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**ENGINEERED FERRITIN NANOCAGE AS A
CHOLESTEROL SEQUESTERING AGENT**

SAMYUKTA RAVISHANKAR

**SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING**

2020

**ENGINEERED FERRITIN NANOCAGE AS A
CHOLESTEROL SEQUESTERING AGENT**

SAMYUKTA RAVISHANKAR

School of Chemical & Biomedical Engineering

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

2020

Statement of Originality

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

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Authorship Attribution Statement

This thesis contains material from 1 paper published as manuscript in the following peer-reviewed journal(s) / from papers accepted at conferences in which I am listed as an author.

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Assoc. Prof. Sierin Lim provided the project direction and edited the manuscript drafts.

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Abbreviations

ABCA1	ATP binding cassette subfamily A member 1
ABCG1	ATP binding cassette subfamily G member 1
ACAT1	Acyl-CoA:cholesterol acyltransferase
ADP	Adenosine diphosphate
<i>Af</i>	<i>Archaeoglobus fulgidus</i>
ANOVA	Analysis of variance
ApoA-1	Apolipoprotein A-I
ApoE	Apolipoprotein
ATP	Adenosine triphosphate
BLI	Bio-layer interferometry
CC	Cholesterol crystals
CCR2	C-C chemokine receptor 2
CD	Cluster of differentiation (CD 64, CD1, CD45, CD11b, CD163)
CE	Cholesteryl esters
CVD	Cardiovascular Diseases
Cy5	Cyannine 5
DAPI	4',6-diamidino-2-phenylindole
DHE	Dihydroethidium (Hydroethidine)
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
EPR	Enhanced permeability and retention
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FDA	U.S. Food and Drug Administration
FITC	Fluorescein isothiocyanate
FITC	Fluorescein isothiocyanate
FITC- M β CD	Fluorescein isothiocyanate conjugated methyl- β -cyclodextrin

FLAP	5-lipoxygenase-activating protein
FX	Free cholesterol
Hb	Haemoglobin
hCD47	Human cluster of differentiation 47
HDL	High-density lipoproteins
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HNO ₃	Nitric acid
ICP-MS	Inductively coupled plasma mass spectrometry
IgG	Immunoglobulin G
IL1 β	Interleukin 1 beta
IPTG	Isopropyl β - d-1-thiogalactopyranoside
IRE	Iron responsive element
IRP	Iron regulatory protein
KCl	Potassium chloride
K _D	Dissociation constant
LAL	Lysosomal acid lipase
LCAT	Lecithin–cholesterol acyltransferase
LDL	Low-density lipoproteins
LDLR	Low density lipoprotein receptor
LOX1	Lectin-type oxidized Low-density lipoprotein receptor 1
LPS	Lipopolysaccharide
LXR	Liver X receptor
MerTK	c-mer proto-oncogene tyrosine kinase
MgCl ₂	Magnesium chloride
MPS	Mononuclear phagocytic system
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
mRNA	Messenger ribonucleic acid
M β CD	Methyl- β -cyclodextrin
NCEH	Neutral cholesterol ester hydrolase
NF- κ B	Nuclear factor kappa B

NLR	Nucleotide-binding domain, leucine rich containing protein
NLRP3	NLR family pyrin domain containing 3
NO	Nitric oxide
NPs	Nanoparticles
O ₂ ⁻	Superoxide anion radicals
Ox-LDL	Oxidised low-density lipoprotein
PEG	Polyethylene glycol
PLGA	poly(lactic-co-glycolic acid)
PLGA	Poly(lactic-co-glycolic acid)
QDs	Quantum dots
QFF	Q Sepharose Fast Flow
RGD	Arginylglycylaspartic acid
rHDL	Reconstituted high-density lipoprotein
ROS	Reactive oxygen species
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
Scara5	Scavenger Receptor Class A Member 5
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	Small interfering ribonucleic acid
SIRPα	Signal regulatory protein alpha
SOD	Superoxide dismutase
SPIONs	Super-oxide iron oxide nanoparticles
SR-AI	Scavenger receptor class A type I
SR-BI	Scavenger receptor class B type I
SREBPs	Sterol regulatory element-binding proteins
TEM	Transmission electron microscopy
Tfr1	Transferrin receptor 1
TNFα	Tumour necrosis factor alpha
UTR	Untranslated region
VCAM1	Vascular cell adhesion protein 1

Summary

Accumulation of lipid laden macrophages (foam cells) is characteristic of atherosclerosis development in the arterial walls. Clinically, the current line of treatment includes using preventive drugs to maintain serum cholesterol levels and emergency surgical interventions at the onset of a heart attack. Therapeutic moieties such as peptides and small molecules directly targeting plaque cholesterol delivered using a transport vehicle has been recognized to potentially be a non-invasive tool for atherosclerosis management. In this thesis, the use of surface modified ferritin protein nanocages is proposed for the display of cholesterol sequestering agents for intracellular cholesterol reduction from atherosclerotic foam cell models.

Ferritin cages have been modified by chemical and genetic methods to incorporate the therapeutic moieties. As a physical sequestration method, the use of hydrophobic cyclodextrin conjugated to ferritin nanocages as cholesterol sequestering agents is proposed. Cyclodextrin molecules are chemically conjugated to the ferritin nanocages surface or encapsulated within the nanocages using metal co-loading methods. The cyclodextrin conjugated ferritin has nanomolar affinity to cholesterol molecules as measured using biolayer interferometry. Treatment of foam cells with the conjugates shows decreased levels of intracellular accumulated cholesterol. Interaction of cyclodextrin with membrane cholesterol are observed to play a crucial role in cholesterol reduction with minimal cholesterol sequestration efficacy observed with cyclodextrins loaded within the inner cavity of ferritin.

As an alternative mechanism of cholesterol sequestration, HDL mimetic peptides were genetically fused to the N-terminus of the cage for surface display. The secondary and quaternary ferritin structures are found to remain intact after peptide modification. Up to 30% reduction in intracellular cholesterol levels were achieved within 24h of treatment with the conjugates in a concentration dependent manner using the foam cell models. Efflux receptor analysis of treated samples demonstrates ABCA1 mediated cholesterol efflux mechanism using mimetic peptides as opposed to a physical sequestration using cyclodextrin molecules.

To further understand the interaction mechanisms of ferritin with macrophages, in-depth internalization and molecular response studies are carried out. Ferritin is observed to be internalized by a transferrin receptor-mediated endocytosis pathway with caveolae and micropinocytosis concurrently playing a role in the uptake. Subcellular localization and transmission electron micrographs reveal endosomal localization with subsequent lysosome induced degradation of the protein shell and release of the iron core in the cytoplasm. mRNA upregulation of iron regulation relevant proteins and iron export is further measured with prolonged incubation. Lastly, preliminary pilot studies with atherosclerotic mice models reveal aortic localization of ferritin within 1 hr with greater than 95% of the macrophages in the lesion site homing the nanoparticles.

This thesis thus includes the successful incorporation of therapeutic agents for cholesterol sequestration from an atherosclerotic marker, foam cells. Coupled with the internalization properties in macrophages and foam cells, this project emphasizes the potential of ferritin protein cages to be used as transport vehicles for cholesterol reduction in atherosclerosis management.

Chapter 1 : Introduction

Data released by the World Health Organization (WHO) report that approximately 31% of deaths globally (17.9 million people) are linked to cardiovascular diseases resulting in heart attacks and strokes. Individuals at a risk of developing cardiovascular disease (CVD) are often diagnosed with the build-up of atherosclerosis plaques. Atherosclerosis is a diseased condition characterized by the deposition of lipids in the arterial lining resulting in lowered blood flow and eventual blockage with continued progression. The physiology of atherosclerosis involves the recruitment of monocytes through the dysfunctional endothelial layer to the lesion sites and their differentiation to macrophages in the presence of pro-inflammatory cytokines. Concurrently, low density lipoproteins (LDL) infiltrated in the plaques undergo oxidative modification to form oxidized LDL (ox-LDL) in the presence of free radicals. Internalization of these modified lipoproteins by macrophages results in the formation of foam cells. Cholesterol loaded foam cells constitute the bulk of the lipid core in the plaque lesion site. The thickening of the lipid core with increased infiltration of monocytes and lipoproteins causes the narrowing of the arterial lumen and reduced blood flow^[1]. Macrophages and smooth muscle cells forming a fibrous connective tissue called a fibrous cap around the lesion undergoes destabilization with increased plaque progression resulting in blood clots and potentially myocardial infarction with the complete blockage of blood flow^[2].

The current line of treatment at the early stages is targeted towards prevention by modulating serum lipid (cholesterol) levels with the use of different generations of statins and thereby slowing the progression of the diseased state by minimizing the infiltration of excess low density lipoproteins to

the lesion site. Long term prescription of these drugs have presented challenges including muscle degeneration and dose adherence^[3]. At the other extreme, surgical interventions include angioplasty and bypass are adopted as treatment during an emergency such as the onset of a heart attack. The current research methods are focussed on the development of therapeutic tools mediating plaque regression without opting for a surgical approach^[4]. Drug eluting stents are often implanted during surgical procedures to open an obstructive artery containing drugs to reduce cell proliferation and prevent restenosis^[5]. Thus, therapeutic modalities directly minimizing plaque area with subsequent atherosclerosis regression has been identified as a gap in clinical atherosclerosis management.

At research stages, several such therapeutic molecules have emerged to address different components of plaque physiology. These include cholesterol sequestering moieties to reduce cholesterol deposition in the lesion site^[6], gene therapy with the use of siRNA controlling transcription factors regulating lipidogenesis^[7] and controlling macrophage polarization to promote the secretion anti-inflammatory factors^[8]. However, the majority of these therapeutic molecules lack cell-specific localization properties, have poor local concentration in lesion sites and their proteolytic instability results in low efficacy of drug interaction with targets^[9]. Improvements in drug penetration to the site of interest can be achieved using active or passive delivery transport system that display or carry these therapeutic molecules with minimal effects on properties whilst localizing in cells or tissues of interest. Nanoparticles (NPs) have garnered significant attention for use as delivery vehicles for small molecules, oligonucleotides, and peptides in inflammatory diseases such as atherosclerosis and tumours. Some of these nanoparticles include silicon NPs^[10], quantum

dots^[11], PLGA^[12] and cell/protein-based nanoparticles^[13]. Although with their individual advantages, protein nanocages being native to the human body rise to the forefront for use in biomedical applications. These include narrow particle size distributions, degradation within biological systems avoiding risks of long-term accumulations and ease of tunability using genetic / chemical methods^[14].

Ferritin is one such protein nanocage that has been proposed as a potential diagnostic tool for atherosclerosis. Ferritins are ubiquitous iron storage proteins present in most life forms. It comprises of 24 polypeptides assembled into a cage structure with 12-15 nm in diameter. Iron present in ferritin have shown MRI contrast agent properties useful in diagnostic imaging and disease progression analysis^[15]. Furthermore, the small yet narrow distribution of particles allows for enhanced permeability effect resulting in passive localization of the particle in diseased sites with endothelial dysfunction, as commonly observed with atherosclerosis and cancerous tissues. Ferritin nanocages have been found to passively accumulate in the atherosclerotic plaque. Terashima et al. have shown enhanced imaging of high-risk atherosclerotic plaques by the delivery of iron contrast and fluorescence imaging agents using human derived ferritin nanocages thus serving as a diagnostic tool^[16].

In this thesis, the possibility of using *Archaeoglobus fulgidus*-derived protein nanocages specifically ferritin as a therapeutic platform by engineering the external surface to display therapeutic modalities is explored. *Archaeal* ferritin presents advantages in terms of tuneable assembly, versatility to surface modification and high temperature stability aiding in the development as a therapeutic tool. Cholesterol sequestration is the chosen mechanism for the

development of therapeutic moieties. The scope of the work and the specific objectives of each of the subsequent chapters have been further elucidated below.

Gap: Conventionally adopted surgical interventions and preventive drugs have presented several drawbacks in the management of atherosclerosis. Thus, direct plaque targeting and cholesterol sequestration from cholesterol laden plaque (dominant macrophage cells) have come to the forefront as a potential approach to develop therapeutic strategies. The proposed small molecular drugs and synthetic peptides present significant challenges due to poor accumulation in the plaque and thereby reduced cholesterol reduction efficacy. Thus, there is a need for the identification of a delivery vehicle that can be engineered with the cholesterol sequestering agents thereby presenting strong therapeutic potential.

Hypothesis: Ferritin protein nanocages can function as a therapeutic platform for cholesterol sequestration in foam cells.

Objectives: To test the hypothesis on using ferritin nanocages as a therapeutic platform, the cages are surface modified to display therapeutic moieties for cholesterol reduction. The cages have been engineered by both chemical and genetic modification techniques whilst maintaining the integrity of the cage structure. The cholesterol reduction efficacy has been demonstrated and compared using *in vitro* foam cell models thus demonstrating therapeutic efficacy. With the objective of therapeutic translation, ferritin cages were further studied to understand its cellular fate (*in vitro* and *in vivo*) and relevant immune response thereby validating its use for therapeutic applications. The objectives of the work to validate the given hypothesis have been divided according to the following chapters.

Chapter 2: Literature review aims to present a comprehensive view of the atherosclerosis diseased models along with an understanding of therapeutic mechanisms presently being developed thus aiding in explaining the concepts used in this project. The global disease burden of atherosclerosis and an in-depth discussion of plaque physiology relevant to the work presented here has been discussed. The current clinical modes of treatment and the associated challenges have been further elaborated. The review also covers new research ideas such as the use of gene therapy, cholesterol sequestering agents, nanoparticles and the potential of these tools to address the challenges of conventional treatment modalities.

Chapter 3: Cyclodextrin conjugated ferritin for direct cholesterol reduction from foam cells shows the possibility of engineering ferritin as an atherosclerotic therapeutic molecule by chemical conjugation techniques. In previous *in vivo* work, cyclodextrin molecules have been shown to reduce plaque area. However, free cyclodextrin suffers from short half-life in the circulation without specific localization resulting in toxicity. Therefore in Chapter 3, cyclodextrin were conjugated both on the inner cavity and outer surface of the ferritin cages to obtain two different engineered molecules. The cholesterol binding efficacy was studied *in vitro* using bio-layer interferometry techniques. The relative cholesterol sequestration efficacy was further tested by measuring intracellular cholesterol levels pre-/post- treatment using fluorescent enzymatic assays. Finally, the advantage of using ferritin conjugated cyclodextrin over bare cyclodextrin molecules is shown by observed increased passive accumulation property in target cells owing to the receptor mediated endocytosis pathway followed by ferritin.

Chapter 4: Genetic fusion of HDL mimetic peptides to ferritin surface

aims to present an alternative pathway of cholesterol reduction as an alternative or complement to the cyclodextrin fusion. Being protein based, ferritin presents itself as a platform for genetic fusion of peptides that are often cost ineffective or difficult to synthesize exclusively. Two different peptide sequences have been chosen and the engineered ferritin proteins have been extensively characterized to ensure minimal impacts on the inherent ferritin self-assembly property. Cholesterol reduction in foam cells treated with these engineered molecules have been estimated. Preliminary studies on the effects of the treatment of efflux receptor expression have further been carried out.

Chapter 5: Cellular fate of ferritin nanocages is not well understood. The

aim of this chapter is to elucidate the uptake and location which will explain the rationale behind choosing ferritin as the nanoparticle that has the potential to be developed as an atherosclerotic therapeutic/drug delivery agent. The *in vivo* and *in vitro* localization of ferritin in macrophage cells (abundant in atherosclerotic disease model) has been shown. The subcellular localization of ferritin in cellular organelles has been demonstrated using both quantitative and qualitative techniques. *In vitro* assays showing ferritin degradation under endosomal/lysosomal conditions have been carried out. The molecular responses of the cells (both immune and iron related proteins) have been evaluated to confirm the compatibility of ferritin to be used for any subsequent treatment. Further engineering of the cage surface with poly-histidine results in alteration of the localization and molecular responses with increased endosomal escape and cytoplasmic localization.

Chapter 2 : Literature Review

With about 18 million deaths each year, CVD is the leading cause of mortality globally. The main underlying cause of CVDs resulting in stroke and myocardial infarction is the formation of atherosclerotic plaques. The next sections highlight the physiology of atherosclerosis and elaborate on the key markers that are garnering research interest as targets for the management of the disease.

2.1 Physiology of Atherosclerosis

Atherosclerosis is characterized by lipid accumulation in the arteries leading to plaque formation. The increased LDL content in the blood results in the accumulation of these molecules in the intima region. The secretion of inflammatory markers and the accumulation of modified LDL (acetylated or oxidized) increases the recruitment of monocytes across the endothelial monolayer into the intima(**Figure 2-1**)^[1]. These monocytes differentiate into macrophages which eventually differentiate to form foam cells upon intracellular lipid accumulation. The different stages of atherosclerosis ranges from early lesions, lesion expansion, development of the necrotic core and subsequent impacts such as plaque rupture or blockage of the arteries. Fibrous cap rupture results in destabilization of the plaque region triggering thrombus formation and thereby could cause myocardial infarction^[2, 17]. Identification and development of treatment strategies targeted towards early lesions could contribute to better management of the disease condition (**Figure 2-1**).

Figure 2-1: Stages in the development of atherosclerosis plaque from recruitment of monocytes to formation of the lipid core in the blood vessel lining^[18].

2.1.1 Macrophages

Macrophages play a key role in all stages of atherosclerosis. Macrophages maintain cholesterol homeostasis by the uptake of cholesterol from LDL and the efflux of excess cholesterol to high-density lipoproteins (HDL). Cholesterol molecules play a crucial role in maintaining the fluidity of the cell membrane thereby maintaining its structural and functional properties. However, excess cholesterol influx in the cell results in lysosomal and cytosolic accumulation and consequently the formation of lipid droplets. This makes the cell prone to apoptosis and compromises native immune function of macrophages.

As seen from **Figure 2-2**, during cholesterol homeostasis, scavenger receptors (SR-A1) and lectin like oxidized low-density lipoprotein receptor-1 (LOX1) macrophages bind to oxLDL transferring the cholesterol esters (CE) inside the lipoprotein to lysosomes followed by late endosomes. CE then gets

hydrolysed to free cholesterol (FX) by lysosomal acid lipase (LAL) followed by transfer and re-esterification by acyl coenzyme A: cholesterol acyltransferase-1 (ACAT1) in the endoplasmic reticulum (ER). During the cholesterol efflux process, CE from the ER is hydrolysed by neutral cholesteryl ester hydrolase (NCEH) and transported to HDL molecules outside the cell via ATP-binding cassette (ABC) transporters (ABCG1) and scavenger receptors (SR-BI). Increased oxLDL and pro-inflammatory cytokines in the intima results in overexpression of scavenger receptors and LOX-1 resulting in increased uptake. This together with the downregulation of ABCG1 and NCEH receptors, involved in cholesterol efflux, increases intracellular cholesterol accumulation^[19]. These cells with high lipid content are referred to as foam cells due to their ‘foamy’ appearance.

Figure 2-2: Mechanism of foam cell formation depicted using a schematic and electron micrograph of human aorta containing lipid droplets and cholesterol crystals indicated with yellow and blue respectively^[19, 20].

Imbalance in the hydrolysis and esterification process of cholesterol results in accumulation of both free cholesterol and CE in the foam cells.

Upregulation of ACAT1 would result in CE accumulation in the ER resulting in the formation of large lipid droplets. Free cholesterol produced by the hydrolysis process in the lysosomes are also shown to be highly toxic. These free cholesterol molecules crystalize and results in the formation of cholesterol crystals that are needled shaped structures made of hydroxyl cholesterol molecules ^[20]. Cholesterol crystals (CCs) have been shown to be pro-apoptotic thereby resulting in cell death and its release in the extracellular matrix (ECM) of the plaque region. Furthermore, CCs in the ECM, trigger systemic inflammation and increases the risk of fibrous cap rupture and thrombus formation ^[21].

2.1.2 Endothelial cell dysfunction

Endothelial cells form the single layer lining of blood vessels and is responsible for tissue growth and repair. The endothelial lining further plays a crucial role in preventing thrombosis by anticoagulant mechanisms and controls clot formation in response to vascular injury thereby maintaining its integrity^[22]. Dysfunction of the endothelium is characteristic of several human diseases such as cancer diabetes mellitus and atherosclerosis^[23]. Endothelial dysfunction occurs at the early stages of atherosclerosis which in turn is suggested to induce vasoconstriction of blood vessels^[24].

The endothelial integrity has been recognized as a marker of individual atherosclerosis risk. Nitric oxide (NO) is synthesized in endothelial cells by L-arginine amino acid catalysed a constitutive nitric oxide synthase dependent pathway^[25]. A fine balance between NO and ROS is required to maintain homeostasis in the vasculature. NO plays a role in minimize leukocyte adhesion, inhibits pro-inflammatory cytokine generation and the oxidation of LDL. Endothelial dysfunction in the early stages of atherosclerosis progression is the

result of a reduction in NO generation and increase in ROS levels^[26]. Although there is still an ongoing debate if defect in NO signalling is the result of inflammatory lesion environment or the cause, the impaired vasodilation coupled with increased ROS could increase the risk of thrombosis and myocardial infarction^[27].

2.1.3 Transcription factors pathways in cholesterol sensing

There are two distinct pathways that play a role in cholesterol sensing followed by the activation of signalling pathways to maintain lipid homeostasis. Cholesterol levels are detected by sterol regulatory element binding proteins (SREBPs) and liver X receptors (LXR). The activation of SREBPs in the nucleus activates the biosynthesis of cholesterol in the cells thereby regulating the lipid levels, fatty acids and triglycerides in the plasma. In the presence of excess cholesterol in the cells, SREBPs is retained on the endoplasmic reticulum thereby being in its inactivated state^[28]. SREBPs is responsible for the activation of hydroxymethylglutaryl-CoA (HMG-CoA) reductase and LDL receptor genes which have been identified as targets for drugs to maintain cholesterol homeostasis in the blood^[29]. LXR on the other hand are responsible for activation of reverse cholesterol transport, cholesterol efflux and its metabolism (conversion to bile acids)^[30]. Binding of endogenous LXR agonists (oxidized forms of cholesterol) results in conformational changes in the ligand binding domain triggering the activation of genes (ATP binding cassette transporters - ABCA1, ABCG1) facilitating cholesterol efflux^[31-33]. Macrophages lacking or with downregulated LXR have higher susceptibility to cholesterol overload and thereby the formation of foam cells contributing to atherosclerosis progression^[34, 35]. The use of synthetic agonists have been shown to have athero-protective

effects by the activation of vascular cholesterol efflux genes however with observed hypertriglyceridemia and lipogenesis effects necessitating the need for further evaluation^[36-38].

