and the measurements were averaged over 5 runs. Data was plotted in OriginPro 9.1 (OriginLab Corporation, MA, USA).

4.3.3.2 *Static Light Scattering (SLS)*

To determine shape ratio (δ) of β LG A-PEG Proteinosome, radius of gyration (R_g) over hydrodynamic radius (R_h), ($\delta = R_g/R_h$) was measured by Static Light Scattering (SLS) and Dynamic Light Scattering (DLS) respectively in a multi angle Brookhaven BI-200SM Goniometer instrument. A dilute solution of β LG A-PEG Proteinosome was used for both SLS and DLS experiment. 0.25 mg/ml mono β LG A-PEG Proteinosome solution was filtered through 0.22 μ m membrane filter before sample analysis. SLS scattering intensity was measured at different angle from 35° up to 145° with a 5° increment, while fixed angle of 90° was used to measure DLS. R_g was obtained from a simple linear fit of a logarithmic plot of the Guinier equation:

$$\ln I(q) = \ln I(0) - (R_g^2/3) q^2$$

Guinier Plot allows the determination of the radius of gyration from the measured scattered intensity $I(q)$ as a function of the scattering angle θ . q defined as the scattering vector $q = 4\pi \sin(\theta)/\lambda$ and, λ = wavelength of X-ray. An average of three runs was used to calculate R_g , R_h and shape ratio.

4.3.3.3 *Transmission electron microscopy (TEM)*

TEM images were collected using a FEI Tecnai G2 200 kV TEM (FEI, Netherlands). 200 mesh carbon type B Cu grid with Formvar coating (Ted Pella Inc, USA) was used to prepare samples. The surface of the grid was hydrophilized before adding 10 μ L sample on the Cu grid. After 10 mins on the grid, the excess solution was wicked-off using a Whatman filter paper. 20 μ L of 2 % Uranyl acetate (UA) was added immediately and after 45 sec. The grid was then allowed to air-dry for at least 1 hr before imaging.

4.3.3.4 *Cryogenic-transmission electron microscopy (Cryo-TEM)*

For cryo-TEM, 4 μ L of sample containing proteinosome were adsorbed onto a holey carbon-coated grid (Quantifoil, Germany). The grid was afterwards blotted with Whatman filter paper (GE Healthcare Bio-Sciences, MA, USA) for 2 seconds and then was vitrified into the liquid ethane at -178°C using a Vitrobot. Frozen grids were transferred into a Carl Zeiss Libra 120 PLUS Transmission Electron Microscope (120 kV, in-column EELS - energy resolution <1.5 eV,

LN2-cooled stage for 1 grids, and imaged using TRS Sharp-EYE 2k x 2k CCD), (Company: Carl Zeiss Microscopy, Germany).

4.3.3.5 Matrix-Assisted Laser Desorption/Ionization – Time of Flight (MALDI-TOF)

Shimadzu AXIMA (Tokyo, Japan) Performance Mass Spectrometer was used to measure the mass of modified β LG A using sinapinic acid as the matrix.

4.3.3.6 Circular Dichroism (CD)

Spectra were collected in a v2circular dichroism spectrometer (Aviv Biomedical, Inc, Lakewood, NJ, USA) by placing 100 μ L of 2.0 mg/mL samples on a 5 mm thick quartz cuvette. The collected spectra were an average of 10 runs with a bandwidth of 1 nm, wavelength scan 260 nm – 190 nm, wavelength step 1 nm, and a scan speed of 2 nm/sec. All measurements were performed at room temperature and the data was plotted in OriginPro 9.1 (OriginLab Corporation, MA, USA).

4.4 RESULTS AND DISCUSSION

4.4.1 Preparation and characterizations of proteinosome

In the first step of proteinosome preparation, PEG3300 was functionalized with tetrazole using a previously reported method [Figure 4.2] [271]. This tetrazole functionalization was done in order to facilitate the conjugation of PEG to supercharged β LG A using simple click chemistry, during the final step.

In the second step, the single addressable thiol group, Cys121 of β LG A, was derivatized with allyl chloride to introduce an alkene moiety to form allyl-modified β LG A (β LG A-Allyl). This alkene moiety serves as a dipolarophile for the photo-induced conjugation of PEG-tz in the final step. The derivatization was confirmed by the loss of the thiol groups on the protein as quantified by an Ellman's test [Table 4.1].

Table 4.1: Ratio of free thiols to β LG A protein before and after reaction with allyl chloride.

	Conc. of β LG A (mg/mL) ^(a,b)	Conc. of Free -SH (μ M) ^(b)	Free -SH / β LG A (mole/mole)
β LG A before reaction	2.5 ($\pm$ 0.3)	3.3 ($\pm$ 0.1)	1.3
β LG A after reaction	0.41 ($\pm$ 0.04)	-0.1 ($\pm$ 0.2)	-0.03

In the third step, β LG A-Allyl was supercharged with positively charged groups by modifying the protein's carboxyl groups to amino groups, achieved by reacting with ethylene diamine (EDA). Together with the amino groups already present on the protein, this resulted in an excess of positive charges on the amino-modified β LG A (β LG A (+)). Similarly, a second batch of β LG A-Allyl was supercharged by reacting amino groups with succinic anhydride to yield carboxyl groups. This produced a highly negatively charged β LG (β LG A (-)). Native β LG A has a total of twenty seven carboxyl groups and sixteen amino groups that, in theory, are accessible for modification. Based on MALDI-TOF analysis, it was possible to modify ca. 75% - 80% of the available sites on β LG A-Allyl conjugate. The remaining residues were most likely residing in solvent-inaccessible regions of the protein and therefore could not be modified. Moreover, complete amidation or succinylation would lead to the protein's denaturation [273].

Figure 4.4: Characterization of supercharged β LG A-PEG conjugates. (A) Fluorescence spectra of positively (dark blue) and negatively (light blue) supercharged β LG A-Allyl with PEG-tz, before (dashed) and after UV irradiation (solid lines) reveals a peak at 495 nm (λ_{max}), generated as a result of the conjugation reaction. **(B)** Mass spectra (MALDI-TOF) of native β LG A (black), β LG A-PEG (+) (dark blue), and β LG A-PEG (-) (light blue) **(C)** SDS-PAGE of native β LG A, β LG A-PEG (+), and β LG A-PEG (-), (lanes 1-3, respectively), (M) Molecular weight markers. **(D)** Circular dichroism spectra of native β LG A (black), β LG A-PEG (+) (dark blue), β LG A-PEG (-) (light blue), and an equimolar mixture of β LG A-PEG (+) and (-) (red).

In the fourth and final step, PEG-tz was conjugated to supercharged β LG A-Allyl via photo-induced click chemistry by irradiating the sample at 302 nm for 15 min. The conjugation was confirmed by the appearance of a fluorescence emission peak at 495 nm [Figure 4.4A]. In this step, photolysis of tetrazole upon UV irradiation yields a pyrazoline cycloadduct that exhibits strong fluorescence at 495 nm (λ_{max}) and serves as a useful indicator for monitoring the progress of the reaction as reported previously [Figure 4.3] [273]. Previously, our group and others have reported that in the absence of an alkene moiety, the tetrazole is capable of conjugating with other functional groups such as the tryptophan moieties in the case of proteins [273]. In the current work, despite targeting the conjugation towards the alkene present on β LG A-Allyl, it was not possible to prevent minor side-reactions with tryptophan residues leading to the formation of di-, and tri-PEGylated products as confirmed by MALDI-TOF analysis [Figure 4.4B]. When compared to the native β LG A [Figure 4.4C, lane 1], SDS-PAGE of the purified β LG A-PEG(-) and β LG A-PEG(+) showed a clear increase in their respective M_w [Figure 4.4C, lanes 2 & 3 respectively]. β LG A-PEG(-), however, migrated to a much higher M_w than expected from MALDI analysis [Figure 4.4C, lane 3]. This abnormal migration is most likely a result of either the excessive negative charge or its interaction with (negatively charged) SDS. Indeed, it is not uncommon for highly charged proteins to show variable electrophoretic mobility [274].