2.1.4 Iron regulation in atherosclerosis

Iron is an essential component in several biological processes such as cellular metabolism, oxygen transport, cellular growth, DNA synthesis. Iron exists in its two predominant oxidation states; ferric (Fe^{3+}) and ferrous (Fe^{2+})^[39]. The redox conversion between the two oxidation states occurring in the presence of enzymes and ADP/ATP plays a crucial role in maintaining cellular functions. Majority of the iron (65%) in the human body exists in the Fe^{2+} oxidation state in bound to Haem molecules and remaining are stored in cells in insoluble Fe^{3+} state. Fe^{2+} ions in haemoglobin that covalently binds to oxygen molecules allowing for transport in the human body^[40]. Thus, iron deficiency could result in symptoms such as shortness of breath, tiredness and extend to tissue damage in various organs over time. However, an overload of iron could result in undesirable Fenton reactions generating reactive oxygen species (superoxide, hydroxyl radicals) inducing oxidative stress, DNA damage, cellular malfunctions, cell death and potentially causing carcinogenesis^[41, 42]. This could occur because of large amounts of dietary iron, genetic disorders, repeated blood transfusions etc^[40]. Iron metabolism is thus tightly regulated across various cell types such as tissue macrophages, hepatocytes, erythrocytes, and duodenal epithelial cells. Particularly tissue macrophages in the spleen, bone marrow and liver play a crucial role in recycling 90-95% of the body iron thereby maintaining iron homeostasis^[43].

2.1.4.1 Endogenous Ferritin (L-Ferritin, H-Ferritin)

Ferritin is a ubiquitous iron storage protein present in most plant and animal tissues. Its main functions are to store insoluble Fe^{3+} ions creating a reserve which can act as a metabolic iron source when necessary as well as to protect the cells from toxic effects of iron due to its ability to catalyse the formation of reactive oxygen species. Though there is insignificant sequence homology between the ferritin species, similarities exist in the overall structure of the ferritin proteins^[46]. Ferritin proteins assemble with 24 sub-units of polypeptides and is primarily (80%) alpha helical with a 12-15nm average hydrodynamic diameter^[47].

Mammalian ferritin is found in the cytoplasmic region of the cells. The 24 sub-units of these ferritin proteins comprise of heavy and light chain sub-units. The proportional ratio of the two sub-units varies across different cells types and tissues. Heavy chain ferritin (H-ferritin) has high ferroxidase activity and is responsible for the conversion of Fe^{2+} ions to the Fe^{3+} oxidation state. Ferritin light chain (L-ferritin) is then responsible for the nucleation of this oxidized iron form and formation of the iron core within the protein shell^[48]. In the human body, light chain ferritin constitutes the major proportion of circulating ferritin that is secreted from cells (predominantly macrophages) following a non-classical lysosomal pathway^[49]. Thus, a combination of heavy and light chain ferritin plays a role in regulating intracellular iron levels.

Both L and H-ferritin are tightly regulated to mitigate the toxic effects of iron overload. Iron overload in tissues and cells results in upregulation of ferritin at transcriptional and translational levels^[50]. One of the mechanisms is following the IRP-IRE system. IRPs (IRP1 and IRP2) are cytoplasmic RNA binding

proteins that bind to the iron responsive elements present on the untranslated regions (UTR) of genes of ferritin and iron influx receptor (Tfr1) thereby controlling their regulation in maintaining iron homeostasis. Under iron overload conditions IRPs actively bind to ferritin mRNA promoting its translation and simultaneously also decrease the translation of Tfr1 to minimize any further iron influx^[51, 52].

2.1.4.2 Ferritin imbalance in disease models

The primarily role of ferritin in iron homeostasis has garnered significant interest in extending its role in diseased models characterized by an imbalance in iron levels. Serum ferritin has been used clinically as a diagnostic marker for several decades. Although not clearly understood as a marker to a particular disease a serum ferritin levels is associated with angiogenesis, inflammation, iron deficiency and cancer^[53].

Tumour: Jezequel et al. have further suggested the potential of using ferritin as a prognostic marker in patients with node-negative breast cancer. Patients with higher tumour growth rates had higher ferritin concentration. The high protein expression levels is seen primarily in macrophages and to lesser extent in lymphocytes, fibroblasts, endothelial cells and tumour cells^[54].

Atherosclerosis: Similar observations were made in pro-inflammatory conditions such as atherosclerosis. Atherosclerosis plaques have been found to contain 10-fold more iron stores than healthy arteries. Increased serum free iron mediated ROS generation causes the modification of LDL to form ox-LDL. This enhance lipoprotein uptake by macrophages to form foam cells ^[44]. During atherosclerosis progression, leaky vessels invading the intima could cause

intraplaque haemorrhage which is characterized by the rupture of the neo-vessels. The red blood cells released in the intima undergo apoptosis or are taken up by macrophages via the phagocytosis pathway releasing containing haemoglobin the extracellular space in the plaque region^[55]. Spontaneous oxidation of Hb results in the formation of heme and free iron that triggers ROS generation and oxidation mediated LDL modification. Iron bound to transferrin receptors is taken up by macrophages by receptor mediated endocytosis and stored in ferritin, the intracellular iron storage protein. Free heme is scavenged by haptoglobin protein (Hp) which is in turn taken up by macrophages upon binding to the CD163 receptor ^[56].

In response to inflammation, macrophages initially play an atheroprotective role by reducing the serum iron concentration levels. Intracellular ferritin is overexpressed to store the excess iron and L-ferritin, with low iron content, is secreted out of the cell. Serum ferritin secreted from macrophages is clinically used as a biomarker for body iron content and to diagnose inflammation. The uptake of iron minimizes the amount of redox active iron (Fe²⁺) in the serum thereby reducing corresponding inflammatory response^[49, 57]. This property of macrophages is however, limited to early lesions. Intracellular iron overload mediates polarization of macrophages from anti-inflammatory M2 to pro-inflammatory M1 phenotype. This triggers the generation of inflammatory markers and promotes plaque progression^[58]. Systemic and intracellular iron/ferritin levels play a crucial role in atherosclerosis progression or regression. Thus, modulating these levels could control the pathogenesis of atherosclerosis. Furthermore, given that ferritin is a iron storage

protein, the characteristic role of iron in atherosclerosis is a crucial aspect to consider when using ferritin as therapeutic for atherosclerosis management.

Ferritin protein, in addition to its iron storage properties has been identified to have anti-inflammatory and consequent immunomodulatory properties. Fan et al. have shown that overexpression of L-ferritin decreased the production of pro-inflammatory cytokines like TNF- α , IL1 β and nitric oxide (NO). Furthermore, a reduction in intracellular iron pool and ROS is observed. These observations are due to the suppression of nuclear factor- κ B (NF- κ B) pathway that is activated in response to LPS in murine macrophages^[48].

2.1.4.3 Ferroportin

It is the only known iron exporter protein found to be present on the cell membrane of iron-processing cells such as macrophages, hepatocytes, enterocytes etc. This transmembrane protein is regulated by the hepcidin hormone. Once hepcidin binds to ferroportin, it is internalized and degraded by the endo-lysosomal pathway such that no further iron export can occur^[59]. High ferroportin levels are observed in patients with high serum ferritin levels and low transferrin levels. The secretion of hepcidin is understood to be regulated by iron. Ferroportin's role in iron homeostasis is suggested to follow a direct (protein upregulation) and/or indirect (hormone stimulation) mechanism. An increase in overexpression of ferroportin in response to intracellular iron overload triggers iron efflux^[60]. High plasma and tissue iron levels could further stimulate hepcidin binding to ferroportin resulting in its internalization and trapping the iron within the cells. The consequent rise in cytoplasmic iron levels then triggers other transcriptional responses to minimize iron influx and increase iron storage^[61].

2.2 Current approaches for the treatment of atherosclerosis

When diagnosed with a narrowed artery condition due to plaque deposition, lifestyle changes are often the proposed treatment method. Additionally, cholesterol lowering drugs such as statins are prescribed with anti-platelet medications to minimize the risk of blockage due to thrombus formation. For advanced stages of atherosclerosis, a surgical approach for the implant of stents is recommended.

2.2.1 Statins

Pharmacological and gene therapies have been used for the reduction of cholesterol levels thereby minimizing the risk of developing cardiovascular diseases. Clinically statins are used in combination with anti-inflammatory therapies for the treatment of atherosclerosis. Statins are molecules that inhibit the HMG-CoA reductase enzyme thus reducing cholesterol biosynthesis and lower serum cholesterol levels. Statins further result in increased LDL uptake by liver cells due to overexpression of low-density lipoprotein receptor (LDLR) receptors thereby further reducing cholesterol and triglyceride concentrations. The decreased lipid production rate and increased catabolism rate has resulted in statins being predominantly used clinically as a preventive drug since the past few decades ^[62, 63]. Additionally, pharmacological agents are being clinically tested to be used in conjunction with statins. These include anti-inflammatory drugs such as methotrexate and allopurinol that inhibit receptor ligand interactions responsible for cytokines and ROS generation respectively ^[64].

2.2.2 Surgical methods: Bypass/angioplasty

For high risk patients with advanced lesions and those who have suffered an onset of a heart attack, surgical intervention remains the only viable solution.

Coronary bypass surgery a blood vessel harvested from the patient's leg, arm or chest is sewed to the coronary artery with the blockage to reroute and restore the blood flow. However, the risks of secondary onset of atherosclerosis in other blood vessels, postoperative symptoms. Poor medication adherence and high costs still remain a cause of concern^[4, 65]. In coronary balloon angioplasty, a balloon catheter is inserted in the blocked blood vessel and expanded to widen the artery and restore the blood flow. Metal stents with an anti-proliferative drug coating are placed to inhibit neointimal hyperplasia and reduce the risk of artery narrowing^[66]. However, there is a risk of restenosis and need for multiple stents especially in lesions with higher complexity. Furthermore, overlapping of multiple stents affects the efficacy of drug release, increases cytotoxicity and increased risk of stent thrombosis^[66, 67]. Both bypass and angioplasty are prescribed only for late stage atherosclerosis patients or those with chest pain to quickly restore the blood flow, thereby increasing the risk of a fatal cardiac event.

2.2.3 Anti-inflammatory drugs

Despite inflammation being one of the primary causes and markers of atherosclerotic lesions, there is no definitive anti-inflammatory drugs based more of treatment being clinically used. This is largely due to the range of possible pro-inflammatory target pathways making it difficult to isolate a single dominant pathway for the development of anti-inflammatory drugs. Furthermore, the role of the individual pathways in disease progression varies across individuals. Some of the markers include TNF, CCR2, interleukins (ILs), p-selectin, 5-lipoxygenase activating protein (FLAP) etc^[68]. Consequently, multiple anti-inflammatory drugs have failed to show positive outcomes of being able to prevent a fatal myocardial infarction in human clinical trials thus garnering the need for new approaches^[69].

2.3 Challenges of treatment methods

Statin is a preventive drug that is clinically prescribed to regulate serum cholesterol levels by intervening the cholesterol biosynthesis process. One of the issues with statin therapy is persistence. Adherence to the prescribed dosage and daily consumption, has been found to be a problem in ~ 50% of the population after 2 years of regular intake ^[3]. Since statins are prescribed to patients with high cholesterol levels, a stop in the medication could increase the cardiovascular risk. Furthermore, long term exposure to the drug has been reported to increase insulin resistance thereby increasing the risk of diabetes incidence. This has been associated with the mechanism of statins wherein the mitochondrial interaction of the drug causes a mitochondrial dysfunction in the cells at high statin tissue concentrations. Additional drugs such as Ezetimibe has been proposed to be used in conjunction with statins so as to reduce the concentration of statins required while maintaining the LDL lowering effect ^[3, 70]. However, further research is being conducted on these treatment methods as an observed increase in carotid intima-thickness has been reported with the use of Ezetimibe. Hence, there has been active research to develop alternative lipid lowering drugs with minimal mitochondrial interaction to avoid adverse effects because of dysfunction. Furthermore, being a preventive drug, although statins have been useful to maintain cholesterol homeostasis, only a small change in plaque area has been observed post statin therapy ^[71],

Recently, gene therapy has evolved as a method to facilitate atherosclerosis regression. An example is the delivery of mRNA using recombinant retroviruses that can incorporate the viral gene into the target genome. This allows for the expression of recombinant proteins such as antioxidants in vivo to inhibit

atherogenesis. Antioxidants like catalase reduce the ROS thereby decreasing ROS mediated LDL modification and thus reduced intracellular cholesterol uptake. Additionally, vectors are being developed to knock down LDLR receptors to achieve the same effect of reduced intracellular cholesterol accumulation and foam cell formation ^[72]. Gene therapy has been demonstrated to mediate plaque regression in mice models. However, its clinical translation remains a challenge due to poor efficiency of gene transfer and high cost of large-scale production of gene vectors. The stability of vectors in the physiological environment is also a cause of concern, wherein, ectopic expression could result in undesired immunological response ^[72, 73].

2.4 Emerging developments in atherosclerosis therapeutic models

2.4.1 Small molecule cholesterol sequestering agent - Cyclodextrin

Cyclodextrins (CDs) were discovered over a 100 years ago and have lately drawn a lot of attention in the pharmaceutical, cosmetic and food sector due to its structural properties. CDs are composed of cyclic oligosaccharides wherein the number of glucose units in each molecule vary depending on the type of cyclodextrin (α , β or γ). CDs in their native state are non-polar in nature with a large toroidal shaped hydrophobic cavity ^[74]. This hydrophobic cavity allows for the encapsulation of hydrophobic guest molecules such as cholesterol. The physiochemical change of the guest molecules once inside the cavity increases its aqueous solubility. The cholesterol molecules remain encapsulated within the cavity as a dynamic equilibrium is maintained due to non-covalent interactions and the formation of CD-cholesterol complex ^[75].

Figure 2-4: A) Chemical structure of β -CD, B) Encapsulation of cholesterol molecule in the toroidal hydrophobic cavity of CD and C) 3D representation of steric hindrance sites on native and derivatives of CDs.

In this thesis, β -CD is chosen as the molecule for intracellular cholesterol sequestration. The hydrophobic cavity volume of β -CD (160 Å³) has been reported to be optimal for sequestration of guest molecules. α -CD (100 Å³) has been found to be too small for the entry of guest molecules and γ -CD (250 Å³) to maintain the equilibrium between the guest molecule and the CD cavity. However, β -CD has the lowest aqueous solubility due to the high Gibbs free energy of the hydrogen bonding between the hydroxyl groups of the glucose units [76]. To enhance the water solubility, derivatives of CDs are synthesized by the addition of methyl or hydroxypropyl groups. These functional groups increase the distance between the CD molecules thus enhancing aqueous solubility. However, the addition of function groups increases the steric hindrance thereby affecting the interaction with the guest molecule as seen from the steric hindrance

sites in **Figure 2-4**. To overcome this limitation, β -CD is conjugated on the protein surface in this thesis. Since the bulk of the solution comprises of the soluble ferritin protein, the overall solubility of β -CD is enhanced.

The exact mechanism of cyclodextrin mediated intracellular cholesterol reduction is largely unknown. There are however two proposed mechanisms. The first is that cyclodextrin acts as a shuttle to extract cholesterol from the plasma membrane. The redistribution of lipid rafts in the membrane triggers the shuttling of cholesterol from the cytosolic space to the membrane and eventually out of the cell. Based on kinetic studies conducted, cholesterol molecules diffuses into the CD cavity without the CD adsorbing onto the plasma membrane owing to the low kinetic energy of the cholesterol transfer process. This proposed hypothesis emphasizes that cyclodextrin does not enter the cell and only directly influences plasma membrane cholesterol ^[77]. Recently however, experiments have shown that some of the cyclodextrin is likely to be endocytosed, sequester endosomal cholesterol, and decrease the overall intracellular cholesterol accumulation. This report tested the cholesterol extraction efficacy of methyl beta cyclodextrin when incubated with Niemann-Pick type C mutant cell that are characterized with high lysosomal cholesterol accumulation. It was observed that there was a prolonged decrease in the cholesterol levels even after cyclodextrin was removed from the media. The study suggested that some of the cyclodextrin is likely to have localized in the endosomes within the 24hr treatment hence resulting in the prolonged cholesterol sequestration after the cyclodextrin was removed. It is recommended that strategies at targeted endocytosis and retention of CDs to improve its efficacy as intracellular cholesterol solubilizing agents ^[78].

Subcutaneous administration of 2-Hydroxypropyl- β -cyclodextrin (HP- β -CD) in hypercholesterolaemic mice models induced atherosclerotic regression by about 45%. Overall, there are three proposed mechanisms suggested for this observation. The first being the physical solubilization of cholesterol from the cell membrane which is followed by excretion of the CD-cholesterol complexes. In addition to this, CD has been found to oxidize intracellular cholesterol to form 27-hydroxycholesterol. This oxysterol is capable of diffusing across the cell membrane thus reducing the overall intracellular cholesterol load. Furthermore, 27-hydroxycholesterol has been found to activate the liver X receptor that encodes transcription factors that play a role in ABCA1 and ABCG1 expression. Thus, the formation of these cholesterol derivatives due to CD treatment results in LXR mediated upregulation of receptors responsible to enhance reverse cholesterol transport. The work thus highlights the potential use of cyclodextrins, an existing FDA approved drug, for the treatment of atherosclerosis ^[79].

However, one of the key limitations is the dosage of HP- β -CD administered. Toxicological reports have shown that the LD₅₀ of β CD is between 450-790 mg/kg when IV administered which is below the dosage of 2000 mg/kg (~22 mM) administered in the study. Toxicity due to CD is associated with membrane damage due to cholesterol extraction, microcrystal formation in the kidneys resulting in necrosis and oxidative stress causing ototoxicity. Although derivatives of cyclodextrin such as hydroxy-propyl substitutions have been shown to cause lower nephrotoxicity, ototoxicity is still a concern at 10 mM range. Furthermore, use of hydroxypropyl derivatives shows lower cytotoxicity in vitro but also lower cholesterol affinity as compared to methyl substituted or native CD ^[80]. In vitro studies have demonstrated cyclodextrin to be an effective

cholesterol efflux agent in the micromolar range however, translation to in vivo requires higher dosage requirements. This could be explained due to the $< 1\text{nm}$ size of CD molecules. Pharmacokinetics studies estimate the half-life ($t_{1/2}$) of CDs to be less than 2h and is expected to be cleared out of the system within a 6hr period ^[81]. Furthermore, CDs are effective in mediating cholesterol efflux, however, are not specific in function to vascular macrophages. Intravenous injection of CDs would result in the CDs to be quickly cleared out by the kidneys before being able to permeate and localize in the lesion site resulting in the need for high dosage requirements to achieve desired therapeutic effects. The above factors contribute to the need for high dosage requirements thereby limiting clinical translation. Thus, there is a need for modulation of native or methylated cyclodextrins to achieve desired cholesterol efflux levels with low dosage requirements potentially by increasing the circulation time and localizing the molecules in the lesion sites.

2.4.2 HDL and HDL-like mimetics therapy

HDL plays a key role in maintaining cholesterol homeostasis by acting as a sink for efflux of excess cholesterol from the cells and transporting the same to the liver for further metabolism. Lately, epidemiologic studies have linked low HDL-C values ($< 40\text{ mg/dL}$) to increased risk of cardio vascular disease such as atherosclerosis ^[82]. Thus, several in vitro and in vivo tests have been conducted wherein HDL or HDL like nanoparticles have been used as agents of cholesterol efflux and overall atherosclerosis regression.

The first of these being the use of radiolabelled HDL for imaging aortic lesions thereby exploiting the characteristic property of HDL complexes to localize in the interstitial space of the plaque owing to its small size (7-13 nm

diameter)^[83]. Apo A-1 mimetic peptides, key structural protein on HDL molecules, were further synthetically synthesized and found to contribute to atherosclerosis regression by about 80% when orally administered to hypercholesterolaemic LDL receptor-null mice ^[6]. These mimetic peptides have also been found to minimize modified LDL levels due to its antioxidant properties. In continuation to these studies, synthetic HDL complexes using polymer lipid hybrids have been developed. These can be incorporated with targeting ligands and Apo A-1 mimetic peptides to target cell ABCA1 and scavenger receptors to mediate intracellular cholesterol efflux ^[84]. Furthermore, contrast agents and PLGA polymers have been incorporated in the hydrophobic core of the HDL complexes facilitating enhanced imaging and sustained delivery of therapeutic drugs such as statins or other anti-inflammatory drugs to the plaque region ^[85, 86]. These nanoparticles typically range from 80-100 nm in size allowing for incorporation of drugs in the core and still being in the range to permeate through the leaky vasculature in the lesion site (**Figure 2-5**).

Figure 2-5: Proposed structure of ideal mimetic lipoprotein with Apo A-1 helical properties for cholesterol efflux and receptor activation and hydrophobic core to act as a cholesteryl ester sink and incorporation of therapeutic drugs^[87].

Isolation of plasma HDL and synthesis of HDL like nanoparticles are associated with significantly high costs. Plasma HDL isolation requires large volume of sample and reagents thus making large scale translation difficult. Furthermore, inconsistency and stability of isolated HDL is a further cause of concern. Synthetically synthesized HDL has several components including multiple types of phospholipids and synthetic peptide chains to mimic the HDL molecules. Synthetic peptides are time consuming and costly to produce. Further, the mimetics are composed of multiple components thus increasing the possibility of undesired interactions with other receptors or molecules when used in physiological systems^[87]. Clinical translation of HDL mimetics specifically in human patients have been found to be affective only with chronic infusion or short term at high doses higher than the recommended levels. CER-001 rHDL mimetics when tested on humans with acute coronary disorder proved to mildly reduce the atheroma size^[88]. A challenge with the use of mimetics is the lack of understanding of the mechanism behind the cholesterol sequestration ability and transport to the liver for further metabolism. There is a need for further enhancement in the cholesterol extraction properties as presently high doses of mimetics are needed to achieve the regression effects. Bi-helical peptides have been proposed to be used for this purpose however still lack studies on them^[87]. The expression of ABCA1 and SR-B1 proteins, which facilitate cholesterol efflux to HDL, is reduced in atherosclerotic lesions. This could potentially explain the poor ability of low concentrations of rHDL or lipoprotein mimetics to sequester cholesterol. Lastly HDL in advanced lesions have been found to be dysfunctional due to oxidation catalysed by myeloperoxidase resulting in the loss of binding ability of HDL to cholesterol efflux or scavenger receptors thus

impairing the atheroprotective property ^[89]. Apo A-1 protein used in HDL mimetics could also be oxidized in the lesions thus affecting its efficacy as a therapeutic agent. To overcome the above-mentioned limitations, other modes to facilitate are being investigated which could be used in conjunction or as an alternative to HDL therapy. Although the above methods have been useful to minimize the cardiovascular risk, presently no clinically approved drug exists that directly targets the plaque region and facilitates regression.