Figure 4.5: Size and shape characterization of supercharged protein-polymer conjugate assemblies. Size distributions as determined by DLS of (A) native β LG A and (B) equimolar mixture of positively and negatively supercharged β LG A-PEG. (C-D) TEM images of an equimolar mixture of oppositely supercharged protein-polymer conjugates. Inset in D, is a magnified image from the same sample. Scale bars: C (100 nm); D (500 nm); Inset in D (50 nm).

Circular Dichroism (CD) spectroscopy measurements of β LG A-PEG (+) and β LG A-PEG (-), indicated a secondary structure similar to that of the native protein, albeit with a small degree of unfolding as observed by a blue-shift in the minima at 216 nm as well as a lower signal at 190 nm for the individual protein-polymer conjugates [Figure 4.4D]. The slight unfolding is most likely due to supercharging, which may disrupt intramolecular hydrogen bonding within the protein, a phenomenon commonly observed with supercharged proteins [275].

Figure 4.6: Individual monomers of β LG A-PEG (+) and β LG A-PEG (-) fail to self- assemble into proteinosomes. (A-B) DLS measurements of β LG A-PEG (+) and β LG A-PEG (-), respectively. (C-D) Zeta potential measurements of β LG A-PEG (+) and β LG A-PEG (-), respectively. (E-F) TEM images of β LG A-PEG (+) and β LG A-PEG (-), respectively. Scale bars in E & F represent 500 nm.

Dynamic light scattering (DLS) measurements of dilute solutions of native β LG A revealed a broad peak centered around 10 nm suggesting the presence of small aggregates [Figure 4.5A]. Mixing of solutions of β LG A-PEG (+) with equimolar amounts of β LG A-PEG (-) gave rise to the appearance of a

major peak at 80 nm [Figure 4.5B]. This suggested interaction between the oppositely charged conjugates and assembly into well-defined protein particles with a narrow size distribution. Individual monomers failed to show any form of assembly on their own [Figure 4.6A-B], most likely because of extensive charge repulsion. Zeta potential measurements of β LG A-PEG (+) and β LG A-PEG (-) monomers indicate their average charge to be +18 and -18, respectively [Figure 4.6C-D]. DLS analysis of an equimolar mixture of supercharged β LG A, lacking a conjugated PEG chain, also resulted in protein assemblies, albeit with a much larger diameter and size-distribution [Figure 4.7A]. This indicates that even though supercharging plays a key role in self-assembly, formation of well-defined assemblies is governed by the presence of a PEG-chain.

Figure 4.7: Self assembly β LG A-Allyl (+) and β LG A-Allyl (-) in MilliQ water. (A) Dynamic light scattering (DLS) of β LG A-Allyl (+)(-) assembly (Green). (B) Comparison of β LG A-Allyl (+)(-) (turbid solution) and β LG A-PEG (+)(-) proteinosome (clear solution).

Subsequent TEM analysis of the supercharged protein-polymer conjugates revealed that individual solutions of supercharged β LG A-PEG remains stable and did not exhibit a propensity to self-assemble into higher-order structures [Figure 4.6 E-F]. However, when solutions of oppositely supercharged β LG A-PEG were mixed in equimolar ratios monodisperse spherical aggregates of 50 - 80 nm in diameter were formed, henceforth referred to as proteinosomes [Figure 4.5C-D]. As observed by electron microscopy these proteinosomes were

more reminiscent of viruses, being typical protein aggregates, than of earlier reported vesicular aggregates formed by self-assembly of protein or peptide conjugates.

Figure 4.8: Separation of mono-PEGylated βLG A-PEG (+) and βLG A-PEG (-). (A-B) Size exclusion column separation profile of βLG A-PEG (+) and βLG A-PEG (-), respectively. Colors indicate the various PEGylated and non-PEGylated species in the sample as indicated in the legend. (C) SDS-PAGE gel of the eluted fractions from A & B.

To further confirm the role of PEGylation in self-assembly, I removed the by-products (di- and tri-PEGylated βLG A) [Figure 4.8] via size exclusion chromatography. DLS data confirms that mono-PEGylation is sufficient to drive self-assembly, yielding proteinosomes with a relatively narrow size distribution when compared to proteinosomes formed with a non-purified-PEGylated βLG A [Figure 4.9A-B]. Cryo-TEM of proteinosomes assembled from oppositely charged mono-PEGylated conjugates are in agreement with DLS data, where most of the assemblies appear to be of a homogenous size distribution [Figure 4.9C].

Figure 4.9: DLS measurements and cryo-TEM of supercharged protein-polymer conjugate assemblies. Size distributions as determined by DLS of (A) an equimolar mix of positively and negatively supercharged mono-PEGylated β LG A, and (B) an equimolar mixture of positively and negatively supercharged mono-, di-, and tri-PEGylated β LG A. Cryo-EM of samples from A (C) and B (D-E). Insets in C and E are magnified images of the same sample. Scale bars: C-E (100 nm).

On the other hand, conjugates of a non-purified PEGylated β LG A, similar to those used to obtain the TEM images, appear to yield assemblies of a relatively heterogeneous nature [Figure 4.9D-E]. This variation in size distribution between proteinosomes formed from mono-PEGylated, and multiple

PEGylated β LG A hints at the possibility of controlling the overall size of the proteinosomes by mixing appropriate ratios of the di- and tri-PEGylated β LG A.

Additionally, these proteinosomes exhibit excellent stability and remained unaffected over a wide range of ionic strengths, over a pH range of 2-11, and when stored at 4° C for a period of 21 days [Figure 4.10 and Figure 4.11 respectively]. Under similar conditions, native β LG A tend to grow into larger aggregates and reach sizes up to 1200 nm or more [Figure 4.10]. These results strongly suggest that β LG A-PEG (+) and β LG A-PEG (-) behave as amphiphilic polyelectrolytes.

Figure 4.10: Effect of pH (A) and ionic strength (B) on the self-assembly of proteinosomes and native β LG A. For pH study (A): 100 μ L of the proteinosome sample in milliQ water was taken in a micro cuvette (Eppendorf® UVette, Sigma-Aldrich, Singapore). pH was adjusted by adding small drops (1-5 μ L) of dilute HCl or dilute NaOH, incubated for 5 min before DLS measurement were collected (5 runs, 1 min/run), average of 5 runs. For ionic strength study (B): 100 μ L sample in milliQ water was taken in a micro cuvette, appropriate volumes of 5 M NaCl solution was added to it. After each addition, the samples were incubated for 5 min before DLS measurement (5 runs, 1 min/run), average of 5 runs. Data was plotted in OriginPro 9.1 (OriginLab Corporation, MA, USA).

Figure 4.11: Time dependent correlation diagram of proteinosomes. Correlation curves of the same sample collected over 21 days shows very little change in the overall size of the proteinosomes. Data was plotten in OriginPro 9.1 (OriginLab Corporation, MA, USA).

With the help of static light scattering (SLS) measurements, the radius of gyration (R_g) for the proteinosomes was calculated to be 47 nm. Using a combination of DLS and SLS data it is possible to determine the δ value ($R_g:R_h$) which can help distinguish between solid and hollow spheres. While solid spheres have a δ value of 0.776, a thin-walled hollow sphere has a δ value of 1 [276]. For the proteinosomes, the δ value was determined to be 0.9062 that suggests thick-walled hollow spheres [Figure 4.12].

Figure 4.12: DLS and SLS of β LG A-PEG proteinosomes. (A) Dynamic light scattering (DLS) data of β LG A-PEG proteinosomes (B-D) Guinier Plot of Static light scattering (SLS) data of β LG A-PEG proteinosomes from each of three runs to determine the radius of gyration (R_g).

4.4.2 Encapsulation

After being able to form nanocapsular assemblies of β LG A-PEG conjugates, I wanted to confirm if these capsules were capable of encapsulating solutes. For this, I attempted to encapsulate plasmid DNA (pDNA) and tested the proteinosomes's ability to offer protection against nucleases added externally. Agarose gel analysis showed that pDNA (pCMVTnT), which migrated as two bands [Figure 4.13, lane 1], was unaffected when mixed with proteinosomes [Figure 4.13, lane 2]. The same pDNA when treated with nucleases (XhoI and NotI), underwent cleavage to migrate as a single band of linearized pDNA [Figure 4.13, lane 4]. However, when the pDNA was encapsulated into proteinosomes, most of it remained in the well and failed to enter the gel. The smear observed in this lane is most likely from pDNA complexed with the protein-polymer conjugates that failed to participate in the self-assembly process. It is interesting to note, however, that the single band observed for the nuclease treated pDNA, completely disappeared when encapsulated and treated with