2.4.3 Use of nanoparticles in biomedical applications

Nanoparticles (NPs) have emerged as materials for active research for biomedical applications such as treatment and biomedical imaging. Various types of NPs is found in nature or synthesized broadly categorized into organic and inorganic NPs. Organic nanoparticles are carbon based particles that include complex proteins (ferritin), lipid vesicles (liposomes), polymeric structures (dendrimers), carbon nanotubes etc. Examples of inorganic NPs include super paramagnetic iron oxide NPs (SPIONs), silver NPs, gold NPs, mesoporous silica NPs etc ^[90].

There are key considerations necessary to develop such nanostructures in terms of size, morphology, surface coating etc. to ensure high efficacy and low toxicity when translated to physiological systems. Particles with hydrodynamic diameter < 6 nm are known to undergo renal excretion and thereby low circulation times. Select inorganic larger particles (> 300 nm) tend to be toxic as they are not easily cleared out by the body (neither cell uptake nor renal excretion) and hence accumulate in the liver and spleen regions ^[91]. Particles with size between 10 - 300 nm are found to be optimal to ensure sufficient circulation time and minimal toxicity.

NPs increase the efficacy of therapeutic molecules encapsulated within by accumulating in the diseased tissue regions due to enhanced permeability and retention (EPR) effects. Following this mechanism, NPs have been extensively tested for delivery of anti-tumour drugs such as doxorubicin, cisplatin etc ^[92]. However, only recently, few articles exploiting its use for atherosclerosis treatment have been published. Vessel walls in atherosclerotic tissues are like tumour tissues wherein gaps in the endothelial lining allows for the extravasation of NPs to the site of interest. Furthermore, NPs are also proposed to enter the interstitial space via the adventitia through the dysfunctional micro-vessel network formed during angiogenesis. Accumulation of liposomal nanoparticles in rabbit aortic arteries induced with advanced atherosclerosis has been demonstrated by multi-modal imaging ^[93]. NPs are known to be passively taken up by macrophages following the mono-nuclear phagocytic system (MPS). This is a disadvantage in case where NPs are required to be delivered to specific tissues such as tumour tissues. However, this feature proves to be advantageous when targeted inflammation sites in atherosclerosis ^[94]. Intracellular delivery of therapeutic molecules to macrophages specifically lipid laden macrophages (foam cells) in the plaque region is thus possible due to this passive uptake property of NPs. This has been previously demonstrated by the intracellular delivery of poly(lactic-co-glycolic acid) (PLGA) NPs loaded pitavastatin into monocytes in mice arteries which decreased monocyte recruitment by inhibiting monocyte chemotaxis and differentiation into macrophages ^[95]. Other examples of use of nanoparticles as carriers in diseased models have been described in the following sections.

2.4.3.1 Liposomes

Liposomes are sphere shaped vesicles of single or multiple phospholipid bilayers encapsulating an aqueous environment within. The size and charge can be modulated by the composition of the lipids^[96]. The hollow core allows for encapsulation of drugs within and the amphipathic bilayer being structurally like cell membranes facilitates cellular uptake. The external surface can further be engineered by chemical conjugation of targeting peptides and antibodies for targeted delivery to the diseased sites. The drug release can then be triggered by pH, heat, magnetic field or light irradiation^[97]. Almer et al. have shown the targeted delivery to atherosclerotic lesion site using recombinant interleukin-10 conjugated liposomes for enhanced in vivo imaging of the lesion sites^[98]. E-selectin binding peptide conjugated liposomes have been designed for the delivery of dual anti-inflammatory drugs (atorvastatin calcium and curcumin). *In vivo* reduction in lesion sites was observed using this combinatorial drug delivery platform targeting dysfunctional endothelial cells^[99]. Though there has been substantial progress with the development of liposomes as delivery vehicles of anti-inflammatory drugs, challenges remain that limit clinical translation. These include limitations with synthesis of lower size diameter particles due to membrane stiffness, reaction efficiencies of chemical conjugation strategies, particle size control mononuclear phagocyte system (MPS) clearance and toxicological concerns of cationic liposomes^[100, 101].

2.4.3.2 Quantum Dots

Quantum dots (QDs) are nanometer sized crystals etched out from semiconductor materials that when excited with a certain wavelength of light produce a single fluorescent colour. They have thus been suggested to have

applications in targeted delivery to diseased sites for targeted imaging and drug delivery^[102]. Jayagopal et al. have utilized the multispectral imaging property of QDs to simultaneously visualize multiple cell types within the same atherosclerotic tissue^[103]. Infrared quantum dots have been encapsulated in simian virus 40 (SV40) for targeted imaging properties. The targeting peptide conjugated on the SV40 virus garners targeting properties to the delivery vehicle for the localization of fluorescent quantum dots in the lesion sites thereby being suitable for *in vivo* imaging^[104]. The targeted fluorescent imaging and multiplexed capabilities of quantum dots present them as promising candidates for early diagnosis, discerning different cell types and staging of atherosclerotic lesions based on the severity.

2.4.3.3 Ferritin protein nanocages

Proteins such as ferritin are ubiquitous in nature and are characterized by suitable physical stabilities. Proteins exist as monodisperse particles that do not aggregate under physiological pH and temperature conditions^[105]. This facilitates minimal immunological responses and ease of predicting optimal dosage requirements of the therapeutic molecules encapsulated within the proteins. Ferritin is an iron storage protein ubiquitous to all life forms including bacteria, archaea, and animal tissues. In physiological systems, ferritin plays a role of an iron storage protein by converting Fe (II) to Fe (III) in the cytosolic and mitochondrial region of the cell^[106]. Irrespective of its source, ferritin exists as a cage structure comprised of 24 polypeptides encapsulating an interior mineral $\text{Fe}_2\text{O}_3 \cdot \text{H}_2\text{O}$ in its cavity with a maximum loading capacity of 4500 Fe atoms. The mineralized iron core is formed by the interaction of Fe^{2+} ions (entering via the ion channels) with the Fe^{2+}/O oxidation (ferroxidase) sites^[47, 107, 108]. This results

in the conversion of Fe^{2+} to the mineral state for storage. Apoferritin refers to ferritin without the mineralized iron core. These unloaded ferritin cages can exist as dimeric sub-units or a hollow cage structure. As described earlier, mammalian source plays a crucial role in the physiology of atherosclerosis. In addition to its physiological role, recombinant ferritin derived from different sources has been shown to be a transport vehicle for use as therapeutic and diagnostic tool for biomedical applications^[109]. The hollow interior of the cage allows for the loading of metal ions as well as drug molecules for biomedical applications. Furthermore, the outer cage surface allows for genetic or chemical modifications with peptides and small molecules that can be used for targeted delivery of these cages to specific sites for therapeutic purposes^[14].

Ferritin has garnered significant attention as a nanocarrier for tumour targeting. Uchida et al. were one of the first research groups to show the modification of the cage surface with targeting peptides whilst keeping the self-assembled cage structure intact. They demonstrated the conjugation of integrin targeting RGD peptide to human ferritin showed enhanced internalization by cancer cells thus presenting the potential of targeted delivery of imaging and therapeutic agents^[110]. *Pyrococcus furiosus* derived ferritin genetically modified to display chimeric F_c binding peptide (derived from IgG) resulted in active targeting of overexpressed folate receptors on breast cancer cells demonstrated by fluorescent imaging^[111].

In addition to tumour targeting, though not to the same extent, ferritin has been more recently identified as a tool for atherosclerosis management. Localization of the human ferritin in macrophages has been demonstrated both *in vitro* and *in vivo*. Recombinant human ferritin used as reaction vessels for the

generation of magnetite iron oxide core (as opposed to the natural mineral core) resulted in comparable T2 relaxivity as iron oxide NPs clinically used in MRI imaging^[112]. These nanocomposites as a result of protein receptor interaction resulted in higher internalization than iron oxide NPs in murine derived macrophages. Intravenous injection of Cy5.5 conjugated human ferritin in mice fed with a high fat diet showed significant fluorescent signal in the carotid arteries in the vascular lesions in comparison with a healthy mice control model. Similar to the observations in the *in vitro* studies, T2 enhancement in macrophages infiltrating vascular lesions were observed after injection with human ferritin with the magnetite core.

This work makes use of ferritin derived from *Archaeoglobus fulgidus* (AfFtn). Unlike animal source ferritin (archaea and bacterial ferritin do not comprise of varying sub-units heavy and light chains). Details of the structural and distinct characteristics of Af ferritin is elucidated in the next section.

2.4.3.3.1 *Archaeoglobus fulgidus* ferritin (AfFtn):

Archaeoglobus fulgidus archaeon thrive in extreme temperature and anaerobic conditions. Thus, ferritin derived from this species are resistant to thermal denaturation at temperature up to 85°C. AfFtn has a high degree of similarity to bacteria (*E.coli*) derived, human and other mammalian source ferritin but differs in overall structural properties. The 24 polypeptides assemble to form a quaternary structure with tetrahedral symmetry as opposed to the octahedral symmetry of other ferritin species. This results in the presence of four large pores of 45Å in size in native AfFtn^[113](**Figure 2-6**). Replacing the non-conserved residues Lys 150 and Arg 151 with alanine results in a symmetry

switch of the open pore structure to a close pore mutant (AfFtnAA)^[114]. The inner diameter of the closed pore mutant is ~ 8 nm and outer diameter is ~ 12 nm as measured by dynamic light scattering and transmission electron micrographs.

Figure 2-6: Schematic of symmetry switch from AfFtn open pore structure to closed pore mutant [Adapted from Ref^[113]].

Conventionally, multi-subunit ferritins as seen in earlier examples exist as assembled cage structures in neutral buffer conditions. Loading of drugs or other cargo in these cages require the structures to be disassembled under low pH conditions and reassembled in the presence of the cargo by slowly increasing the pH to near neutral conditions. Although high loading efficacy can be achieved with this method, there is a significant loss in protein yield due to improper re-folding and pH induced irreversible denaturation^[115]. AfFtn on the other hand exist as dimeric units at neutral pH conditions that can be assembled *in vitro* in the presence of metal ions or high salt concentrations (500mM NaCl). Addition of reducing/chelating agents and lowering the salt concentrations causes the cages to disassemble thus releasing the cargo within^[116]. Co-loading of drugs with

metals during the cage assembly step results in regulation of cargo uptake and release with minimal loss in protein yield.

Chapter 3 : Cyclodextrin conjugated ferritin cages for direct cholesterol sequestration

Abstract:

Accumulation of lipid-laden macrophages (foam cells) is characteristic of atherosclerosis development in the arterial walls. Ferritin nanocages have been found to passively accumulate in the atherosclerotic plaque. Ferritin has been actively investigated as a carrier for contrast agents in atherosclerosis diagnosis. The potential of ferritin as a carrier for therapeutic molecules to mediate cholesterol reduction from foam cells is demonstrated in this chapter. Cyclodextrin molecules are chemically conjugated to the ferritin nanocages surface or encapsulated within the nanocages using metal co-loading methods. The cyclodextrin-conjugated ferritin has nanomolar affinity to cholesterol molecules. Treatment of foam cells with the conjugates shows decreased levels of intracellular accumulated cholesterol. The preferential localization of ferritin to foam cells is due to transferrin receptor-mediated endocytosis process. These findings show that ferritin nanocages as carriers localize cyclodextrin molecules to foam cells which mediate intracellular cholesterol reduction, thus highlighting its potential use as a therapeutic agent.

3.1 Introduction:

“Atherosclerosis is the leading cause of mortality globally ^[117]. Changes in diet, exercise and cholesterol lowering drugs such as statins, are prescribed in response to atherosclerosis diagnosis. Statins, being a preventive drug, maintain cholesterol homeostasis but contribute minimally to plaque area reduction and cause side effects such as muscle degeneration with chronic use ^[71]. Other experimental therapeutic approaches include gene therapy to knock down LDLR

to minimize intracellular cholesterol accumulation and use of recombinant retroviruses to express antioxidants thereby reducing ROS mediated atherogenesis. However, stability of the vectors, scalability issues, and cost of these methods have limited their clinical translation ^[72]. To overcome these limitations, other approaches for foam cell targeting and atherosclerosis regression that could be used in conjunction or as an alternative to current therapeutic approaches are being investigated in this thesis.

Atherosclerosis is characterized by the accumulation of oxidized low-density lipoproteins in the arterial wall and the recruitment of monocytes in developing plaques. The synergistic effect of increased expression of adhesion molecules and the influx of cholesterol containing lipoproteins results in the transmigration of the monocytes into the intima, its differentiation into macrophages, and the formation of foam cells ^[118, 119]. These lipid laden foam cells have been recently identified as biomarkers for the progression of atherosclerosis and are being explored as potential targets for atherosclerosis treatment ^[64].

Nanoparticles for biomedical applications such as treatment and biomedical imaging have been an active research area. Particles with sizes between 10 - 300 nm are found to be optimal to ensure sufficient circulation time and minimal toxicity ^[91]. Examples of nanoparticles that have been introduced to address atherosclerosis include reconstituted high-density lipoproteins, PLGA nanoparticles, lipid carriers and protein-based nanoparticles ^[84, 85, 120, 121]. Distinct from other nanoparticles that require capping agents, proteins naturally exist as monodispersed particles without aggregating under physiological conditions ^[105]. This facilitates the ease of predicting optimal dosage requirements of the therapeutic molecules co-delivered with the proteins. Ferritin, a protein

composed of 24 polypeptides forming a cage structure, is an iron storage protein that stores insoluble ferric compounds intracellularly thus maintaining cellular iron homeostasis. Human ferritin has been found to localize in the plaque region and be up-taken by macrophage cells by receptor-mediated endocytosis upon intravenous administration. The ferritin is found to localize in the lesion sites for up to a duration of 48 hours before being degraded by proteases ^[122]. Vessel walls in atherosclerotic tissues have gaps in the endothelial lining allowing extravasation of nanoparticles to the site of interest. Nanoparticles are proposed to enter the interstitial space via the adventitia through dysfunctional microvessel network formed during angiogenesis. They subsequently localize in the interstitial space via EPR effects ^[16, 93]. Ferritin has been previously proposed to be used as potential candidate for diagnostic imaging with properties desired for MRI contrast agents ^[15, 16, 123, 124].

Taking advantage of ferritin's natural preferential accumulation in the plaque, its' therapeutic potential can be thus harnessed. In this thesis, *Archean* derived ferritin is modified by chemical and genetic modification techniques to mediate cholesterol efflux from foam cells and contribute to atherosclerosis management. This work makes use of *Archaeoglobus fulgidus* derived ferritin with two amino acid modifications (K150A/R151A; AfFtnAA) that are formed by the self-assembly of the 24 polypeptides into a nanocage of ~ 12 nm ^[114]. AfFtnAA is a closed pore mutant of wildtype ferritin with controllable self-assembly property in response to ionic strength or metal loading. Co-loading of small molecules such as drugs with metal ions results in encapsulation of high concentration of the drugs in its hollow core of ~ 8 nm in diameter. Ease of surface modification allows for the conjugation of small molecules or antibodies

using chemical conjugation or genetic fusion strategies ^[14]. In this study, cyclodextrins (CDs) are chosen as the candidate to mediate cholesterol reduction *in vitro*. CDs have been known to have high affinity to cellular cholesterol and thereby can promote solubilization or removal of cholesterol from foam cells ^[125, 126]. CD and its derivatives are US FDA approved natural glucose oligomers (found as by-products of starch degradation) used as complexing agents to solubilize and deliver hydrophobic drugs ^[127, 128]. Subcutaneous administration of 2-hydroxypropyl- β -cyclodextrin in hypercholesterolaemic mice models induced atherosclerotic regression by about 45% ^[79].

This chapter is a proof-of-concept study demonstrating the use of cyclodextrin-ferritin nanocage conjugates (AfFtnAA-CD) as a cholesterol sequestering agent from foam cells. Here, molecular interaction studies using biolayer interferometry shows nanomolar range binding affinities between free cholesterol molecules and AfFtnAA-CD. The intracellular cholesterol reduction capacity of the construct is quantified using enzymatic assay. Uptake studies using ICP-MS and fluorescence imaging confirm the passive localization of ferritin to cholesterol loaded foam cells in comparison with methyl- β -cyclodextrin (M β CD) molecules. Using *in vitro* foam cell models, the potential use of engineered protein nanocages as theranostic carriers for cyclodextrin to mediate atherosclerosis regression is highlighted.

3.2 Experimental

3.2.1 AfFtnAA modification and production

Cysteine substitution was made at the 94th position in the AfFtnAA plasmid sequence and is verified to be located on the outer cage surface using PyMol simulations. The 94th amino acid residue is a non-interacting residue on the

external surface of ferritin avoiding any steric hindrance with the ferroxidase sites of the cages (APPENDIX, **Figure 7-1**). The substitution in AfftnAA plasmid (pET11a vector) is carried out using site directed mutagenesis following protocols mentioned by Bachman ^[129]. Briefly, forward, and reverse primers are designed to include the codons corresponding to the mutation with ~ 30 amino acid extensions binding to AfftnAA (template) plasmid. The linear amplification of the plasmid is carried out for a total of 40 cycles and a final extension of 15 mins. The template plasmids were digested with Dpn1 and transformed into *E. Coli* DH5 α competent cells for colony screening. The isolated plasmid was then transformed into a *E. coli* BL21(DE3) C+RIL strain and overexpressed in medium containing Ampicillin (100 mg/ml) and Chloramphenicol (50 mg/ml). The overexpression and protein purification was performed as previously described ^[15, 114]. Optical density measurements were taken during cell growth. Gene expression was induced by the addition of 1mM isopropyl β -D-thiogalactopyranoside (IPTG) when OD₆₀₀ reached between 0.6 - 0.8. Post 4 hours of growth after induction the cells were harvested using a centrifuge at 8000xg for 5 mins. Cell pellets were then lysed using a ultrasonicator followed by heat treatment at 85°C for 10 mins. Hydrophobic interaction chromatography (HIC) was performed on a AKTA-Explorer FPLC System (GE Healthcare) using a HiPrep Phenyl FF (high sub) column equilibrated with 25 mM HEPES, 50 mM NaCl and 500 mM (NH₄)₂SO₄ buffer at pH 7.4. The desired protein was eluted out of the column with 25 mM HEPES, 50mM NaCl. The eluted proteins were buffer exchanged with 25 mM HEPES, 50 mM NaCl, pH 7.4 buffers using a Amicon Ultra-15 Centrifugal Filter Units (10,000 Da) to remove any (NH₄)₂SO₄ present in the eluted fractions. Protein concentrations were measured using

Bradford Assay with bovine serum albumin (BSA) as the standard and the Quick Start™ Bradford 1X Dye Reagent.

3.2.2 Conjugation of cyclodextrin

Cyclodextrin was conjugated to the protein cage using copper (I) catalysed azide/alkyne cycloaddition reaction (CuAAC) following the methods described previously with slight modifications ^[130]. The introduced cysteine mutations on the cage was reduced by the addition of 15 molar excess of tris(2-carboxyethyl)phosphine (TCEP) and incubated for 1hr at RT on a 3D shaker. Propargyl maleimide (MW ~ 130.12, Kerafast, Inc., USA) was used as the linker to react with the sulphydryl groups of the cysteine. AfFtnAA was incubated with 100 molar equivalents of propargyl maleimide for 3 hours at 25°C with vigorous shaking at 200rpm. Unreacted propargyl maleimide was then removed using a PD-10 column using 25 mM HEPES, 50 mM NaCl, pH 7.4 as the buffer. The CuAAC reaction was then performed using 10 µM of AfFtnAA-PM, 15mM β-CD-N₃ (MW ~1.1kDa, Cyclolab, Hungary), 0.25mM copper (II) sulphate, 2.5mM Tris(3-hydroxypropyltriazolylmethyl)amine, 5mM sodium ascorbate and 5mM aminoguanidine hydrochloride (Sigma) Incubated at 25°C for 12 hours. Unreacted components were then removed using a PD-10 column and desalted AfFtnAA-CD stored in 4°C for further characterization.

3.2.3 Loading of cyclodextrin

Equivalent of 1000 atoms of fluorescently labelled randomly MβCD (FITC-MβCD) (MW ~ 13.kDa, CycloLab Ltd, Hungary) and 600 Fe atoms per ferritin cage was co-loaded at RT and incubated for 2 hours. Excess FITC-MβCD was desalted using a PD-10 column. The number of FITC-MβCD molecules encapsulated was characterized by fluorescence measurements at Ex495/Em519

nm and correlated with a standard curve plotted with known concentrations of FITC-M β CD.

3.2.4 Protein size determination

The hydrodynamic size of the protein was determined by dynamic light scattering (DLS) (Zetasizer Malvern Instruments) by assaying a 0.2 mg/ml protein sample in 25 mM HEPES (First Base), 50 mM NaCl (Sigma), pH 7.4. The intact assembly of the protein cages after conjugation with cyclodextrin was confirmed using transmission electron microscopy (TEM) (JEOL JEM1400). Samples of 0.1mg/ml were stained with 2% uranyl acetate and imaged at 120 kV and 25,000X magnification.

3.2.5 Biolayer Interferometry

The interactions between AfFtn-CD and unesterified cholesterol molecules was measured by biolayer interferometry using the ForteBio BLItz unit. Before the experiment, Streptavidin biosensors (ForteBio) was hydrated in MiliQ water for 10 mins. 100 μ g/ml of cholesterol-PEG-Biotin (MW ~ 5000 Da, NanoCS) was immobilized onto the streptavidin probe for 150 s and the baseline was generated in MiliQ water for 30 s. The probe was dipped in varying concentrations of the protein followed by MiliQ water during the association and dissociation step respectively for 300 s each. The binding of AfFtnAA to the probe without immobilized cholesterol molecule was also tested as a negative control. The dissociation constants (K_D) were derived using the BLItz ProTM software. Langmuir model is used by the software to determine the dissociation constant assuming a 1:1 binding of ligand and anylate. Since the K_D is independent of concentration of protein the probe is dipped in, error estimations can be obtained

by determining the standard deviation between the K_D values obtained for the different protein concentrations.