nucleases [Figure 4.13, lane 5]. The pDNA when encapsulated within the proteinosome is too big to enter the gel, as seen from the strong bands observed in the wells of the gel. If the pDNA remained unencapsulated, a migration pattern similar to that observed for samples where the pDNA was mixed with preformed proteinosomes was expected [Figure 4.13, lane 2], but this was not the case. I neither saw the two characteristic bands for supercoiled and circular pDNA nor did I observe the linearized pDNA, as seen for digested pDNA [Figure 4.13, lanes 2 and 4 resp.].

Figure 4.13: pDNA encapsulation and protection from nuclease. 0.8 % agarose gel electrophoresis of naked pCMVTnT (pDNA) (lane 1), pDNA added to preformed proteinosomes (lane 2), pDNA encapsulated in proteinosomes (lane 3), naked pDNA digested with Xho1 and Not1 (lane 4), and a sample containing encapsulated pDNA that was digested with restricted enzyme Xho1 and Not1 (lane 5). DNA marker (M).

This suggests that the pDNA is entrapped within the proteinosomes and is protected from nuclease activity. Overall, these results indicate that the

proteinosomes are capable of encapsulating large molecular weight species, while offering some form of protection to the contents within.

Even though proteinosome shows a clear indication of DNA encapsulation it is possible to exclude that the highly charged DNA molecule can wrap over (attach to) the proteinosome surface and results in the same response in the gel [Figure 4.13]. As a confirmation, I attempted to encapsulate non-charged protein, GFP from jellyfish, *Aequorea victoria*, and dye-labelled polysaccharide, dextran into proteinosomes. Proteinosomes prepared in the presence of GFP [Figure 4.14A, Brown bar] exhibited ~ 50 % greater fluorescence intensity when compared to empty proteinosomes to which GFP was added on the outside [Figure 4.14A, Blue bar]. Similar encapsulation results were obtained with FITC labelled dextran [Figure 4.14]. This difference in fluorescence intensity suggests encapsulation and the low encapsulation efficiency can be explained by the inability to prevent non-specific interactions between GFP and the proteinosome surface. DLS data confirms that the presence of GFP or FITC-dextran did not interfere with the self-assembly of proteinosomes [Figure 4.14B-C].

Figure 4.14: Encapsulation of GFP and FITC-Dextran into proteinosomes. (A) Normalized fluorescence intensities of GFP or FITC-dextran encapsulated proteinosomes (Brown) and control-empty proteinosomes to which GFP or FITC-dextran were added after proteinosome formation (Blue). (B) Size distributions as determined by DLS of GFP encapsulated proteinosome (Blue line), GFP added to empty proteinosome (Brown line), and empty proteinosomes to which no GFP was added (Black line). (C) Size distributions as determined by DLS of FITC-dextran encapsulated proteinosome (Blue line), FITC-dextran added to empty proteinosome (Brown line), and empty proteinosomes to which no FITC-dextran was added (Black line).

To explain the self-assembly of oppositely charged β LG A-PEG conjugates into defined capsules, I propose a process driven by a contribution from (1) surface hydrophobic patches present on the native protein, (2) electrostatic interactions contributed via the introduction of excess charges, and (3) the amphiphilic nature of the protein-polymer conjugate by the introduced PEG moiety. Native β LG A, on its own, is well known to self-associate into various shapes under harsh denaturing conditions [277-279]. Weak hydrophobic forces