3.2.6 Foam cell formation

RAW 264.7 murine macrophage cells were cultured in Dulbecco's modified Eagle's medium (DMEM), 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin media (all from Gibco™) in T-75 flasks. 20,000 cells were seeded in 96 well plates and grown overnight in 37°C. The medium was then changed to a serum free media for 12 hours followed by 24 hours incubation with 40 µg/ml oxLDL. oxLDL was synthesized by the oxidation of LDL (Lee Biosolutions, USA) with 5 µM of CuSO₄ in PBS (Gibco) for 16 hours at 37°C. Unreacted CuSO₄ was removed using a PD-10 column.

3.2.7 Cell staining and imaging

Cells were fixed with 4% (Paraformaldehyde, Sigma-Aldrich) by incubation in 4°C for 15 mins. After washing with PBS, cells were incubated with DAPI 30 mins at 37°C in darkness. The cells were then washed with PBS and imaged using an inverted Zeiss Axiovert S100 fluorescence microscope (Carl Zeiss, Germany) with a 20X objective at excitation wavelength of 405 nm for DAPI stained samples.

For filipin staining, a working solution of 0.05 mg/ml is prepared in PBS diluted from the 1 mg/ml filipin III stock solution in DMSO (Sigma). Cell media is replaced with this stock solution and incubated for 1h at 37°C in darkness. The cells were then washed with PBS and imaged using an inverted Zeiss Axiovert S100 fluorescence microscope (Carl Zeiss, Germany) at excitation wavelength also at 405 nm.

Fluorescence imaging of other samples including cells incubated with both FITC-M β CD and dye conjugated AfFtnAA were imaged using the same equipment parameters at excitation wavelength of 488 nm. Quantification of fluorescence intensities were carried out using ImageJ software. Mean intensities and standard deviation was measured from Intensities measured were a mean of three wells with cells treated with the indicated samples imaged at the same brightness and objective. Intensity measured was normalized to the number of cells counted within the image.

3.2.8 Uptake study using inductively coupled plasma mass-spectrometry (ICP-MS)

Cells were seeded in 24 well plates at a seeding density of 0.1×10^6 cells/ml with 1ml volume added to each well. Some wells were incubated with oxLDL to form foam cells following the protocol mentioned earlier. Post incubation with AfFtnAA for varying time points (2 hours, 6 hours and 24 hours), cells were scraped and resuspended in 250 μ l of phosphate buffer saline (PBS). 20 μ l was used for cell counting and the rest was digested with nitric acid overnight at room temperature to prepare samples for ICP-MS analysis. Samples were sent to the Central Environmental Science and Engineering Laboratory to carry out the measurements. Briefly, ICP-MS measurements were taken using PerkinElmer, Model: Elan DRCe system. Fe concentrations were determined against a calibrated standard curve between 1ppb to 100ppb.

3.2.9 Cholesterol enzymatic assay

Cholesterol was extracted from the cells using hexane:isopropanol (3:2, v/v) solution by incubation at RT for 1 hr. Enzymatic assay was conducted using Amplex Red Cholesterol Assay kit (Thermo Fisher Scientific, Singapore)

following the protocol provided by the supplier. Briefly, samples were diluted with the reaction buffer and incubated with 0.2 U/ml Cholesterol esterase, 2 U/ml Cholesterol oxidase, 2 U/ml Horseradish peroxidase and 300 μ M Amplex Red for 1 hr at 37°C. Fluorescence was measured at Ex560/Em590nm. Cell protein from the cells were extracted using 0.2 M NaOH and quantified using Bradford assay.

3.2.10 Error analysis

All error analysis in this and the subsequent chapters have been carried out using Analysis of Variance (ANOVA). Unless otherwise stated, one-way ANOVA test is utilized given the single independent and dependent variable. Parametric test has been used in this thesis to test the null hypothesis (the means of the two groups being compared are equal) and validate the “significance” of difference between the data.

3.3 Results

3.3.1 Conjugation and loading of cyclodextrin in ferritin protein nanocages

Small molecules can be incorporated onto protein nanocages on the external and internal surfaces. **Figure 3-1** shows the two approaches used in this work where CDs are chemically conjugated to the external surface of AfFtnAA nanocages or loaded in the inner cavity. Conjugation of β -CD to the AfFtnAA has been achieved by copper(I)-catalyzed azide/alkyne cycloaddition (CuAAC) reaction). The conjugation requires modification of AfFtnAA external surface to incorporate cysteine amino acids for reaction with maleimide based linker. The cysteine-modified AfFtnAA (AfFtnAA/E94C) has been produced and purified showing self-assembly characteristics upon iron loading that is consistent to

previously reported data on AfFtnAA ^[114] (**Figure 3-2**). Basic characteristics of the unloaded protein subunits (Apo-AfFtnAA) is verified using SDS-PAGE presenting a thick band at 20 kDa. The self-assembly property is observed to remain intact after the cysteine mutation with the resulting hydrodynamic diameter of ~ 14 nm after iron-mediated cage formation.

Figure 3-1: Display of cyclodextrin on the external surface and loading in the cavity of AfFtnAA (A) Propargyl maleimide linker is reacted with the reduced cysteine groups on Apo-AfFtnAA following thiol-maleimide reaction chemistry. Linker conjugated protein sub-units (Apo AfFtnAA/E94C-PM) are reacted with azide modified β -CD molecules in the presence of copper (II) sulphate as catalyst and sodium ascorbate as the reducing agent. Protein nanocages are assembled using the β -CD conjugated AfFtnAA sub-units (Apo AfFtnAA-CD) by the addition of 2400 Fe^{2+} atoms to form the cyclodextrin-ferritin nanocage conjugates ((Fe2400) AfFtnAA-CD). (B) AfFtnAA subunits is assembled to form the nanocages following a co-loading method with 1000 M β CD molecules and 2400 Fe^{2+} atoms incubated for a period of 1 hr under static conditions to form the CD encapsulated AfFtnAA nanocages.

Figure 3-2: Verification of self-assembly property of AfFtnAA/E94C using A) SDS-PAGE indicating band at 20 kDa corresponding to monomer units of the protein, B) Dynamic light scattering, and C) Electron micrograph of 2400 Fe loaded AfFtnAA/E94C protein nanocages indicating hydrodynamic diameter of ~ 13 nm.

The relatively insoluble β -CD has been shown to have the highest affinity to cholesterol molecules compared to methyl- and hydroxypropyl-substituted β -CDs due to the absence of steric hindrance from the additional substituent groups typically incorporated to enhance solubility ^[76]. The conjugation of β -CD to hydrophilic AfFtnAA enhances its solubility and stability in aqueous solutions allowing the use of native CDs. M β CD is chosen for loading within the nanocage internal cavity and for *in vitro* cholesterol reduction assays due to its comparable cholesterol affinity and high aqueous solubility. A size increase from 5 nm for Apo-AfFtnAA to 12.5 nm for (Fe2400) AfFtnAA-CD is observed after conjugation and iron loading. Mass spectrometry measurements show the conjugation of AfFtnAA with the linker and subsequently with β -CD (**Figure 3-3A, B**). Two significant peaks at 20.3 kDa and 21.4 kDa correspond to the expected molecular weight of AfFtnAA-PM and AfFtnAA-CD. There are 24 cysteines available for conjugation in a single ferritin protein cage. Two peaks are observed on the mass spectrometry data corresponding to unconjugated and

conjugated protein determined by the increase in mass of the monomer protein unit upon CD conjugation. Peak integration is carried out on the mass spectrometry results to measure the ratio of the area under the two peaks to determine the degree of conjugation. The ratio obtained shows that ~75% of the cysteines are modified corresponding to 17-18 molecules of cyclodextrin conjugated to each ferritin cage.

Encapsulation of CDs inside AfFtnAA is achieved by co-loading with its natural ligand. Fe^{+2} has been reported to drive the self-assembly of the AfFtnAA subunits during which other molecules could be encapsulated ^[116]. The encapsulation efficiency was quantified using fluorescence measurements of FITC tagged M β CD. The fluorescence measured from the CD encapsulated ferritin samples is correlated with a standard curve to estimate the amount of CD molecules loaded per cage (**Figure 3-3E**). This yields a result of ~ 35 cyclodextrin molecules loaded in each ferritin nanocage. A CD/ Fe^{2+} loading ratio of ~ 1:15 is achieved. To further get an accurate estimate of CD loading, additional studies on potential quenching of FITC-M β CD fluorescence upon loading in the cavity needs to be accounted for. Quenching would result in an underestimation of the loading values calculated here. Lastly, TEM images of cyclodextrin conjugated and encapsulated nanocages show intact cage structures thereby validating the retention of the self-assembly property of AfFtnAA post modifications with cyclodextrin molecules (**Figure 3-3C, F**).

Figure 3-3: (A) Increase in size observed at individual reaction steps using DLS of Apo AfFtnAA, Apo AfFtnAA-PM, Apo AfFtnAA-CD and (Fe2400) AfFtnAA-CD nanocage. (B) Mass spectrometry graph of cyclodextrin conjugated nanocages with peaks corresponding to unconjugated AfFtnAA-PM and conjugated AfFtnAA-CD (C) Electron micrograph of (Fe2400) AfFtnAA-CD. (D) DLS of AfFtnAA (MβCD) with hydrodynamic diameter at 13.5 nm. (E) Standard curve of FITC-MβCD with the position of AfFtnAA (FITC-MβCD) fluorescence indicated in blue (F) Electron micrograph of AfFtnAA (MβCD).

3.3.2 Cholesterol binding affinity and intracellular cholesterol reduction studies

Surface plasmon resonance approaches have been used to demonstrate high affinity of cyclodextrins to cholesterol molecules ^[131]. To ensure that cyclodextrin remains effective in binding cholesterol, their affinity has been determined using biolayer interferometry (BLI) with streptavidin-functionalized probe. Biolayer interferometry is a technique used to monitor interactions

between molecules based on the interference pattern generated due to changes in optical thickness of the molecular layer caused by the binding of molecules to the probe ^[132, 133]. After ensuring the absence of unspecific binding of AfFtnAA-CD to the streptavidin tip, biotinylated free cholesterol was immobilized onto the probe following high affinity streptavidin-biotin interactions. The relative ligand interaction of unconjugated and cyclodextrin conjugated protein to the immobilized cholesterol molecules is studied (**Figure 3-4**). Apo instead of iron loaded AfFtnAA was used for the analysis due to the interference of metal ions with the BLI system. An increase in optical binding thickness is observed with CD conjugated nanocages. It is seen that the association rate of cyclodextrin conjugated was higher than the unconjugated protein indicating the role of cyclodextrin in increasing the affinity of AfFtnAA to the cholesterol molecule. Affinity measurements were conducted with varying concentrations of AfFtnAA-CD to determine the range of dissociation constant values. Unconjugated Apo AfFtnAA has a K_D of 100 μ M. In contrast, Apo AfFtnAA-CD shows higher specific binding towards free cholesterol with a K_D of $\sim$ 5 nM. Interactions between M β CD were not quantified due the limitation of the instrument being unable to measure small molecular (less than 10kDa) interactions. These results confirm the functionality of cyclodextrin upon conjugation with AfFtnAA and suggest its usage for cholesterol sequestration in physiological systems.

On confirmation of molecular binding of AfFtnAA-CD to cholesterol molecules using interferometry, the cholesterol sequestration capacity of AfFtnAA-CD has been tested *in vitro*. Foam cells generated by incubation with 40 μ g/ml oxLDL under serum free media conditions shows intracellular

cholesterol accumulation (APPENDIX, **Figure 7-2**). Cholesterol enzymatic assay (Amplex red assay) is used as a quantitative assay to measure cholesterol reduction in foam cells treated with the different conditions for 24 hours. Macrophage cells without oxLDL treatment and untreated foam cells have been used as controls. A 38% reduction in accumulated intracellular cholesterol is observed with AfFtnAA-CD treatment which was comparable to cholesterol levels in macrophage cells (**Figure 3-5A**). AfFtnAA loaded with M β CD shows an insignificant decrease in accumulated cholesterol levels. A significant decrease was observed with AfFtnAA-CD treatment. Cells treated with native AfFtnAA did not show any marked decrease in % of accumulated cholesterol as compared to the untreated samples.

Figure 3-4: Biolayer interferometry results showing the binding affinity of A) 5 µg/ml AfFtnAA, AfFtnAA-CD and B) 0 µg/ml (Water), 0.625, 1.25, 2.5, 5 µg/ml AfFtnAA-CD to cholesterol immobilized on the streptavidin probe sensor.

The effect of AfFtnAA-CD was further tested with treatment in a time dependent manner varying from 1 to 24 hours (**Figure 3-5B**). A decrease in cholesterol levels within 2 to 6 hours of treatment with AfFtnAA-CD is observed. The results validate that the CD remained functional upon conjugation and mediated

cholesterol sequestration property of AfFtnAA-CD. The overall cholesterol levels of treated cells dropped to the level of untreated macrophage cells within 6 hours of treatment thereby, highlighting the effectiveness of AfFtnAA-CD in reducing intracellular cholesterol level. Furthermore, given the intracellular cholesterol levels remain unchanged after 6hrs, this suggests probable saturation of CD mediated cholesterol sequestration or a transient state of cholesterol uptake by cells vs. sequestration by CDs wherein a cholesterol homeostasis is being reached.

Figure 3-5: A) Quantification of intracellular cholesterol accumulation in foam cells treated with 130 µg/ml AfFtnAA, 65 µg/ml AfFtnAA (MβCD), 130 µg/ml AfFtnAA-CD, 5µM MβCD (equivalent to CD loaded within the cages and conjugated on the surface) using Amplex Red Cholesterol assay. Cholesterol amounts per µg of cell protein has been normalized with untreated cells to obtain the % of accumulated intracellular cholesterol. B) Intracellular cholesterol reduction upon treatment with 130 µg/ml AfFtnAA-CD for varying treatment durations of 1, 2, 6 and 24 hours. ± SEM. (n = 2, where n is the least number of independent experiments performed in triplicate). Statistical analysis has been performed using one-way ANOVA test. * P < 0.05.

3.3.3 Preferential accumulation of ferritin in foam cells

To gain an insight into the uptake specificity of AfFtnAA and FITC-M β CD to foam cells over macrophage cells, quantitative uptake tests have been carried out using ICP-MS and imaged using fluorescence microscopy. Utilizing the presence of iron atoms within ferritin nanocages, quantification of total iron content inside cells using ICP-MS has been used as a method to monitor cellular uptake levels of AfFtnAA (**Figure 3-6A**). The iron concentrations are normalized against cell number to obtain the relative amount of iron per cell thus correlating with the relative AfFtnAA accumulated per cell. Over increasing duration of treatment, a steady increase in accumulated iron content is observed with foam cells demonstrating greater uptake relative to macrophage cells. After 24 hours, foam cells show almost triple increase in uptake of AfFtnAA as compared to unloaded macrophage cells. The increased uptake levels in foam cells is further visualized using fluorescence imaging (**Figure 3-6B**). A similar increase in uptake of protein conjugates is observed in macrophage cells and foam cells treated with AfFtnAA-CD for a duration of 24h (**Figure 3-6C**).

Human ferritin undergoes transferrin receptor mediated endocytosis in mitogen activated T and B cells ^[134]. A co-incubation test with transferrin, a natural ligand of transferrin receptor, is conducted to further confirm a similar uptake mechanism in macrophage cells incubated with the ferritin cages studied here. As expected, accumulated iron amounts in cells incubated only with transferrin is found to be lower than when incubated with AfFtnAA. This is due to the lower iron content of 10 Fe atoms per molecule of transferrin as compared to AfFtnAA with 1700 Fe atoms per cage. Normalization with the Fe atoms per cage shows up to 3 times higher uptake of transferrin than ferritin thus validating

it as a natural ligand. Co-incubation of 5 times (by mass) higher amount of transferrin with AfFtnAA results in only a slight increase in total cellular iron content. Thus, indicating the inhibition effect on ferritin uptake as a result of co-incubation. The transferrin uptake serves as the baseline for subsequent calculations. Subtraction of iron content due to transferrin uptake from the total intracellular iron measured after co-incubation shows a 62% inhibition in AfFtnAA uptake (**Figure 3-6D**). The decrease in uptake in the presence of transferrin is also visualized using fluorescence imaging of AfFtnAA tagged with AlexaFluor 488 (AF488) in the inner cage surface (APPENDIX, **Figure 7-3**). An inhibition of AfFtnAA uptake is observed in the presence of excess transferrin thereby suggesting the role of transferrin receptor mediated endocytosis pathway in ferritin uptake.

The absence on metal ions from cyclodextrin cavity requires an alternative method to monitor the intracellular iron levels. Fluorescence imaging has been used to observe the localization of FITC conjugated M β CD in macrophage and foam cells at 2 and 24-hour time points (**Figure 3-7A, B**). The values have been corrected for any autofluorescence observed in untreated cells. Increase in fluorescence intensities are observed after 24 hours as compared to a 2-hour treatment period for both macrophage and foam cells. However, no significant difference is observed between the fluorescence intensities in the two cell types for each of the time points measured.

Figure 3-6: Cellular uptake of AfFtnAA and AfFtnAA-CD (A) Quantification of cellular uptake of macrophage cells and foam cells treated with 130 μ g/ml AfFtnAA for 2h, 6h, 12h and 24h. Macrophage cells without incubation with AfFtnAA are used as control. (B) Uptake study using dye conjugated ferritin nanocages (AfFtnAA-AF488) incubated with RAW264.7 macrophage cells (left) and foam cells (right) for 24h. Cells are stained with DAPI (blue) and the dye conjugated protein appear green (C) Cellular uptake of 130 μ g/ml AfFtnAA-CD quantified using ICP-MS with cells treated for 24h (average $\pm$ SD) (D) Identification of transferrin receptor as a receptor for uptake of AfFtnAA. Cells were incubated with 325 μ g/ml Transferrin, 65 μ g/ml AfFtnAA and co-incubation of the same concentration of transferrin and AfFtnAA for 90 minutes. Iron content inside the cells was quantified using ICP-MS and normalized with iron content per molecule to correlate with protein uptake and inhibition. Statistical analysis has been performed using one-way ANOVA test. (n = 3, where n is the number of independent experiments with triplicate measurements taken for each). *** P < 0.005 and **** P < 0.001.

Figure 3-7: Cellular uptake of FITC-M β CD (A) Fluorescence images of macrophage and foam cells incubated with 1 mM FITC-M β CD for 2h and 24h. (B) Quantification of fluorescence intensities normalized with cell number per frame. $\pm$ SD for all quantified images. ($n = 3$, where n is the number of wells from which the images were acquired for each sample). Statistical analysis has been performed using one-way ANOVA test.

3.4 Discussion

In this work, the cholesterol sequestration capacity of ferritin nanocages that have been modified with β -cyclodextrin both on the internal and external cage surface is compared. Similar cholesterol sequestration efficacy of conventional atherosclerosis first-line drug, simvastatin, and methyl beta cyclodextrin (M β CD) molecules using the foam cell models developed for this work is further demonstrated (APPENDIX, **Figure 7-4**). Eighteen molecules of cyclodextrin were conjugated to the cage using CuAAC reaction. Previous studies using CuAAC reaction method makes use of long linkers such as N-propargyl-3-maleimidopropionamide as an alternative to the propargyl maleimide linker used in this study ^[130]. Although resulting in slightly enhanced conjugation efficiency, additional reaction steps for synthesis of the long linker

can be avoided while maintaining at least 75% conjugation using propargyl maleimide. Approximately 35 molecules of M β CD were encapsulated within the cage cavity following the co-loading method with metal ions. This corresponds to a loading capacity of 11.7% which resulted in higher number of cyclodextrin molecules per cage in comparison to external surface conjugation. In addition to the metal-induced assembly, AfFtnAA comprises of hydrophobic residues on the inner surface which could further contribute to the encapsulation affinity of CDs.

Intracellular cholesterol reduction has been observed following treatment with AfFtnAA-CD. Recent studies by other research groups have demonstrated prolonged CD mediated cholesterol efflux after endocytosis by Niemann Pick Type C mutant cells, a disorder characterized by the accumulation of lysosomal cholesterol ^[78]. The comparison between AfFtnAA-CD and AfFtnAA (M β CD) in mediating intracellular cholesterol reduction highlights the dual importance of cell membrane interaction of CDs obtained from the conjugated nanocages along with possible interactions with the intracellular cholesterol pool after endocytosis of the conjugates.

A 65% inhibition in AfFtnAA uptake in macrophage cells is observed upon co-incubation with 5 times molar excess of transferrin. Thus, it is concluded that endocytosis of ferritin in macrophage cells is most likely mediated by transferrin receptors which are known to be overexpressed in human carotid plaque foam cells ^[135]. Therefore, ferritin can serve as a useful delivery platform owing to its passive uptake property and preferential localization in foam cells overexpressing the receptor of interest.

Unlike the receptor mediated endocytosis pathway of AfFtnAA, uptake of FITC-M β CD has been shown to be mediated via micropinocytosis pathway in Caco cells ^[136]. This pathway, also known as cell drinking, does not involve receptors specific to the ligand (CDs in this case) and results in uptake of the ligands along with other extracellular matrix proteins. Since this pathway is common to both foam cells and macrophage cells, no appreciable difference in FITC-M β CD uptake is observed between the two cell types as shown here using fluorescence imaging.

The high dosage requirement of CDs has limited its clinical translation as a bare drug despite its high cholesterol affinity. This could be explained by the <1 nm size of CD molecules. CD half-life ($t_{1/2}$) is estimated to be less than 2 hours and is expected to be cleared from the system within 6 hours ^[81]. CDs administered through intravenous injection are rapidly cleared by the kidneys preventing them from permeating and localizing in foam cells in lesion sites. The passive accumulation property of ferritin in foam cells demonstrated in this work suggests that protein cages could act as mediators of specific delivery of CDs to foam cells. This could result in enhanced localization of CDs in the plaque region *in vivo*, thus potentially reducing CD dosage requirements to below sub-toxic levels. The future direction of the work conducted aims to determine optimal dosage requirement *in vivo* and harnessing the potential of cages as a versatile platform for atherosclerosis diagnosis and therapy.