due to partial or complete unfolding of the protein, along with electrostatic attractive forces between oppositely charged amino acids are the two main driving forces behind such aggregations [280]. In the case of our supercharged β LG A-PEG conjugates, the protein undergoes slight unfolding as suggested by CD data, and is likely to expose some of the hydrophobic patches on the protein. However, supercharging β LG A prevents its self-assembly due to charge repulsion, but when mixed with oppositely charged conjugates, they self-assembled into nanosized capsules. This is not unlike viral self-assembly where the positively charged protein subunits are prevented from aggregation due to charge repulsion, but are driven to self-assemble in the presence of a negatively charged ssRNA, combined with specific interactions between certain amino acids termed caspar pairs that are usually two adjacent carboxylate groups [281, 282]. The important difference when comparing viral self-assembly with the self-assembly of β LG A - PEG protein conjugates, is the presence of PEG which directs the assembly into hierarchically ordered structures. Without the PEG, the supercharged protein mixtures are still capable of self-assembly, but only to form large aggregates that exhibit a less defined structures [Figure 4.7A]. PEGylation makes the protein-polymer conjugate slightly amphiphilic, with the protein and the PEG acting as the hydrophobic and hydrophilic moieties, respectively. This is similar to the role of PEG in the construction of giant amphiphiles.

4.5 CONCLUSION

In conclusion, I have described the synthesis and characterization of a novel protein-polymer conjugate that spontaneously self-assembles into nanosized spherical capsules by utilizing interactions not unlike those seen in viral assemblies. Since, the self-assembly occurs in an aqueous environment and only upon addition of the oppositely charged species, it offers better control over the initial step of self-assembly process. The selectively modified β LG A simultaneously take advantage of both hydrophobic and electrostatic interactions that drive the self-assembly. This is not the case with other protein-polymer conjugates that rely on emulsions for solubilizing and their removal for initiating self-assembly. Moreover, the proteinosomes described herein are generally more

stable than those prepared by alternative techniques. These are the strategic advantages of the proposed proteinosome assembly process over alternative approaches. It is also worthwhile emphasizing that β LG is a readily available (inexpensive) milk protein that opens up for scale-up and industrial applications. I also expect this approach of supercharging and conjugating with a polymer, for self-assembly, to be applicable to other globular proteins as well.

CHAPTER 5 SUMMARY AND FUTURE PROSPECTIVES

5.1 SUMMARY

In this thesis, I have addressed two self-assembled systems 1) hybrid lipid-polymer vesicle and 2) protein-polymer conjugated vesicle, proteinosome to mimic cellular membrane and viral capsids' architectures respectively. In the hybrid vesicle system, I show that mixing of low molecular weight (M_w) biodegradable amphiphilic copolymer with phospholipid POPC spontaneously form nano- and micron size vesicles by film rehydration process followed by extrusion and electroformation methods, respectively. I furthermore observed that the polymers on their own do not form any vesicle nor are capable of encapsulating hydrophilic molecules. However, the presence of lipid facilitates the vesicle formation and enables encapsulation of a hydrophilic dye. The major contribution of this work is, as hypothesized, that low M_w polymer provides an energetically more stable membrane by reducing the membrane mismatch between lipid and polymer. Thus, it yields a bilayer with a thickness, $\sim 5\text{-}7$ nm that is very close to that found in natural lipid membranes. The surface properties of these vesicles are lipid-like, but with a higher resistance to enzymatic digestion as compared to POPC vesicles. These vesicles also show improved semipermeable property as compared to POPC vesicles. In addition, a hydrophobic model drug (e.g., Curcumin, a natural compound extracted from *Curcuma longa* (turmeric), an excellent anti-inflammatory agent [283] and anti-cancer agent [284]) has been encapsulated into the bilayer membrane of the hybrid vesicle. Its release profile has been recorded for 30 days at 4 °C (data not shown here) as a proof of concept to a model drug delivery vehicles. Although I have shown the hybrid vesicle with POPC, other phospholipid types should be in principle expand this phospholipid-assisted hybrid vesicle-forming strategy and contribute to the design of functional vesicles for future applications.