3.5 Conclusions

Foam cells in atherosclerosis have been identified as key targets to mediate atherosclerosis regression by facilitating a reduction in intracellular accumulated cholesterol. In previous works, ferritin protein nanocages have been proposed to

be used as contrast agents due to EPR mediated preferential accumulation property in the plaque region. The use of cyclodextrin conjugated ferritin nanocages as a therapeutic molecule to reduce cholesterol from foam cells is reported. High affinity of cyclodextrin-ferritin conjugate to cholesterol molecules and subsequently observed cholesterol reduction in *in vitro* foam cell models has been demonstrated. Furthermore, ferritin has been shown to accumulate in foam cells with 3-folds higher affinity than in macrophage cells thus demonstrating specificity. This study opens perspectives on the use of ferritin nanocages as a delivery vehicle for cyclodextrin mediated atherosclerosis regression.”

Chapter 4 : Genetic fusion of HDL mimetic peptides to ferritin cages promote ABC receptor mediated cholesterol efflux

Abstract:

HDL particles are physiological complexes responsible to mediate efflux of excess cholesterol from the cells. ApoA-1, a key constituent of HDL particles, interact with cholesterol efflux receptors located on the cell membrane to mediate cholesterol efflux. ApoA-1 mimetic peptides, in particular 4F-AA, has been previously shown in literature to demonstrate similar cholesterol efflux receptor interaction and anti-inflammatory properties as the whole apoA-1 protein. In this chapter, ferritin nanocages are conjugated with the 4F peptide and its variant (2X-4F) on the N-terminus thus mediating external surface display of the mimetic peptides. Intracellular cholesterol reduction in foam cells was observed upon treatment with the engineered protein conjugates. Studies on the effect of protein treatment on cholesterol efflux receptors has further been demonstrated to elucidate possible mechanisms of cholesterol reduction in response to protein conjugate treatment.

4.1 Introduction

HDL particles are comprised of 70% apoA-1 protein, a 243 amino acid α -helical amphipathic protein that mediates cholesterol efflux upon interaction with ABC and SR-B1 receptors on the cell surface ^[137]. While maintaining cholesterol homeostasis, ApoA-I proteins sequester both free and esterified cholesterol from the cell membrane and develop to form nascent to mature HDL particles. ApoA-1 proteins undergo lipidation to form nascent HDL which then acquire free cholesterol from macrophages in the peripheral tissues via ABCA1 and ABCG1

transporters located on the cell membrane. The free cholesterol transferred onto HDL are esterified by lectin:cholesterol acetyltransferase (LCAT). The resulting mature HDL molecules are metabolized by the liver. In addition to their cholesterol efflux properties, HDL particles have also been identified to have anti-inflammatory, antioxidant and anti-thrombotic properties thereby reducing the risk of atherosclerosis plaque formation and progression^[138].

Atherosclerosis risk is identified with an increase in LDL cholesterol with an often observed decrease in HDL cholesterol levels in the serum. However, clinical studies using existing drugs like Niacin which increases serum HDL levels have largely shown to be ineffective highlighting the importance of the functionality of the molecule rather than the mature molecule itself^[139]. Synthetically derived HDL taps into this functionality of HDL molecules. Reconstituted HDL (rHDL) are synthetically prepared using phospholipids and ApoA-1 proteins modelled according to different HDL species (found at different stages of HDL pathway). The constructs could be altered by varying the type of phospholipids and ApoA-1 variant used. In both mice and human studies, rHDL has previously shown promising results by stabilizing existing plaques, reducing inflammation and in some cases demonstrated plaque regression^[140]. However, the 243-residue sized ApoA-1 protein presents difficulties in synthesis using solid state method and high costs for scale-up production. ApoA-1 mimetic peptides presents itself as an elegant solution to overcome key limitations with the use of the whole protein such as cost of reconstitution from plasma and laborious chemical synthesis steps^[141].

Five human clinical trials have been conducted with ApoA-1 mimetic peptides such as 4F and 4DF peptides presenting positive results with significant

reduction in atherosclerotic lesion size ^[137]. ApoA-1 peptides, *in vitro* and *in vivo*, have been shown to mediate cholesterol efflux from cells by sequestering cholesterol and phospholipid to form HDL-like particles along with exhibiting anti-inflammatory properties thereby minimizing lipid oxidation. However, oral delivery of these peptides requires high dosage of up to 100mg infused for 2 weeks due to the associated rapid clearance rate owing to the small size. A large portion of injected peptides have also been found to degrade within one hour of injection *in vivo*, with the maximum $t_{1/2}$ being about 2.25 hours ^[142]. Furthermore, unlike ApoA-1, glomerular filtration minimizes the localization of the peptides in the lesion site and limits primary place of action to the intestines wherein the oxidized lipids are inactivated ^[137]. However, the efficacy of 4F peptides in mediating cholesterol efflux presents itself as a potential candidate in the development of a therapeutic molecule using ferritin as a display scaffold. This could overcome challenges of synthetic peptide synthesis, lesion site accumulation and early protease degradation by the extended circulation times using the peptide-ferritin conjugates *in vivo*.

In this study, engineered ferritin cages genetically fused with 4F peptide and its variant 2X-4F peptide have been synthesized (**Figure 4-1**). Extensive characterization is performed to ensure the structural integrity of the cage structure and the secondary structure of the fused peptide. The cholesterol sequestration efficacy of the construct has been demonstrated using *in vitro* foam cell models as used in the previous study. Preliminary studies on the mechanism of action has further been discerned by transcriptional analysis of cholesterol efflux receptors (ABC transporters) in the presence and absence of treatment with the ferritin conjugates.

Figure 4-1: Schematic of fusion of HDL mimetic peptides to the ferritin AfFtnAA for cholesterol sequestration

4.2 Materials & Methods

4.2.1 Protein nomenclature

The annotation 4F-AA refers to 4F peptide conjugated the ferritin protein. As in Chapter 3, the ferritin protein utilized is the closed pore mutant (AfFtnAA) of the *Archean* derived wild-type ferritin. The annotation for 2X4F-AA refers to 2X4F (Dual 4F sequence repeats with a linker) conjugated to AfFtnAA protein.

4.2.2 Plasmid construction

GBlock sequences that codes for the two ApoA-1 domain (4F-AA: DWFKAFYDKVAEKFKEAF and 2X4F-AA: DWFKAFYDKVAEKFKEAF KVEPLRADWFKAFYDKVAEKFKEAF) were digested using XbaI and BamHI (**Figure 4-2**) and ligated into pET28a plasmids digested using the same endonucleases for 1 hr at 37°C. The construct will be His-tagged at the N-

Terminus. Digested vectors were separated using agarose gel electrophoresis (1% agarose gel) and extracted from the gel using QIAquick Gel Extraction Kit (Qiagen). The ligation was performed overnight at 4°C in the presence of T4 DNA ligase (NEB). DH5α competent *E. coli* cells were transformed with 100 ng of ligated plasmids and the plasmid-containing colonies were selected in the presence of 50µg/ml Kanamycin antibiotic (Gold Biotechnology). The sequence was confirmed using commercial service (Fist Base).

Figure 4-2: Plasmid design of genetic fusion of mimetic peptides to ferritin sequence.

4.2.3 Protein expression & purification

E. coli BL21(DE3) C+RIL strain was transformed with the plasmid construct and the gene was overexpressed in medium containing Kanamycin (50 µg/ml) and Chloramphenicol (25 µg/ml) (Sigma). The overexpression was performed following protocols similar to those mentioned in Chapter 3 (Section 3.2.1). Gene expression was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside (Gold Biotechnology) when OD₆₀₀ reached 0.6 - 0.8. Post

4 hours of growth after induction the cells were harvested using a centrifuge at 8000xg for 5 mins. Cell pellets were resuspended in the respective buffers (25mM HEPES, DI water, 20mM Tris-Cl) and lysed using a ultrasonicator. Upon clarification and separation of the insoluble fractions, the 4F-AA was purified using HisTrap chromatography. The purification was performed on AKTA-Explorer FPLC System (GE Healthcare) using a HiTrap HP column (GE Healthcare) equilibrated with 50 mM Tris-Cl (Sigme), pH 8.5. The desired protein was eluted from the column with 50 mM Tris-Cl, 500 mM Imidazole (Sigma), pH 8.5. The eluted proteins were buffer exchanged with 50 mM Tris-Cl, pH 8.5 buffer using Amicon Ultra-15 Centrifugal Filter Units (10,000 Da) (Merck) to remove imidazole present in the eluted fractions. Protein concentrations were measured using Bradford Assay with bovine serum albumin (BSA) as the standard and the Quick Start™ Bradford 1X Dye Reagent. (Bio-Rad) Optimization of purification method was required for 2X4F-AA purification. The 3-step chromatography used and the respective buffers have been tabulated (**Table 4-1**). The purified proteins were assembled to form the cage structure in the presence of 2400 Fe atoms. Excess iron was desalted using PD-10 desalting columns (GE).

Table 4-1: Chromatography techniques used for 2X4F-AA purification

Chromatography method	Column	Equilibration buffer	Elution buffer
HisTrap (Ni affinity)	HiTrap HP	50 mM Tris-Cl, pH 8.5	50 mM Tris-Cl, 500mM Imidazole, pH 8.5
Hydrophobic interaction chromatography	HiTrap Phenyl FF (high sub)	50 mM Tris-Cl, 500 mM Ammonium sulfate, pH 8.5	50mM Tris-Cl, pH 8.5

Ion-exchange chromatography	HiTrap QFF (high sub)	50 mM Tris-Cl, pH 8.5	50mM Tris-Cl, 1M NaCl, pH 8.5
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4.2.4 Protein size determination

Protein size was estimated following protocols mentioned in Chapter 3 with slight variations. The hydrodynamic size of the protein was determined by dynamic light scattering (DLS) technique (Zetasizer Malvern Instruments) by assaying a 0.2 mg/ml protein sample in 50mM Tris-Cl, pH 8.5. The measurement was taken at 25°C with the refractive index of the buffer set at 1.33. Size estimation of both Apo proteins (without iron loading) and (Fe 2400) loaded caged proteins structures were conducted. For transmission electron microscopy, samples of 0.15 mg/ml were stained with 2% uranyl acetate (Sigma) and imaged at 120 kV.

4.2.5 Circular dichroism measurements

The measurements were performed on 50 μ L aliquots (0.5mg/ml) in a quartz cuvette with 0.1 mm pathlength. The temperature of circular dichroism spectrophotometer (Chirascan Photophysics) was set at 25°C. The spectra for the protein samples were obtained across a range of 180 to 250 nm with a resolution of 0.5nm. The measurements were normalized with buffer (50 mM Tris-Cl, pH 8.5) as a blank.

4.2.6 Foam cell treatment and cholesterol enzymatic assay

Cells were treated with the protein conjugated following the methods described in Chapter 3. Briefly, foam cells were differentiated from RAW 264.7 macrophage cells upon treatment with oxidized low-density lipoprotein for 16h (Section: 3.2.6). Varying concentrations of protein conjugates were incubated at

different time-points as indicated in the respective experiments. Intracellular cholesterol accumulation was measured using cholesterol enzymatic assays (Section: 3.2.9) to observe for cholesterol reduction upon treatment.

4.2.7 Real-time PCR

To analyse the effect of ferritin on the mRNA expression of cholesterol efflux receptors in response to protein conjugate treatment, Real time-qPCR was performed. Foam cells were incubated with 200 nM 4F-AA for 24 h. The fold change in mRNA levels of efflux receptors (ABCA1, ABCG1 and SR-BI) were individually normalized with mRNA levels in untreated foam cells. The change in ABCA1 mRNA levels in foam cells was also compared with macrophage cells. Total RNA was isolated from the cell pellets using EZ-10 DNAaway RNA Miniprep Kit (Biobasic) following the instructions by the manufactures. RNA concentration was measured using NanoDrop Spectrophotometer (ND-1000) at $\lambda = 260\text{nm}$. 100ng equivalent RNA from each sample was used along with Luna® Universal One-Step RT-qPCR Kit (NEB) to perform the RT-qPCR reaction. The forward and reverse primers used for the various target genes are summarized in **Table 7-1**. Forward and reverse primers were obtained from IDT DNA. The reactions were performed with 20 μL reaction volume using STepOne plus Real time-PCR system (Applied Biosystems). The cycle threshold values (C_T) for the target genes were determined and normalized relative to β -Actin (Abcam). The fold change in mRNA expression was calculated following the $2^{-\Delta\Delta C_T}$ method.

4.3 Results

The mimetic peptide sequences (4F and 2X4F) were genetically fused to the N-terminus of the ferritin sequence in a pET28a vector and are displayed on the surface of the protein cage. A GGGS linker is introduced between the mimetic

peptide sequence and ferritin to allow for flexibility of the peptide on the cage surface and aid in receptor interaction by minimizing the potential steric hindrances caused as a result of rigid peptide fusion. Pairwise sequence alignment between the amino acid sequences (translated from nucleotide sequencing data) of synthesized plasmid and the design (template) using ClusterW2 software demonstrate successful mimetic peptide-ferritin gene fusion for both proteins (APPENDIX, **Figure 7-5**, APPENDIX, **Figure 7-6**).

4.3.1 **Buffer compatibility of the protein constructs**

The mimetic peptide sequences comprise of both charged and hydrophobic amino acid residues. Thus, a change in the overall solubility properties is expected upon conjugation of these peptide sequences to ferritin thereby needing an optimization of resuspension and purification buffers. To determine the optimal resuspension buffer for the protein constructs a buffer optimization study is conducted by resuspending cell pellet in different buffers, followed by cell lysis (by ultrasonication), and differential ultra-centrifugation which allows for separation of the cytoplasmic proteins from the cellular components. Centrifugal speeds of 1,000xg, 10,000xg and 100,000xg result in the separation of the protein from the insoluble fraction comprising of nucleus/mitochondria, endo/lysosomal compartments and membrane fractions at the respective speeds. Higher speeds allow for higher protein purity in the soluble fraction reducing the number of steps required for purification and indicative of higher protein compatibility with the resuspension buffers. Poor protein solubility in the buffer would result in a significant fraction of the protein being obtained in the insoluble fractions. The 4F-AA and 2X4F-AA are resuspended in three distinctly different buffers in terms of properties and centrifuged at the

above-mentioned speeds after cell lysis. SDS gel electrophoresis of the soluble (supernatant) and insoluble (pellet) fractions is carried out to visualize the presence of the desired proteins in the isolated fractions (**Figure 4-3**).

Both 4F and 2X4F-AA are found to be present in the insoluble fractions at lower speeds indicative of poor solubility in HEPES buffer. In contrast, resuspension in DI water and Tris buffers demonstrate that majority of the proteins remain in the soluble fractions post centrifugation at 100,000xg with similar solubility characteristics. HEPES is often the buffer of choice for native AA resuspension as also seen in Chapter 3.

Figure 4-3: SDS-PAGE analysis of solubility of 4F-AA and 2X4F-AA resuspended in 25mM HEPES, DI and 20mM Tris-Cl buffer conditions yielding supernatant (S) and pellet fractions (P) after differential centrifugation at 1,000xg (S1, P1), 10,000xg (S2, P2) and 100,000xg (S3, P3) speeds. The expected

location of the band has been indicated with a blue and red arrow for 4F-AA and 2X-4F AA respectively.

4.3.2 Optimization of multi-step purification strategies

Ferritin as seen in Chapter 3 and published studies can be purified with one-step hydrophobic interaction chromatography^[143]. However, the N-terminus fusion of the 4F peptides results in additional exposed hydrophobic sites thereby affecting the binding properties to the chromatography column. To maintain a one-step chromatography purification, the sequences have been incorporated in a pET28a plasmid vector as opposed to the conventional pET11a, to incorporate histidine tag to the engineered ferritin. The tag enables affinity-based chromatography purification of both protein constructs. HisTrap purification yields a ~ 95% pure high concentration of 4F-AA fraction eluted with the addition of 500 mM imidazole as seen from the chromatogram and corresponding SDS-PAGE image (**Figure 4-4A-C**). However, this method was not suitable for 2X4F-AA purification (data not shown). HisTrap purification involves coordination of the imidazole rings on the histidine tagged protein to the Ni²⁺ packed column. However, it is observed that this interaction is hampered with the dual 4F peptide (2X4F) fusion. This is likely because of the formation of additional secondary structures in 2X4F-AA affecting the interaction of the histidine imidazole rings with the Ni charged column. The increased number of positively charged residues (lysines) on the 2X4F peptide sequence as compared to the 4F peptide (also reflected in the less negative zeta potential value indicated earlier) could result in electrostatic repulsion between the protein and charged chromatography columns thereby resulting in poor binding.

As an alternative, other chromatography methods such as ion exchange chromatography and hydrophobic interaction chromatography have been attempted. Anion exchange chromatography (QFF) is the chosen method wherein the protein is obtained from the flow through fractions. Since desired protein is obtained in the flow through fractions, multi-step purification is thus required to obtain high purity proteins. Size exclusion chromatography (SEC) yielded protein fractions with high purity confirmed using SDS-PAGE (APPENDIX, **Figure 7-7**). Dual peaks are observed in a single fraction for 2X4F-AA indicating possible aggregations and housekeeping proteins of similar sizes. Mass spectrometry on the isolated SEC fractions were performed for both constructs (**Figure 4-4D**). Both chromatogram and mass spectrogram demonstrate a size difference between both the protein constructs. Peaks from mass spectrometry are obtained at 25,075 Da and 28,147 Da for 4F-AA and 2X4F-AA which matched the expected molecular weights for the respective proteins. Thus, indicating the successful genetic fusion of the mimetic peptide and its variant to ferritin and subsequent purification.

Figure 4-4: Chromatogram of A) 4F-AA and B) 2X4F-AA purified using HisTrap and QFF respectively. 4F-AA is eluted in the elution fractions whereas 2X4F-AA is obtained from the flow through fraction of IEX chromatography. C) SDS-PAGE analysis of 4F-AA (1) and 2X4F-AA (2) proteins purified using the above-mentioned methods. The bands correspond to the expected molecular weights of the respective proteins. D) Mass spectrometry analysis (MALDI-TOF) of 4F-AA and 2X4F-AA.

4.3.3 Secondary structures & self-assembly properties of ferritin remain intact after peptide fusion

To determine the effect of peptide fusion on protein self-assembly properties and cage characteristics, the engineered cages were characterized

using DLS, CD and TEM. The 4F-AA and 2X4F-AA apo proteins were assembled in the presence of 2400 Fe atoms.

DLS analysis showed significant variation in the hydrodynamic diameters of apo 4F-AA and 2X4F-AA resulting in peaks at 6.5 nm and 165 nm respectively (**Figure 4-5A**). The average particle size of unmodified AA ranges between 6 – 8 nm (**Figure 3-3A**). Self-assembly in the presence of iron resulted in particles with sizes close to 13.5 nm and 32.7 nm for 4F-AA and 2X4F-AA, respectively. The average particle size of self-assembled native cages is usually close to 12 nm as seen from Chapter 3. The increased particle sizes observed for the engineered construct thus corresponds to the extended peptides fused to the protein surface.

The significantly larger particle sizes of apo 2X4F-AA could be explained by the presence of high molecular weight housekeeping proteins or the presence of self-aggregated 2X4F-AA dimeric proteins. The two possibilities can be distinguished based on the particle sizes measured after protein assembly into cage structures. In the presence of housekeeping proteins, cage formation would result in no observable changes in the measured particle sizes in DLS since larger particle results in higher scattering intensity further affecting the derived volume based particle size distribution thus not resulting in visible peaks for lower particle sizes. Since this was not the case as seen from the particle sizes of the assembled structures, it is inferred that there is a tendency for apo 2X4F-AA to aggregate. However, the change in sizes after iron addition is indicative of the higher thermodynamic stability and interaction of the iron induced self-assembly in comparison to protein-protein aggregation.

In order to determine the effects of peptide fusion on the secondary structure of the proteins, circular dichroism spectra are obtained (**Figure 4-5B**). The signature α -helix troughs are expected at $\lambda = 208$ nm and 220 nm which were observed for native AA and the engineered constructs. HDL mimetic peptides are expected to adopt an α -helix dominant secondary structure as simulated using Robetta software (APPENDIX, **Figure 7-8**). Apo proteins prior to iron loading are predicted to exist in a dimeric and trimeric state for 4F-AA and 2X4F-AA, respectively. Since ferritin also has a ~60% dominant α -helix morphology, no significant changes in location of the troughs were observed for the constructs. Slight variations in the magnitude of molar ellipticity at the indicated wavelengths could be associated with poor signal to noise ratio (despite subtraction of the buffer background) due to protein aggregations altering the interaction with the polarized light. Overall, the secondary structure is observed to be preserved after peptide fusion. Visible cage structures on transmission electron micrographs indicate that the self-assembly characteristic of ferritin remains intact despite the peptide modification (**Figure 4-5C,D**). However, it is noted from the electron micrographs, chromatography and DLS results, there is a significant aggregation tendency observed for 2x-4FAA.

The addition of mimetic peptides was further attempted on human ferritin proteins. Conjugation of the mimetic peptides to human ferritin however did not yield a soluble protein construct despite resuspension in a of buffers with differing polarity, salt/sugar concentrations, pH and denaturing properties (APPENDIX, **Figure 7-9**). Majority of the proteins, both 4F-HsFtn and 2X4F-HsFtn, were obtained in the pellet (insoluble fraction). The proteins were soluble only under total protein unfolded conditions in the presence of 8M urea indicating

the effect of peptide fusion on protein folding during the overexpression step. Protein unfolding and refolding often results in greater than 80% loss in protein yield thereby not being suitable for robust applications.

Figure 4-5: DLS, CD, and TEM characterization of HDL mimetic peptide fused ferritin. A) Hydrodynamic diameter of Apo 4F-AA, (Fe2400)4F-AA, Apo 2X4F-AA and (Fe2400)2X4F-AA measured using dynamic light scattering. B) Circular dichroism measurements of 2400 Fe loaded AA, 4F-AA, 2X4F-AA. Transmission electron micrographs of C) (Fe2400)4F-AA and D) (Fe2400)2X4F-AA stained with 1.5% uranyl acetate.

4.3.4 Treatment with engineered proteins show intracellular cholesterol reduction in foam cells within 24h

To confirm the cholesterol reduction efficacy foam cells were treated with 4F-AA and 2X4F-AA cages according to protocols mentioned in Chapter 3. Briefly, RAW264.7 macrophages were loaded with low density lipoproteins in serum free media to induce foam cell formation. The cholesterol loaded media was replaced with 200 nM of either 4F-AA or 2X4F-AA and the treatment was continued for 4 h and 24 h timepoints. Post-treatment intracellular cholesterol was extracted using a hexane/isopropanol solvent extraction method and quantified using Amplex Red enzymatic assay. The intracellular cholesterol levels measured were normalized with the total cell protein content. These levels are compared with the cholesterol levels in untreated cells to obtain % intracellular accumulated cholesterol.

No visible reduction in cholesterol levels is observed after 4 h of treatment with the protein constructs with cholesterol accumulation percentage measured at $116 \pm 55\%$ and $117 \pm 62\%$ in cells treated with 4F-AA and 2X4F-AA, respectively. The cholesterol efflux and influx process in the cells is understood to be a transient in nature factoring in the cells' tendency to ensure homeostasis. Since the time was insufficient to observe the sequestration effects of the cages, the transient effects could have resulted in the variation between experiments hence resulting in a large SD. The % intracellular accumulated cholesterol measured were $69 \pm 15\%$ and $90 \pm 32\%$ post treatment with 4F-AA and 2X4F-AA, respectively (**Figure 4-6A**). Significantly higher reduction is observed with 4F-AA treatment in comparison with 2X4F-AA despite the lower

number of mimetic residues. These differences could be potentially due to the lower stability of the 2X4F-AA protein constructs as seen from the size exclusion chromatograms and visible aggregations in the transmission electron micrographs. The aggregations could affect the receptor interaction properties thereby resulting in lower cholesterol reduction efficacy. A concentration dependent study with 4F-AA further showed similar cholesterol reduction capacity of ~ 65 – 70% could be achieved with 100 nM proteins within the 24 h incubation (**Figure 4-6B**).

mRNA expression levels of cholesterol efflux receptors (ABCA1, ABCG1 and SR-BI) were measured in foam cells treated with 200 nM 4F-AA for 24 h. With respect to untreated macrophage cells, a 2-fold increase in ABCA1 expression is observed likely in response to maintaining intracellular cholesterol homeostasis (**Figure 4-7A**). Greater than 5-fold increase in mRNA levels are observed in foam cells treated with the protein conjugates normalized with respect to untreated foam cells (**Figure 4-7B**). A similar increase in expression levels, though to a lesser magnitude, is observed for ABCG1 and SRB-BI mRNA levels with treatment. Interestingly, AfFtnAA without the peptide modification also demonstrated an upregulation in ABCA1 efflux receptor implying the possible role of ferritin in cholesterol efflux receptor regulation (APPENDIX, **Figure 7-10**).

Figure 4-6: A) Intracellular accumulated cholesterol in foam cell treated with 4F-AA and 2X4F-AA for a duration of 4h and 24h. B) Concentration dependent cholesterol reduction in foam cells treated with 200 nM AffFnAA (no peptide), Untreated (0nM), 50nM, 100nM and 200nM 4F-AA. The % intracellular cholesterol levels are represented relative to untreated foam cells (at 100%). (n = 2, where n is the least number of independent experiments performed in triplicates). Statistical analysis has been performed using one-way ANOVA test. * P < 0.05.

Figure 4-7: Fold change mRNA expression levels. A) Relative expression of ABCA1 in foam cells normalized to untreated macrophage cells. Statistical significance has been indicated with respect to macrophages estimated using one-way ANOVA test. (n = 3, where n is the least number of independent experiments performed in triplicates) B) Relative fold change of mRNA expression levels of

ABCA1, ABCG1 and SR-BI in foam cells treated with 200 nM 4F-AA normalized to untreated foam cells. Statistical significance has been represented with respect to untreated foam cells for the respective genes estimated using one-way ANOVA test. ($n = 2$, where n is the least number of independent experiments performed in triplicates). * $P < 0.05$, ** $P < 0.01$.

4.4 Discussion

HDL therapy and associated HDL mimetic peptides have emerged as potential candidates to mediate cholesterol reduction in atherosclerotic lesion sites thereby aiming to reduce the overall plaque burden. To overcome inherent challenges of synthetic peptide production, the use of ferritin as a form of delivery vehicle for these peptide sequences has been proposed. In this work, successful fusion 4F HDL mimetic peptides and its variant to the external surface of ferritin by N-terminal genetic fusion methods is carried out. The overall self-assembly properties remained intact after peptide fusion with some alterations to its solubility properties. Cholesterol reduction efficacy was further demonstrated *in vitro* wherein concentration and time dependency of treatment was observed.

With the surface display of either peptides of 25 residues (4F) or 50 residues (2X4F) with a GGGS linker, ferritin retained its secondary and tertiary structures as seen from light scattering, circular dichroism, and transmission electron micrographs. However, the buffer compatibility and purification optimization studies suggest that there is an alteration to the protein surface properties including a shift towards a more positively charged surface of the engineered proteins relative to unmodified ferritin. Variation chromatography

column binding was further observed between 4F-AA and 2X4F-AA highlighting the role of peptide amino acid residues in determining the overall charge and surface properties of the fused protein constructs. The alternation of properties is expected to be the result of additional charged residues (lysine, glutamic acid and aspartic acid) resulting in higher sensitivity to the use conventional cationic buffers such as HEPES.

The difference in buffer compatibility can be explained by the distinctively differing properties of HEPES and Tris. HEPES is an organic chemical with a relatively lower pKa of 7.45 than Tris (8.2). Thus, at pH of 7.5 HEPES buffer tends to have higher cationic properties in comparison to Tris. The cationic nature would thus result in electrostatic induced interactions between buffer salts and the fused mimetic peptides comprising of several positively charged residues resulting in an overall poor buffer compatibility. The effect of the progressively increased positively charged residues in the respective protein conjugates were reflected in the zeta potential values of AA, 4F-AA and 2X-4F-AA which were measured to be -10.5 mV, -4.1 mV and -1.1 mV, respectively. Tris-Cl is therefore chosen as the resuspension buffer owing to higher protein stability and broad range buffering capacity required for subsequent cage formation and *in vitro* experiments. Higher concentration of 50mM is used to adjust for any changes in pH during the iron loading step. Additional incorporation of hydrophobic residues (alanine, valine, and phenylalanine) would further contribute to reduced aqueous solubility and increase the possibility of aggregation as seen from the broader particle size distribution of 2X4F-AA.

Similar to ferritin protein used in this study, protein cages have been demonstrated as an effective platform for the display of therapeutic diagnostic

peptides/proteins^[144, 145]. In this chapter, the peptide fusion strategy was also attempted with *Homo sapiens* derived ferritin. Human ferritin, like the archaeal ferritin are 12 – 15 nm sized protein cages self-assembled from 24 polypeptides. Approximately 60% homology exists between the two ferritin proteins. However, significant differences exist in hydrophilicity and polarity of the iron entry and exit channels^[146]. Human ferritin has been shown to have a significantly higher cellular internalization rate thereby having higher therapeutic potential^[147]. Furthermore, minimal immunological responses are expected since these proteins are naturally present in the body. The conjugation of the mimetic peptides to human ferritin, however, did not yield stable/soluble protein constructs. Therefore, despite sequence homology and structural similarities, archaeal ferritin presents itself as a more promising candidate for the display of fusion peptide moieties in comparison to human ferritin.

In vitro foam cell models to demonstrate the cholesterol sequestration efficacy of the fused protein constructs has been used in this chapter. This study showed a ~30% reduction in cholesterol levels in foam cells treated with 4F-AA for a 24h time duration. A concentration of 100 nM or higher of the engineered proteins is determined to be optimal to achieve the desired cholesterol reduction. No significant cholesterol reduction was measured with 2X4F-AA treatment. The difference in treatment efficacy could be explained by the poor stability of the proteins and protein folding affecting receptor interaction.

The cholesterol reduction achieved in this work is in line with cholesterol efflux measured in previous studies conducted in murine macrophage cells induced with high ABCA1 receptors. Lu et al. have shown cholesterol efflux using a “peptibody” generated by the fusion of 4F peptide to mouse IgG antibody

[148]. A maximum of 10% cholesterol efflux was observed within a 4 h time duration after treatment with 1 μ M of peptibody. Size exclusion chromatograms of the medium after cholesterol efflux demonstrated the formation of HDL-like particles generated by the adsorption of cholesterol on the peptibody. Therefore, in this chapter, in addition to validating the cholesterol efflux properties of HDL mimetic peptides as previously shown in literature, the efficacy of cholesterol reduction in foam cells (resembling diseased cells present in atherosclerotic lesions) has been demonstrated. Thus, emphasizing the potential of using these protein constructs as an atherosclerotic therapeutic molecule. mRNA expression studies revealed an upregulation of cholesterol efflux receptors in foam cells after treatment with the peptide conjugates. ABCA1 gene upregulation is further observed with ferritin treatment independent of the mimetic peptide modification, thus indicating that the ferritin-cell interaction is likely contributing to the upregulation. However, from Chapter 3 (**Figure 3-5**), it is understood that ferritin without modification does not result in an overall intracellular cholesterol reduction. Thus, indicating the role of 4F peptide in acting as a cholesterol sink resulting in an overall reduction in intracellular cholesterol. However, more work is required to verify the efflux pathway and the role of the peptide modification mRNA expression analysis can be performed with synthetic HDL mimetics and compared to the molecular responses observed after treatment with the protein conjugates in this study.

4.5 Conclusions

In Chapter 3, a physical method of cholesterol sequestration following small molecule interaction with the lipids in the cell membrane with subsequent intracellular cholesterol reduction is employed. In this chapter, the use of HDL

mimetic peptides for intracellular cholesterol reduction from foam cell models is reported. The genetic fusion of the peptides was successfully achieved with minimal effects on cage structure integrity. Intracellular cholesterol reduction was observed within a 24h period in a treatment concentration dependent manner. 4F-AA conjugates demonstrated higher intracellular cholesterol reduction in comparison to 2X4F-AA. This is likely associated with lower protein stability and protein-protein aggregations in the larger protein conjugates. Furthermore, an upregulation of cholesterol efflux receptors especially ABCA1 receptor were observed in foam cells after treatment with the protein conjugates indicating the potential role in efflux receptor interaction in intracellular cholesterol reduction. The study thus demonstrates an alternative mechanism of mediating cholesterol reduction from foam cells by using genetic engineering of the cage surface. Thus, placing further emphasize on the use of ferritin as a therapeutic delivery vehicle in atherosclerosis with the potential to mediate cholesterol reduction following multiple pathways.

Chapter 5 : Cellular Fate and Responses of Ferritin Nanocages in Murine Macrophages

Abstract:

Macrophages act as the first line of defence in the human body upon introduction of foreign antigens, including nanoparticles in a biological environment. As suggested in the previous chapters, macrophages differentiation to foam cells are a key component of atherosclerosis progression. However, understandings of the cellular fate and trafficking of organic NPs such as ferritin in macrophages as well as the triggered molecular responses are limited. In this work, in-depth study on the intracellular fate and immune relevant responses of a model organic NP, ferritin, in murine macrophage cell lines is performed. Concentration dependent internalization mechanism and quantify the ferritin NPs subcellular localization in various organelles is demonstrated. Iron (present in ferritin core) export is observed to occur over extended period along with the activation of mRNA expression of iron relevant proteins upon exposure. A study to modulate the intracellular location of the NPs by incorporating endosomal escape characteristics and its molecular effects using transcriptional analysis were conducted. The in-depth analyses of cellular uptake and responses to a versatile protein NP such as ferritin would provide basic principles for the design and engineering of other protein NPs with similar properties for future biomedical applications.

5.1 Introduction

Nanoparticles (NPs) have been recognized for their use as therapeutic agents, imaging agents, and as part of scaffolds for biomedical applications^[149]. Macrophages constitute the first line of defense in response to the introduction of

foreign antigens in a biological environment such as the human body. The activation of these innate immune cells results in the uptake of NPs and subsequent clearance or inflammatory responses ^[150-152]. Macrophages are being actively studied as targets for the development of therapeutic NPs for the regression of tumours and atherosclerosis due to their activation in the lesion sites ^[153, 154]. Thus, an understanding of the uptake pathways of the NPs in macrophages, their intracellular trafficking and the resulting cellular responses remains a key area of research. Understanding the mechanisms would aid in improved tuning of physiochemical properties of the NPs to achieve the desired bioavailability, ensure safety and fulfil the intended therapeutic or diagnostic properties with greater efficacy.

Internalization pathways of synthetic nanoparticles like super paramagnetic iron-oxide nanoparticles (SPIONs) and gold nanoparticles have been extensively studied in macrophages and similar immune cell lines^[155]. It has been shown that SPIONs are internalized by time-dependent vesicle-bound pathways resulting in the formation of multivesicular bodies in the cytoplasm. It is accepted that some of the internalized SPIONs undergo lysosomal degradation while others are released in the cytoplasmic compartments for further metabolism. Furthermore, studies have demonstrated that size and shape of nanoparticles affect the rate of uptake of gold nanoparticles in HeLa cells ^[156, 157]. Naturally derived NPs, such as protein NPs, lipoproteins and viral capsids are being actively developed as drug carriers in cancer and atherosclerosis wherein the leaky vasculature allows for the permeation of the NPs to the sites of interest ^[158-160]. Organic NPs degrade *in vivo* in the presence of proteases and lysosomal enzymes thus overcoming the challenge of nephrotoxicity and hepatotoxicity

associated with nanoparticle accumulation. The uniform size distribution and facile production methods presents organic NPs, especially ferritin, as an attractive molecule for applications in nanomedicine ^[161]. Like inorganic NPs, organic NPs are taken up by the reticuloendothelial system wherein macrophages play the key role in engulfing and breaking down the injected nanoparticles. However, quantitative studies on the cellular fate of organic nanoparticles have been largely limited due to challenges with detection of the particles *in vitro*.

This work addresses this gap by using a quantitative method to investigate the mechanisms of uptake and trafficking of organic NPs, specifically ferritin protein cages. Ferritin in nature exists as an iron storage protein that stores insoluble ferric (Fe^{3+}) state iron in a soluble and bioavailable state in the internal core of its cage-like structure. The encapsulation of iron in the core of the protein shell prevents exposure of the ferric iron to reactive oxygen species thereby minimizing iron-induced oxidative damage. The protein comprises of 24 polypeptides that self-assemble to form a ~12 nm cage structure in the presence of iron or high salt concentrations ^[114, 116]. In the past years, due to their inherent and tuneable iron storage properties ferritin protein nanocages have been actively studied for its use as a nanocarrier for iron-based contrast agents. Redox sites in the inner cage surface serves as the platform for iron core formation with super paramagnetic properties with comparable T1 and T2 relaxivities to SPIONs ^[123, 143]. There have been several studies demonstrating the passive localization property of ferritin to lesion sites such as in the case of tumour and atherosclerotic plaques ^[16, 162]. Ferritin entry into cells has been shown to be mediated by transferrin receptor (Tfr1) and scavenger receptors (Scara5) resulting in its localization in the endosomal and lysosomal compartments ^[134, 163]. However,

questions remain regarding the fate of ferritin after internalization in cells ^[161]. It is commonly accepted that the protein shell undergoes lysosomal degradation in the presence of proteases and low pH conditions. The iron core, however, is expected to undergo minimal degradation under these conditions. To the author's knowledge, there are no studies elucidating the cellular fate of these iron nanoparticles encapsulated within the cage after internalization. Furthermore, in diseases such as atherosclerosis, uptake of nanoparticles by vascular macrophages has been shown to facilitate the localization of nanoparticles to the lesion site ^[16].

In the present studies, *Archaeoglobus fulgidus* derived ferritin with two amino acid modifications (K150A/R151A; AfFtnAA) is used. The subunits are assembled using 2400 Fe atoms ((Fe2400)AA). AfFtnAA is a closed pore mutant with tuneable self-assembly and facile surface modification properties. The thermophilic source of the protein imparts high thermostability and is reflected in the protein stability post modifications. The Fe atoms located in the core of the ferritin are used as quantification markers (measured using intercoupled plasma mass spectrometry) to monitor the cellular trafficking of the protein NPs and its subsequent degradation. This chapter show concentration-dependent ferritin internalization in RAW264.7 murine macrophage cell line. Dominant lysosomal and cytoplasmic localization of iron followed by export from cells in a time-dependent manner is observed. It is further shown that internalization of ferritin affects the iron homeostasis in the cells at a transcriptional level with observed changes in iron relevant proteins and endogenous ferritin levels. Further attempts have been made in this chapter to modify the cages to facilitate rearrangement in its subcellular localization to further understand the impact of the localization

region on the cellular iron homeostasis mechanisms. Lastly, the assessment of immune relevant gene expression and oxidative stress in cells generated as a response to ferritin treatment has been conducted and suggestions for ways to counter the same have been discussed.

5.2 Materials & Methods

5.2.1 AfFtnAA protein production

The protein was overexpressed and purified according to methods previously described in literature and in Chapter 3 (Section: 3.2.1)^[114]. Briefly, AfFtnAA plasmid gene transformed in BL21 (DE3) C+RIL *Escherichia coli* was induced with 1mM IPTG and grown for 4h for protein production. The cells were harvested and lysed by sonication (Sonics Ultrasonic Processor VCX500). The lysates were heat treated at 85°C for 10 minutes and ultra-centrifuged (Beckman Coulter Optima L-100XP) at 40,000 rpm for 1hr. Hydrophobic interaction chromatography (HiTrap Phenyl FF (High Sub) , GE) using AKTA Pure (GE) was carried out on the supernatant fraction to obtain pure proteins. 2400 Fe atoms were used to mediate self-assembly of Apo AfFtnAA to form the cage structures. Excess iron was removed using a PD-10 desalting column (GE).

5.2.2 Cell culture

RAW264.7 murine macrophages (ATCC) were grown in DMEM (Life technologies) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco).

5.2.3 Confocal Imaging

AfFtnAA is labelled with fluorescein isothiocyanate (FITC, Sigma) in a 100 molar excess reaction (1h reaction at RT with constant shaking) and dialysed with

25mM HEPES (First Base), 50mM NaCl (Sigma) pH 7.4 to remove unreacted dye. AfFtnAA is incubated with the cells mounted on a slide for 24h. Lysosomes are labelled with LysoTracker™ Red DND-99 (Life technologies) and the nucleus with 4',6-diamidino-2-phenylindole (DAPI, Life Technologies). Confocal Microscope LSM800 (Carl Zeiss) is used to image the mounted samples with a 100x objective at excitation $\lambda = 408\text{nm}$, 488nm and 594nm corresponding to DAPI, FITC and LysoTracker™ respectively.

5.2.4 Endocytosis pathway

Cells are pre-treated with 15 μM Chlorampzine hydrochloride, 385 μM Genistein and 165 μM Amiloride hydrochloride hydrate as clathrin-, caveolae-, and micropinocytosis pathway inhibitors respectively (prepared in cell culture media) for 1 h under typical cell culture conditions. The media is then aspirated, cells are washed with PBS and treated with the indicated concentration of AfFtnAA for 2 h. Amount of internalization of AfFtnAA is quantified using ICP-MS (following protocol mentioned in Chapter 3: Section 3.2.8). Briefly, ICP-MS measurements were taken using PerkinElmer, Model: Elan DRCe system. Fe concentrations were determined against a calibrated standard curve between 1ppb to 100ppb. The amount of Fe detected in the cells is normalized to cells treated with AfFtnAA (without pre-treatment with inhibitors) to obtain % of uptake inhibition for each of the conditions.

5.2.5 Subcellular fractionation

Density-based centrifugation was performed to isolate the cellular organelles following the protocols described previously ^[164, 165]. Briefly, differential centrifugation at 1000xg (5 minutes), 10,000xg (10 minutes) and 100,000xg (1h) was performed to isolate debris, lysosomes/endosomes and cell membrane in the

pellet fraction respectively. 250mM sucrose, 20mM HEPES, 10mM KCl, 1.5mM MgCl₂, 1mM EDTA, 1mM DTT and 1mM protease inhibitor cocktail is used as the lysis buffer. The supernatant from the final centrifugation is collected as the cytoplasmic fraction. Lysosomes are further isolated from the lysosome/endosome mixture by centrifugation in the presence of Triton X-100 and 1mM DTT followed by centrifugation at 15,000xg for 15 minutes. Pellets and supernatant fractions were digested with 3% v/v HNO₃ in MiliQ water to determine iron concentration using Inductively coupled plasma mass spectrometry (ICP-MS) following the protocol stated in section 5.2.4. Total cell pellets and amounts of iron in the culture medium were also prepared for ICP-MS with this procedure.

5.2.6 Transmission electron microscopy

After treatment, the cells were washed 3 times with PBS and collected using trypsin. The cell pellets were fixed overnight with a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde in PBS. After fixation, the pellets were washed with PBS 3 times prior to post-fixing using 2% Osmium tetroxide (OsO₄, Sigma Aldrich) at room temperature for 2 hours. They were further washed with DI water to fully remove the fixative. Dehydration of the pellets was done with an ascending series of ethanol (25%, 50%, 75%, 95% and 100%) and pure acetone, for 40 minutes each at room temperature. Infiltration with resin (Araldite 502 kit, Ted Pella) was started at room temperature with the mixture of acetone and resin at the ratio 1:1, 1:3 and 1:6 for 2 hours, 1 hour and overnight respectively. The next day, cell pellets were transferred to the fresh resin at room temperature and incubated for 1 hour. They were then transferred again to another fresh resin and incubated for 1 hour at 40°C. The same process was repeated at

45°C and 50°C, using fresh resin each time. The cell pellets were finally embedded into polyurethane mold incubated at 60°C for at least 48 hours. The cell pellet embedded capsules were sectioned at thickness 70 to 100 nm through ultra-microtome using a diamond knife and collected on copper grids (EMS). The sections on copper grids were stained with 2% uranyl acetate and lead citrate for 8 minutes each at room temperature. The sections were viewed using Carl Zeiss Libra with an accelerating voltage of 120 kV.