In the protein-polymer vesicle system, I have developed a novel and simplified method to prepare proteinosome, a nano-sized spherical capsule, spontaneously self-assembled from an aqueous medium. A milk protein, β -

lactoglobulin A (β LG A) and poly(ethylene glycol) were used as starting materials. Two supercharged PEGylated protein moieties, β LG A (+) and β LG A (-) were first synthesized by surface functionalization via amination and succinylation followed by PEGylation. Mixing the two oppositely charged protein-polymer conjugate results in hierarchical nano-capsules. Since, self-assembly begins in an aqueous environment and only upon addition of the oppositely charged species, it offers better control over the initial step of self-assembly. It takes advantage of amphiphilic nature of protein-polymer conjugates, protein's hydrophobic patches, and electrostatic interactions of opposite charge species to strengthen its overall final assembly. This is not always the case with other protein-polymer conjugates that rely on emulsions as supporting templates and/or for removal of organic phase upon initiating self-assembly. Moreover, the proteinosomes described herein are generally more stable than those prepared by alternative techniques. These are the main strategic advantages of this process over alternative approaches. Moreover, proteinosome reminiscent of viral capsid architecture and able to encapsulate charged genetic materials (e.g., DNA) and other non-charged (e.g., dextran) or moderate surface charged species (e.g., green fluorescence protein), inside their core. I envisage understanding this approach of supercharging and conjugating with a polymer, for self-assembly, to be applicable to other globular proteins as well. Finally, β LG is a readily available inexpensive milk protein that opens up for industrial applications.

5.2 FUTURE PROSPECTIVES

As described in the earlier paragraphs that lipid-polymer hybrid vesicle possesses lipid-like properties with improved permeability and higher resistance to enzymatic digestion even at the high content (50 %) of the block copolymer. However, it is not yet known, how these low M_w BCPs influence the membrane physical properties such as line tension at the lipid/polymer boundary, bending rigidity, stretching modulus, membrane surface shear viscosity, lateral diffusion coefficient etc. light scattering, Cryo-EM, enzymatic degradation assay has been exploited during this PhD work, but a thorough physio-chemical characterization is also necessary (e.g., micropipette aspiration etc.). Stable lipid-like surface topology, thin bilayer membrane facilitates trans-membrane protein insertion and proper function [56] which opens up the horizon for membrane protein research and membrane protein based drug discovery. An improved understanding of the membrane properties including permeability would undoubtedly accelerate future applications in the era of drug delivery with controlled fashion release over burst release.

In addition, this novel protein-polymer self-assembly unleashes a new branch of building block design and nanostructure development. In fact, protein-polymer conjugates are not new and other research groups (e.g., Olson *et al.*, Mann *et al.*) have also reported different nanostructures in literature (such as cylinders, lamellae, micelles, and vesicular) with numerous applications in biotechnology, medicine, and industry [285, 286] and they possess their own pros and cons. However, the newly developed method overcomes some of the limitations with earlier preparations (e.g., self-assembly in complete aqueous medium etc.) and further strengthen the field of protein-polymer based assemblies for the better future application. Upon understanding the physical and chemical parameters of both the system allow us to pursue this idea to other system and to create a toolbox in medicine and biotechnology.

OUTCOME

PUBLICATIONS

1. Controlled supramolecular self-assembly of “supercharged β -lactoglobulin A – PEG conjugates” into nanocapsules.

Amit Kumar Khan, Sushanth Gudlur, Hans-Peter de Hoog, Winna Siti, Bo Liedberg, and Madhavan Nallani, *Angewandte Chemie International Edition*, 2017, 56, 11754–11758. (DOI: 10.1002/anie.201704298).

2. Photo-induced cyclo-addition of tetrazoles to native proteins.

Winna Siti, Amit Kumar Khan, Hans-Peter de Hoog, Bo Liedberg, and Madhavan Nallani, *Organic & Biomolecular Chemistry*, 2015, 13, 3202-3206. (DOI: 10.1039/c4ob02025a).

MANUSCRIPT IN PROGRESS

Phospholipid-assisted self-assembly of biodegradable polymers into a modulated lipid-like biosensing topology for facile membrane protein incorporation.

Amit Kumar Khan, James Ho, Susmita Roy, Bo Liedberg, and Madhavan Nallani.

PATENT

Tubular and vesicular architectures formed from polymer-lipid blends and method for forming the same.

Madhavan Nallani, Bo Gunnar Liedberg, Atul Parikh, Hans-Peter De Hoog, Amit Kumar Khan, Seng Koon Lim, Pub. No.: WO/2016/159879, International Application No: PCT/SG2016/050152, Publication Date: 06.10 2016.

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