5.2.7 *In vitro* simulation of lysosomal degradation of AfFtnAA

Lysosomal degradation of AfFtnAA was simulated and measured using a iron release kinetics assay. The degradation was simulated at low pH conditions (25mM HEPES pH 7.4, 25mM HEPES 4.5), reducing conditions and in the presence of lysosomal enzyme (1-unit Cathepsin-B). The rate of iron release with the degradation of the core was studied by the reaction of ferrozine and the reduced Fe^{2+} (formed after core degradation) resulting in a complex formation with absorbance peak at $\lambda = 562\text{nm}$. 400nM of (Fe2400) AfFtnAA was incubated in the different buffers in the presence of 1mM ferrozine (Sigma). Iron release was monitored for 1h in the presence of reducing agents (20mM Sodium acetate (NaAC) (Sigma), 5mM Dithiothreitol (DTT) (Sigma), 5mM Ethylenediamine tetraacetic acid (EDTA) (Sigma) pH 4.5) and for 14h in the presence of protease at different pH conditions (25mM HEPES 1unit Cathepsin B pH 7.4/pH 4.5), . Degraded iron cores and protein shell structures were further examined using transmission electron microscopy with and without uranyl acetate staining.

5.2.8 Real-time PCR

To analyse the effect of ferritin on the mRNA expression of various proteins involved in iron metabolism, Real time-qPCR was performed. Cells were

incubated with 200 nM AfFtnAA for 12h, 24h and 48h. Untreated cells were used as the negative control. Total RNA was isolated from the cell pellets using EZ-10 DNAaway RNA Miniprep Kit (Biobasic) following the instructions by the manufactures. RNA concentration was measured using NanoDrop Spectrophotometer (ND-1000) at $\lambda = 260\text{nm}$. 100ng equivalent RNA from each sample was used along with Luna® Universal One-Step RT-qPCR Kit (NEB) to perform the RT-qPCR reaction. The forward and reverse primers used for the various target genes are summarized in **Table 7-1**. The reactions were performed with 20 μL reaction volume using SStepOne plus Real time-PCR system (Applied Biosystems). The cycle threshold values (C_T) for the target genes were determined and normalized relative to β -Actin (Abcam). The fold change in mRNA expression was calculated following the $2^{-\Delta\Delta C_T}$ method.

5.2.9 Expression and cloning of poly-histidine modified AfFtnAA

AfFtnAA plasmid was genetically modified with the fusion of (HHE)₉ at the C and the N-terminus to construct the two plasmids. GGGS is inserted as a flexible linker between the native protein and the additional fragment. The plasmids are transformed in BL21 C+RIL *Escherichia.Coli.* strain. Proteins are produced following the same protocols as the native protein with slight variations. Protein containing supernatants after cell lysis were heat treated at 75°C for 10minutes before ultracentrifugation. HisTrap chromatography (HP) was performed using AKTA Pure fast protein liquid chromatography system (GE) to purify the proteins. Proteins were eluted with 25 mM HEPES, 50 mM NaCl, 500 mM Imidazole at pH 7.5 and buffer exchanged to remove the excess imidazole. Self-assembly for cage formation is mediated using 1200Fe atoms. Lesser (compared to 2400Fe for previous studies in this chapter) Fe atoms are used to ensure

stability of the assembled conjugates. Control AfFtnAA samples for the endosomal escape experiments are also self-assembled in the presence of 1200Fe atoms for consistency.

5.2.10 Reactive Oxygen Species Measurement

After treatment with ferritin under different conditions, the cells were washed 3 times with PBS. This was followed with incubation with Dihydroethidium Hydroethidine (DHE) (Life Technologies) diluted in cell culture media to a 5 μ M working concentration for 1 hr at 37°C. 10mM stock concentration of DHE was prepared in DMSO solvent. Excess dye was washed with PBS (3 times). Mean fluorescence was measured in the scraped cell samples using BD Fortessa X20 at Ex 518/Em 606 nm.

5.2.11 Animal Details

Approval for animal model was obtained from the Institute Animal Care and Use Committee (IACUC) at Nanyang Technological University (NTU) and all procedures were performed in accordance with the protocols and guidelines for the Use of Animals. All 5 ApoE^{-/-} mice used for the IVIS and flow cytometry were more than one year old. The first mice harvested 1hr after injection was imaged under IVIS and the rest of the mice were used for flow cytometry.

5.2.12 *In vivo* biodistribution (IVIS) of aorta, blood and urine

ApoE^{-/-} mice were administered a single bolus dose of Cy5 labelled ferritin through a lateral tail vein at dose of 18mg/kg. After 1hr from the injection, the mouse was euthanized and perfused with 20 mL of PBS/mouse through cardiac puncture, and aorta was excised. Blood and urine of the injected mice were also sampled 1hr after injection. The Liquid (Blood and Urine) samples and aorta were imaged using *ex vivo* near-infrared fluorescence (NIRF) imaging system.

5.2.13 Flow cytometry of digested aortas

Aortas extracted from 5 mice were digested with 1mg/ml collagenase IV in hank's balanced salt solution (HBSS) at 37 °C for 60 min in room temperature and filtered through a 160 µm nylon mesh to obtain cell suspension. The cells were washed with FACS buffer solution before being suspended and incubated with a mixture of macrophage specific antibody cocktails for 30 min at 4 °C. A cocktail of antibodies (BioLegend) include CD45 (clone 30-F11), CD11b (clone M1/70), CD64 (clone X54-5/7.1), F4/80 (clone BM8), MerTK (R&D Systems) and CD31 (clone ER-MP12) (Bio-Rad) which are tagged with non-overlapping fluorescent particles at different wavelengths. Fluorescence was detected using a flow cytometer (BD Biosciences LSRFortessa X-20), and the data were analysed using BDFacsDiva and FlowJo software. Macrophages from aortas were identified as Trypan Blue- CD45+ CD11b+ MerTK+ CD64+ F4/80+. Population identified as CD31+ was used to identify the group of cells where the Cy5 fluorescence was present excluding the Cy5 fluorescence presence in the population identified as macrophages.

5.3 Results

5.3.1 *In vitro* demonstration of ferritin internalization in macrophages

In order to visualize the internalization of ferritin in macrophages, confocal imaging was carried out using conventional lysotrackers and FITC conjugated AfFtnAA (**Figure 5-1**). The images reveal localization of ferritin in the endosomal/lysosomal compartments given the overlapping regions of red and green fluorescence resulting in an overall yellow fluorescence appearance. However, unspecific staining around the cellular compartments results in difficulties to distinguish these individual organelles. Treatment of the cells with

ferritin for 1 h at 4°C results in localization of the cages on the membrane along with some internalized particles (APPENDIX, **Figure 7-11**). The binding of ferritin to the cell membrane under stunted uptake conditions is indicative of internalization pathways being mediated by membrane interactions/binding. To further discern the NPs in cellular compartments, ICP-MS is used to study the internalization mechanism by monitoring the amount of Fe present in these organelles that were individually isolated from the whole cell lysate.

Figure 5-1: Confocal imaging of 200 nM FITC (Green) conjugated AfFtnAA uptake in RAW264.7 cells after 24 h incubation. Cells stained with DAPI (Blue) and LysoTracker (Red) to visualize nuclei and lysosomal compartments, respectively.

5.3.2 Ferritin internalization involves more than a single dominant pathway

Human and horse spleen source ferritin has previously been shown to undergo transferrin mediated endocytosis in lymphocytes, reticulocytes, and macrophages ^[134]. Furthermore, competitive inhibition in AfFtnAA uptake in the presence of transferrin, a natural ligand to transferrin receptors in Chapter 3 ^[166]. To determine the concentration dependency of the dominant endocytosis pathway, cells were incubated with 2 nM and 200 nM AfFtnAA in the presence of inhibitors for cavolae-, clathrin- mediated endocytosis and micropinocytosis for 2h under typical cell culture conditions. The percentage of cellular uptake was normalized to AfFtnAA uptake in the absence of the inhibitors (**Figure 5-2A**). For the lower concentration of ferritin, the highest uptake inhibition of 29.4% in the micropinocytosis pathway and the least inhibition of 8.2% was measured in the clathrin-endocytosis pathway. In contrast, at higher ferritin concentration, inhibition of uptake was observed using all three inhibitors to similar extents of ~22%. Thus, at both concentrations the internalization of ferritin appeared to be mediated by more than one pathway. However, a concentration of about 200 nM is required for clathrin coated pits to play a role in mediating uptake along with the cavolae pits and macropinosomes.

Figure 5-2: Endocytosis pathway and localization. A) Endocytosis pathway determined in cells incubated with 200 nM AfFtnAA for 2h in the presence of inhibitors to test for clathrin-, cavolae- mediated endocytosis and macropinocytosis. B) Amount of Fe accumulated per cell and C) Total amount of Fe per well in cells treated with 200 nM AfFtnAA for 12h, 24h and 48h. Untreated cells correspond to the absence of treatment with 200 nM AfFtnAA (n = 3, where n the least number of independent experiments measured in triplicates). D) Percentage of Fe distribution measured in endosomes, lysosomes, mitochondria, and cytoplasm in cells incubated with 200 nM AfFtnAA for 12h, 24h and 48h (n = 2, where n the least number of independent experiments measured in triplicates). The percentage localization is calculated based on the total Fe amount detected per cell. Statistical analysis has been performed using one-way ANOVA test. (*p<0.05, **p<0.01, ***p<0.005).

The overall AfFtnAA uptake in cells was further monitored by treatment over extended periods of time. A steady rise was observed in the Fe accumulation

per cell over 24h in comparison to untreated cells (**Figure 5-2B**). A significant drop was seen at 48h suggesting the rate of mitosis exceeded the rate of uptake of AfFtnAA. The rising cell numbers of time has been shown in APPENDIX, **Figure 7-12**. Additionally, a decrease in total Fe levels in cells treated with ferritin for 48h was observed (**Figure 5-2C**). Thus, indicating the possibility of iron export occurring with increased exposure times in response to maintaining iron homeostasis.

Similar to the modulation of subcellular localization, Turris et al. have previously shown the overall internalization amounts can be improved in HeLa cells by introduction of residues analogous to heavy chain human ferritin to the exposed 12 amino acid loops on the native *Af* ferritin (AfFtnWT) sequence. A similar 4-fold increase in ferritin internalization using this construct (HuAftn) in RAW264.7 after 24h of treatment when compared to the amount of internalization of AfFtnWT and AfFtnAA has been observed (APPENDIX, **Figure 7-13**).

To understand iron trafficking in the cellular compartments after ferritin internalization, density ultracentrifugation to isolate endosomes, lysosomes, cell membrane, and cytoplasm is carried out (**Figure 5-2D**). The percentage distribution is calculated with respect to the total Fe measured from these organelles combined. After 24h of treatment, 43% of the Fe was present in the endosomal vesicles followed by 27% in the cytoplasmic region. Within 24h minimal changes in localization was observed across the organelles. After 48h, a significant increase in the endosomal iron to 57% and a decrease in the cell membrane accumulation was obtained. Membrane interaction/binding has been previously identified as the initial step prior to internalization ^[149, 167]. Lowered Fe

localization is observed in the membrane preparation at 48h. This can be explained by potential intracellular Fe saturation resulting in lowered membrane interaction of ferritin/Fe with the cells to reduce further Fe internalization. Furthermore, an increase in endosomal Fe levels is suggestive of formation of endosomal sized vesicles to mediate iron export.

5.3.3 Ferritin visualized in early to late endosomal vesicles and undergoes degradation in lysosomal environment

Localization of ferritin NPs in cellular compartments were further visualized using transmission electron microscopy (TEM). Cells treated with ferritin NPs for 24h were compared to untreated cells (**Figure 5-3**). Electron-dense black particles corresponding to the iron cores of ferritin NPs were observed in the cytoplasm but were absent in the untreated cells. However, there were no such particles observed in the endosomal, lysosomal or other cytoplasmic compartments although the presence of iron was detected in these organelles from previous ICP-MS measurements. This is likely due to the degradation of the protein and iron core structures in the lysosomal compartments resulting in a pool of free Fe ions which cannot be seen under the electron microscope.

To further study the lysosomal degradation mechanism of ferritin, *in vitro* simulations of the lysosomal environment was carried out. The fusion of endosomes with the lysosomal compartments typically entails a decrease in pH, presence of lysosomal enzymes and reducing conditions ^[168, 169]. Since the endo-lysosomal fusion is often a dynamic process the above three variables are studied independently to understand the ferritin degradation pathway. The structural integrity of the cage structure was visualized using transmission electron

micrographs under the different buffer conditions (**Figure 5-4A, 4B**). Stained and unstained images are used to visualize the intactness of the protein shell and iron core, respectively. As seen from the electron micrographs, in a reducing environment the protein shell tends to remain intact despite the dissolution of the iron core. The dissolution rate of the iron core was further studied by measuring the iron release kinetics. As the iron core (in Fe^{3+} form) was reduced to the Fe^{2+} state and dissolved, the released free ions formed a complex with ferrozine. The fluorescence signal resulted from the complex was monitored to determine the iron release rate (**Figure 5-4E**). The iron core dissolution was observed to occur within 10 minutes of exposure to the reducing environment (in the presence of DTT and EDTA) with a maximum of 600 μM Fe released in low pH (pH 4.5) and reducing conditions. No iron is observed to be released from ferritin at neutral (pH 7.4) and low pH conditions in the absence of reducing agents indicating intactness of the core structure within. Cathepsin-B is the lysosomal enzyme chosen to mimic the lysosomal environment due to its dual nature of endo- and exo- peptidase activities in neutral and low pH environments, respectively ^[170]. The iron release kinetics is observed to occur at a slower rate than under reducing conditions wherein the degradation of the core plateaued after 12 h (**Figure 5-4F**). Higher amount of released Fe ions was detected during exposure of ferritin to the enzyme at pH 7.4. This was further validated using transmission electron micrographs where higher numbers of intact cages and corresponding iron cores were observed under low pH conditions (**Figure 5-4C,D**). Additional experimental repeats and degradation study at under different buffering conditions needs to be conducted to compare the effects of endopeptidase and exopeptidase activity of cathepsin-B on ferritin. Since in all conditions iron cores

were not observed to remain intact after protein shell degradation, it was concluded that the high contrast particles observed in the transmission electron micrographs correspond to intact ferritin protein nanocages in the cytoplasmic compartment.

Figure 5-3: Transmission electron micrographs of RAW264.7 cells A. Untreated and B. with 24h AfFtnAA incubation.

A

B

C

D

Figure 5-4: Transmission electron micrographs (top row – stained, bottom row – unstained) to demonstrate lysosomal degradation of AfFtnAA in vitro. AfFtnAA in the presence of A. 25mM HEPES pH 7.4, B. 20mM NaAc, 5mM DTT, 5mM EDTA pH 4.5 C. 25mM HEPES, 1-unit cathepsin-B, pH 4.5 D. 25mM HEPES, 1-unit cathepsin-B pH 7.4. E. Cumulative iron release of AfFtnAA at incubated at different reducing/non-reducing conditions for 1 h (n=3, where n is the no. of repeats at the stated buffer conditions). F. Cumulative iron release of AfFtnAA in the presence of cathepsin-B lysosomal enzyme at pH 7.4 and pH 4.5 for 14 h.

5.3.4 Iron homeostasis in cells treated with ferritin

The effect of exogenous ferritin treatment on endogenous proteins involved in maintaining iron homeostasis was investigated using real time quantitative polymerase chain reaction (RT-qPCR) (**Figure 5-5A**). The primers used for all reactions have been summarized in Table 1 of the supplementary information. The mRNA expression levels of endogenous mammalian L- and H-ferritins were monitored. The fold change in the mRNA expression of the target proteins was normalized to untreated cells. A 7- and 9-fold increase in L-ferritin and H-ferritin at the mRNA levels was measured after 48 h of treatment.

Although not to the same extent, an upregulation of ferroportin, iron exporter protein was also observed.

Figure 5-5: A) mRNA expression levels of ferroportin, L-ferritin and H-ferritin in macrophages in the presence of 200nM AfFtnAA for 12h, 24h and 48h. The mRNA expression levels are calculated relative to control samples without AfFtnAA treatment. B) Decrease in Fe levels in the pellet (intracellular) and in the medium measured at 4h, 16h and 24h. The change is calculated in reference to cells and medium at the 0h timepoint. (n = 3, where n is the least number of independent experiments performed in triplicate). Statistical analysis has been performed using one-way ANOVA test. (**p < 0.01, ***p < 0.005, ****p < 0.001). Significance values have been represented with respect to the untreated control samples for A and with respect to the 0h timepoint for B.

In order to investigate the possibility of Fe export, Fe levels were monitored intracellularly and in the medium (**Figure 5-5B**). Following cell treatments with AfFtnAA, the culture media was replaced with that without AfFtnAA. The iron levels were monitored for a duration of 24 h after the medium change. An overall 30% decrease in intracellular Fe levels was observed to

gradually occurs over 24 h. Correspondingly, an increase in Fe levels in the culture medium was also measured, indicating that iron was released from the cells to the extracellular environment likely by exocytosis.

5.3.5 Ferritin treatment induces oxidative stress and cellular responses

In order to elucidate the immune response of RAW 264.7 cells to AfFtnAA treatment, the mRNA expression levels of immunology relevant genes were determined (**Figure 5-6A**). These included pro-inflammatory cytokines (TNF α and IL1 β), chemokine (CCR2) and cell adhesion molecule responsible for leukocyte signalling and adhesion. The genes were selected to represent cellular stress and responses in macrophage cells in response to ferritin incubation^[171]. The AfFtnAA treatment was found to have no significant effects on CCR2, VCAM1 and TNF α . However, within the 24 h exposure time, a significant upregulation of IL1 β was observed. To further study the upregulation of this cytokine, a time-dependent exposure study to AfFtnAA was carried out for 12, 24 and 48 h (**Figure 5-6B**). There were no significant changes in mRNA expression observed at varying times with the expression levels peaking within 12 h of treatment.

Figure 5-6: A) mRNA expression levels of cytokines and chemokines (CCR2, VCAM1, TNFα and IL1β) in RAW 264.7 cells treated with AfFtnAA for 48 h. B) IL1β mRNA expression level changes in cells treated with AfFtnAA in a time-dependent manner (12 h, 24 h, 48 h). The expression levels of the target genes were normalized to that of untreated cells to determine the fold change indicated above. Reactive oxygen species generation measurement in cells treated with C) AfFtnAA for varying time durations (12 h, 24 h, 48 h). D) Apo AA and iron loaded AfFtnAA for 24 h. The ROS generated (measured as fluorescence mean) is expressed as a percentage with respect to the mean fluorescence from the untreated cells. (n = 3, where n is the least number of independent experiments measured in triplicates). Statistical analysis has been performed using one-way ANOVA test. (*p<0.05, **p < 0.01, ***p < 0.005).

To further understand the cellular stress in cells with AfFtnAA treatment, the generation of reactive oxygen species (ROS) was quantified using dihydroethidium (DHE) as a fluorescence probe. The mean fluorescence

intensity obtained for the treated samples were normalized with that of untreated samples to determine the % ROS generated. A time-dependent increase in ROS was observed with the highest increase reaching up to 324% with prolonged AfFtnAA treatment for 48 h (**Figure 5-6C**). A possible cause of the generation of free radicals was hypothesized to be due to the Fe^{3+} ions loaded in the ferritin core undergoing Fenton reaction after protein shell degradation in the lysosomes. To confirm that the presence of iron caused the increase in ROS, a comparison was drawn between Apo AA and (Fe2400)AA (**Figure 5-6D**). Apo AA are ferritin protein cages assembled in the presence of 500 mM sodium chloride instead of Fe^{2+} ions. A significantly lower ROS, close to untreated samples, were observed with Apo AA treatment after 24 h exposure compared to (Fe2400)AA treated samples. A similar trend was observed with lowered mRNA expression levels of $\text{IL1}\beta$ with Apo AA compared to iron loaded ferritin treatment (APPENDIX, **Figure 7-14A**) indicative of the role of Fe within the cages in inducing cellular stress and immune related response.

Inflammasome pathway is identified to be the most common signalling pathway correlating between ROS formation and subsequent upregulation of $\text{IL1}\beta$. However, in this study, the induced oxidative stress by ferritin treatment was instead determined to be the result of an inflammasome independent pathway as elucidated with no significant changes in regulation of NLRP3 gene (APPENDIX, **Figure 7-14B**) ^[172, 173]. This highlights the need for further understanding the pathway ferritin specific mode of acting in causing cellular responses and oxidative stress.

5.3.6 Poly-histidine mediates endosomal escape of ferritin and subsequent transcriptional effects on endogenous ferritin levels

Ferritin protein is genetically engineered to display poly-histidine peptides on the outer surface (HHEN-AA) and within the inner cavity (AA-HHEC). The poly-histidine peptide comprises of six repeats of (HHE) amino acid residues. The intermittent placement of the glutamic acid residues lowers the hydrophobicity of histidines thereby enhancing the overall protein solubility^[174]. The engineered proteins were expressed and purified following that of the unmodified protein with slight modification at the heat treatment step to ensure protein solubility (APPENDIX, **Figure 7-15** A-C). Twelve hundred Fe atoms were used to mediate cage formation as opposed to 2400 Fe atoms in the previous experiments to minimize protein instability. Transmission electron micrographs and ICP-MS revealed that the poly-histidine placed near the inner cavity aids in higher Fe loading per cage which was likely due to the electrostatic interactions between the glutamic acid residues and Fe²⁺ ions at the iron loading step. The Fe core sizes were correspondingly larger for these conjugates as compared to HHEN-AA and unmodified AfFtnAA (referred to as AA in this section) (APPENDIX, **Figure 7-16**). This could be linked to charge-mediated interaction between the Fe ions and glutamic acid residues. Cells were incubated with 75 nM of poly-histidine modified AA with the unmodified AA as the control for 24h and 48h. Fe accumulation was measured in the lysosomal/endosomal components (according to the protocol described in section 5.2.5) and quantified with respect to the combined total Fe measured from cellular organelles such as the vesicular compartments, membrane, and cytoplasm.

A 10% and 5% decrease in endosomal/lysosomal Fe accumulation was observed in cells treated with HHEN-AA and AA-HHEC, respectively, as compared to unmodified AA (**Figure 5-7A**). The longer treatment time resulted in greater endosomal/lysosomal escape and redistribution to the cytoplasmic compartments. The protocol (Section 5.2.8) is followed to determine the effect of 200 nM poly-histidine modified AfftnAA (HHEN-AA and AA-HHEC) on L-ferritin expression levels after treatment with the protein conjugates for 24 h. A 6-fold expression in L-ferritin mRNA levels were observed after 48 h treatment with both the poly-histidine modified protein conjugates. (**Figure 5-7B**). The fold change is estimated based on cells treated with equimolar amounts of unmodified AA for 48 h. The RT-qPCR thus showed that a slight increase in cytoplasmic Fe levels could alter the mRNA expression levels of endogenous ferritin.

Figure 5-7: Poly-histidine tagged ferritin protein nanocages. A) Percentage of Fe accumulation in the endosomal/lysosomal vesicular compartments in cells incubated with AA, HHEN-AA and HHEC-AA for 24h and 48h. The percentage is calculated relative to the total amount of Fe measured in the endosomal/lysosomal, cytoplasm and membrane compartments. B) mRNA expression levels of L-ferritin and H-ferritin in macrophages in the presence of 75nM HHEN-AA and HHEC-AA for 48h. The mRNA expression levels are calculated relative to cells with AfftnAA treatment. (n = 3, where n is the least

number of independent experiments measured in triplicates). Statistical analysis has been performed using one-way ANOVA test. (#p < 0.1, **p < 0.01).

5.3.7 ***In vivo* ferritin localization in macrophage-rich atherosclerotic lesion sites**

Localization of ferritin nanocages in macrophages was further investigated *in vivo* using apolipoprotein E (ApoE) knockout (ApoE^{-/-}) mice models induced for atherosclerotic lesions. The lesion sites are expected to comprise of pro-inflammatory macrophages in the aortic region. ApoE^{-/-} mice were induced to form atherosclerotic plaques by feeding a high-fat diet. Ferritin conjugated with Cyanine 5 (Cy5) dye was used to visualize ferritin localization. Within 1h of injection, fluorescent signals were observed in the lesion site in the aorta (**Figure 5-8A**). Minimal fluorescence signals were measured in blood and urine with the injection time. Preliminary biodistribution studies elucidated that renal and hepatic clearance of ferritin occurred as visibly high fluorescence intensity measured from liver, intestine, and kidneys (APPENDIX, **Figure 7-17**). Flow cytometry analysis of the digested aorta showed that 82% percent of the total event population identified as cells were detected to be positive for Cy5. 70% of these Cy5 positive cells were CD31 positive (endothelial cells, leukocytes etc.) and 30% were identified as macrophage population. 95% of the event population identified to be macrophages was positive for the presence of Cy5 (**Figure 5-8B**). Thus, observing similar internalization trend of ferritin by murine macrophage cells observed *in vitro*. Atherosclerotic mice models are characterized with an increased macrophage content in the lesion sites. Uptake of ferritin NPs in the lesion macrophages are observed to occur within a short period of injection.

However, time-dependent ferritin NP localization studies and degradation behaviour in blood plasma need to be conducted to discern the uptake mechanism and long-term biodistribution of ferritin NPs *in vivo*.

Figure 5-8: A) Optical photograph and ex-vivo fluorescence image obtained from IVIS of the aorta of mice injected with Cy5 conjugated AfFtnAA after 1hr. IVIS fluorescence image of injected sample as reference per drop. B) Flow cytometry of the aorta samples indicating Cy5 positive cells (blue) containing CD31 positive cells (red). Total macrophages present and Cy5 positive macrophages have been shown in green and shaded blue area, respectively.

5.4 Discussion

Ferritin is observed to be internalized by macrophages both in *in vitro* and *in vivo* models. Transferrin-mediated endocytosis is commonly understood as the uptake pathway of ferritin nanocages. This was demonstrated using ferritin from

different sources and in different cell types ^[134]. In agreement with these results, the work conducted in this chapter show that pre-treatment of cells with chlorpromazine resulted in ~22% reduction in ferritin uptake suggesting clathrin- or receptor-mediated uptake. However, no single dominant pathway appears to play a role in the internalization of ferritin as inhibition of uptake to a similar extent is observed in cells pre-treated with caveolae- and micropinocytosis pathway inhibitors. The role of clathrin pits is observed at ferritin concentrations of 200nM thus, suggesting the need for a minimum NP concentration for specific receptor interaction and subsequently endocytosis to occur. Similarly, in a previous work, the internalization of superparamagnetic iron oxide nanoparticles (SPIONs) have been shown to be dependent on all the three pathways to similar extents ^[155]. Treatment of macrophages with chlorpromazine, M β CD and amiloride all dramatically inhibited uptake of the nanoparticles. Since ferritin contains an iron core similar to that of SPIONs in terms of size and structure, the data in this chapter thus indicate that protein shell and bare iron oxide NPs interact with the cell membrane following similar mechanisms thus resulting in internalization following multiple pathways. An initial increase of Fe accumulation followed by a decrease after 48 h exposure was observed with the prolonged treatment of cells with ferritin NPs. Of particular relevance to the observations in Chapter 5, Kim et al. have suggested the result of dilution of NP concentration in the daughter cells as compared to the parent cells ^[175]. Thus, resulting in a non-linear trend in internalization wherein the net NP accumulation in the cells eventually plateaus with time. Since the measured Fe accumulation in this study is an average across cell types of different generations, a similar deviation from the linear NP uptake trend is observed. Furthermore, a significant

reduction in accumulated Fe per cell as well as total cellular Fe levels is indicative of iron export occurring with prolonged incubation.

Ferritin nanocages are found to localize in the endosomal compartments following the clathrin-mediated uptake pathway. *In vitro* simulation of lysosomal conditions shows the digestion of ferritin protein shell occurs in the presence of lysosomal enzymes. Iron core solubilisation by Fenton reaction is further observed to occur under strong reducing conditions with the protein shell remaining intact under these conditions. Subcellular quantification demonstrated a significant presence of iron atoms in the cytoplasmic compartments which likely corresponds to the ferrous (II) ions formed by iron core dissolution. A small proportion of intact ferritin cages were visualized as high contrast particles in the cytoplasm using electron microscopy in this chapter. Ferritin localization was observed to be slightly different from the cellular fate of SPIONs previously studied with RAW264.7 cells. Iron oxide nanoparticles have been previously shown to undergo similar lysosomal degradation pathways with the free iron pool released in the cytoplasm ^[155].

Similarities of the data in this study can be drawn to the localization and degradation pathway of endogenous ferritin synthesized in the cytoplasm in response to Fe incubation ^[164]. Synthesized ferritin is found to enter the lysosomes by binding to the cargo receptor (NCO4A) in response to iron deprivation. The iron inside these ferritin molecules are then released in lysosomes in the presence of high concentration of chelating agents like glutathione and ascorbic acid ^[176]. Furthermore, like endogenous ferritin degradation pathway, the results in this chapter suggest that protein shell degradation is not necessary for the dissolution of the iron core.

In response to iron released from ferritin, mRNA transcriptional analyses show significant upregulation of endogenous ferritin levels at 48h incubation. Thus, suggesting the transfer of iron from exogenously incubated ferritin to endogenous heavy- and light-chain ferritins. Furthermore, ferroportin upregulation is observed validating the activation of iron export mechanism over 48 h. The regulation of ferroportin, the only known iron exporter protein, is indicative of the effects of ferritin on iron homeostasis of the cells. Excess iron released from ferritin degradation induces the production of iron regulation proteins to maintain iron homeostasis ^[177, 178]. Interestingly, the upregulation of L-ferritin mRNA for ferritin NP-treated samples in this study is found to be 10-fold lower than that treated with SPION for a similar 24h exposure time^[155]. This is despite the higher molar iron content present in the ferritin used in this study. The exogenous ferritin is thus found to have less impact on iron homeostasis in cells by providing protective effects likely by slowing the iron-core dissolution process. The significant upregulation of L-ferritin and H-ferritin levels is indicative of potential oxidative damage in the cells due to the production of hydrogen peroxide that occurs during iron core formation in endogenous ferritin. Although such oxidative damage may not result in cytotoxicity, it could have secondary effects on cellular activities and cause genetic disorders ^[179]. Lower iron relevant molecular responses with ferritin treatment thus presents as an advantage over SPIONs when used for biomedical applications. Additionally, the importance of modulating the iron content and treatment duration using ferritin nanocages is understood to minimize the impacts on iron homeostasis and potential oxidative stress. This has been further elucidated in this study by the analysis of immune relevant genes as a response to ferritin treatment.

Macrophages being the first line of defence involved in immune response makes it crucial to study their molecular responses to nanoparticles and to monitor the regulation of immunologically relevant genes because of the treatment. To monitor these responses, the fold change in mRNA expression of CCR2, VCAM-1, IL1 β and TNF α were studied. CCR2 and VCAM-1 are responsible to mediate increased requirement of leukocytes, macrophage polarization, cell migration and cell adhesion in vivo^[180, 181]. Furthermore, the upregulation of pro-inflammatory cytokines namely IL1 β and TNF α in response to ferritin treatment is observed in this work. Additionally, due to the possibility of the Fe³⁺ core undergoing a change in oxidation state and inducing oxidative stress, the ROS levels were monitored over different exposure time. Out of the four genes analysed, a significant upregulation of IL1 β observed along with a time-dependent increase in ROS levels. A correlation between the core Fe content and the molecular responses was drawn with an observed decrease in ROS and immune markers observed with ApoAA treatment. This study further emphasizes the possibility of increased oxidative stress as a result of prolonged exposure to the iron atoms wrapped in the core of the protein shell. Thus, it is important to tune the iron content in ferritin NPs considering the possible oxidative and immunological response. This can be particularly important in the case of ferritin NP targeting to diseased tissues wherein an increase in free radical species or cytokines could result in further progression of the disease due to inflammation.

Ferritin protein nanocages have garnered significant research interest as a drug delivery vehicle for small molecules, siRNA, short peptides, among many others^[144, 182]. The target intracellular space for a large proportion of these

therapeutic molecules is the cytoplasm ^[183]. Based on this study, majority of the cargo in ferritin (in this case Fe) localizes in the endosomal compartments followed by release of the cargo in the cytoplasmic compartments. However, this mechanism of cargo delivery to the cytoplasm would not be feasible with cargo that is sensitive to low pH and proteolytic enzymes in the endosomes and lysosomes. The two key mechanisms proposed to mediate endosomal release are proton sponge theory and charge mediated membrane disruption ^[184]. Proton sponge theory refers to the disruption of endosomal compartments due to osmotic swelling by recruitment of protons for the protonation of non-exposed amino acid residues in response to the low pH in these organelles ^[185]. Membrane disruption on the other hand is thought to occur due to the interaction of the cationic residues with the endosomal membrane surface resulting in holes in the lipid bilayer thereby causing the disruption ^[186]. To overcome the barrier of endosomal/lysosomal compartments, the use poly-histidine peptides to mediate escape of ferritin from these regions is attempted here. Previous studies have demonstrated the use of histidine-based peptides for the delivery of siRNA and toxins using chitosan, gold nanoparticles and ribonucleotide octamers ^[174, 187, 188]. To the author's knowledge, the study conducted in this chapter remains one of the first to attempt endosomal escape of protein nanocages. Two conjugates of ferritin with poly-histidine peptides genetically engineered to the N and C-terminus of the polypeptides have been developed. N-terminus of polypeptides is exposed on the outer surface and the C-terminus faces the inner cavity thus differentiating the location of the peptide on ferritin. This allowed determination of the favoured mechanism of endosomal escape between cationic residue mediated membrane disruption and proton sponge effect. A decrease was

observed in endosomal/lysosomal iron accumulation at 48h with a higher decrease observed with HHEN-AA. Thus, indicating the greater role of surface poly-histidine peptides in membrane disruption and thereby mediating endosomal escape. It is further understood that the protonation of the histidine residues is not sufficient a driving force for the rupture of the endosomal compartments. However, the lower percentage of endosomal escape compared to previous studies is likely due to the higher vulnerability of proteins to lysosomal degradation than hybrid nanoparticles ^[174]. Transcriptional analysis revealed ~6-fold upregulation of L-ferritin in cells incubated with the poly-histidine mutants in comparison to unmodified AA. Despite the higher cytoplasmic iron percentage accumulation with HHEN-AA treatment, greater fold increase of mRNA was likely observed due to the higher iron content loaded per cage of AA-HHEC. The further enhancement of the endosomal release property presented in this work by the addition of amino acid residues is envisioned. The stronger cationic property is expected to further enhance endosomal membrane disruption.

5.5 Conclusions

In this study, the uptake pathways, cellular localization of ferritin nanocages in murine macrophage cell line followed by determining the molecular response in cells to the treatment is analysed. It is determined that although no single pathway is responsible for ferritin uptake in macrophages, local concentration of the particles plays a crucial role in determining the dominant the uptake pathway. Furthermore, the process of protein shell degradation *in vitro* and observed the molecular responses of macrophage cells to the free Fe ions released from the core is investigated. Modulation of intracellular localization of the cages have been further attempted with engineering the cages with peptide

modalities in this chapter. Such an understanding of ferritin NPs internalization and its effects on cellular iron homeostasis could potentially impact the development of diagnostic and therapeutic tools using ferritin nanocages for different diseased models.

Chapter 6 : Concluding Remarks & Future Works

Engineered ferritin nanocages have been proposed to be used as a therapeutic agent in atherosclerosis diseased conditions by mediating cholesterol sequestration from foam cells. Chemical and genetic engineering strategies have been adopted to modify the cage surface and internal cavity to display cholesterol sequestering moieties. Therapeutic efficacy studies have been carried out using *in vitro* lipid-laden macrophages.

Beta cyclodextrin conjugated to the cage surface by copper catalysed click chemistry reaction showed cholesterol sequestration efficacy up to 38% from the foam cell models within 2 – 6 hours of treatment time. A 5nM K_D value was measured between conjugated cyclodextrin and cholesterol molecules using bio-layer interferometry. The cholesterol reduction efficacy was observed to be higher than cyclodextrin molecules loaded in the inner cavity indicating the importance of the interaction of cyclodextrin molecules with membrane cholesterol compared with intracellular lysosomal or cytoplasmic cholesterol molecules.

As an alternative mode of cholesterol removal, HDL mimetic peptides were used as the therapeutic moiety. 4F HDL mimetic peptide and its variant 2X4F (2 repeats) were genetically fused to the N-terminus of ferritin displaying the peptides on the cage surface. Protein characterization using SDS-PAGE, DLS and TEM demonstrated intact secondary and quaternary cage structures after peptide modification. A 30% reduction in cholesterol levels were measured using the foam cell models after 24 h of treatment with 4F-AA. As opposed to the physical cholesterol sequestration using cyclodextrin, cholesterol reduction by

4F-AA is expected to be mediated by cholesterol efflux receptors. A 10-fold mRNA upregulation of ABCA1 receptor was measured after 4F-AA treatment indicating a ABC-transporter mediated cholesterol reduction pathway.

Both cholesterol sequestration modalities, highlighted the higher importance of membrane interaction of the ferritin particles rather than internalization. However, *in vitro* fluorescence imaging and TEM of ferritin showed internalization in macrophages within 12 h of treatment. Thus, the final chapter delved deeper into understanding the cellular fate of ferritin nanocages in the macrophage cell model and studying the molecular responses to the treatment. Additionally, a 3-fold higher preferential internalization was observed in foam cells compared to macrophage cells following the transferrin receptor mediated uptake pathway. Subcellular fractionation of cellular organelles coupled with TEM imaging revealed that the majority of the ferritin were localized in the early and late endosomes. The lysosomal degradation of the protein shell in the presence of enzymes and the reduction of the iron core of the ferritin particle under the reducing environment results in the release of the reduced Fe form in the cytoplasmic region. Iron relevant molecular response analysis showed that the iron released from the degraded ferritin particles are likely transferred to endogenously expressed light and heavy chain ferritin as seen from upregulated mRNA expression of these iron related proteins in response to exogenous ferritin treatment. Additionally, an efflux of excess iron was measured by quantifying the cellular iron levels as well as an observed upregulation of the mRNA expression levels of the iron transport protein, ferroportin.

Finally, preliminary ferritin localization experiments were performed *in vivo* using ApoE^{-/-} atherosclerotic mice models. More than 90% of the macrophages in the lesion site were found to internalize the Cy5 labelled ferritin cages as quantified using antibody specific flow cytometry. However, majority of the ferritin particles were observed to be cleared out of the system following the hepatic metabolism pathway. Thus, there is a need for further tuning of the cages to increase the systemic circulation time so as to make full use of its therapeutic potential in atherosclerosis management.

6.1 Dual surface modification

The present dosage of treatment of foam cells being used is 130 µg/ml of AfFtnAA-CD which is equivalent to 5 uM of cyclodextrin molecules. Although, this dosage is not cytotoxic, the dosage requirements are expected to be higher when used *in vivo* due to the tendency of nanoparticles to localize in the spleen and liver upon intravenous injection. Thus, there is a need to enhance the cholesterol sequestration capacity of the nanoconjugates so as to maintain the dose within subtoxic levels.

AfFtnAA can be used as a platform to present both cyclodextrin and ApoA-1 mimetic peptides on its external surface to mediate enhanced cholesterol sequestration following two independent mechanisms (**Figure 6-1**). The efficacy of the CD conjugation can be controlled using varying linker lengths (by introducing PEG groups in the maleimide linker). This would minimize any steric hindrance presented by the conjugated peptide during the reaction steps. The efficacy of the construct as a cholesterol efflux agent would be tested using RAW 264.7 cells treated with oxidized lipoproteins. In addition, formation of HDL-like

particles can be monitored by isolating the medium and running size exclusion chromatography to observe changes in size of the nanoparticles.

Figure 6-1: Schematic of engineered AfFtnAA chemically conjugated with β -CD on cysteine residues (highlighted in blue) and genetically modified with 4F peptide on the N-terminus (indicated in red).

6.2 Self-peptide conjugation to improve systemic circulation

As discussed earlier, ferritin cages passively accumulate in the plaque by EPR effects and localize to the macrophages following transferrin mediated endocytosis. However, protein cages due to its characteristic are prone to hepatic metabolism and thereby suffering from reduced systemic circulation times. Thus, therapeutic applications using protein cages would involve multiple injections to observe the desired effects.

To enhance the local ferritin concentration in the lesion sites, the two alternative strategies commonly adopted is the conjugation of plaque specific targeting ligands (VCAM1, p-selectin) and the conjugation of polymers or

peptides to minimize non-specific phagocytosis by liver macrophages. The conventional strategy to improve systemic circulation times involves the surface PEGylation of the cage structures^[189, 190]. However, PEGylation has been found to alter the surface properties of the nanoparticles thereby resulting in reduced cell membrane interactions and cell internalization mediated retention in the lesion site. As seen from both the therapeutic moieties for cholesterol sequestration studied in this work, membrane interaction plays a crucial role in mediate cholesterol reduction. Thus, an alternative method to PEGylation that has garnered significant research attention is the use of self-peptides.

hCD47 is a membrane protein present on most mammalian cell membranes that prevents the engulfment of healthy cells present in the body by macrophages. The membrane binding and interaction of hCD47 with SIRP α receptor on macrophages results in a chain of cellular signalling pathways that inhibits the myosin II production pathway^[191, 192]. Myosin II plays a critical role in the regulation of multiple endocytosis pathways thus resulting in a negative feedback signal inhibiting the internalization of macrophages^[193]. The incorporation of hCD47 mimetic peptide on nanoparticles have been demonstrated as an effective way to result in a 4-fold increase in systemic circulation rates in comparison to PEGylation along with the subsequent increase in accumulation in tumour sites following EPR effect^[191]. Thus, conjugation of self-peptides on ferritin could potentially have similar benefits *in vivo* with increased accumulation in lesion sites thereby aiding in therapeutic applications. A reduction in uptake and protein cage degradation by liver macrophages with minimal effects on cell membrane interaction properties would allow for

enhanced ferritin localization in the lesion sites with subsequent cholesterol sequestration from the diseased sites.

6.3 Co-delivery of antioxidant and anti-inflammatory drugs

The therapeutic moieties proposed in this study are expected to aid in the management of atherosclerosis progression by mediating cholesterol reduction from foam cells in the lesion sites. A therapeutic modality that would complement this therapeutic approach would be with the use of antioxidant or anti-inflammatory drugs.

Figure 6-2: A. Mechanism of intracellular SOD mediated reduction of free radicals^[194]. B. Schematic of ferritin surface engineered with cholesterol sequestering peptides and loaded with antioxidant hydrophobic drugs in its inner core.

Statins are the clinically prescribed drugs used to maintain serum cholesterol levels by regulating the cholesterol synthesis process. Most generations of statins have also shown to demonstrate anti-inflammatory properties when delivered to the lesion sites. Such anti-inflammatory properties

result in the inhibition of cytokine production responsible in increasing T-cell migration and endothelial dysfunction. Antioxidant drugs have also evolved to be in the forefront as a tool to control plaque progression. Free oxygen radicals serve to enhance neutrophil migration to the lesion sites and play a role in the oxidation of LDL molecules. The uptake of these modified LDL by macrophages plays a crucial role in the formation of foam cells. Enzymes such as super oxide dismutase (SODs) have been shown to catalyse the oxidation of superoxide free anion radicals (O_2^-) to form oxygen and hydrogen peroxide thus decreasing the free oxygen radicals and subsequent inflammation and foam cell formation caused as a result^[194-196] (**Figure 6-2A**). However, the hydrophobicity and the absence of specificity to accumulation in the diseased tissues and cells limits its therapeutic purpose. The co-loading of these kinds of anti-inflammatory and antioxidant drugs in ferritin could serve as a delivery mechanism.

The loading of hydrophobic molecules such as cyclodextrin was successfully achieved in this work following the metal co-loading method. The inner cavity minimizes contact with the external aqueous environment thus maintaining an intact structure of a transport vehicle. The external cholesterol sequestering moieties and the loading of drugs would result in the reduction of cholesterol levels whilst inhibiting the formation of radicals responsible for the formation of the cholesterol loaded cells (**Figure 6-2B**). Such dual approach of prevention and therapeutic approach targeting lesion site foam cell levels using the same platform would contribute to better management of atherosclerosis progression.

Bibliography

1. Lusis, A.J., *Atherosclerosis*. Nature, 2000. **407**(6801): p. 233-241.
2. Arroyo, L.H. and R.T. Lee, *Mechanisms of plaque rupture: mechanical and biologic interactions*. Cardiovasc Res, 1999. **41**(2): p. 369–375.
3. Spence, J.D. and G.K. Dresser, *Overcoming challenges with statin therapy*. J Am Heart Assoc, 2016. **5**(1): p. e002497.
4. Schulz, P., et al., *Medications and associated symptoms/problems after coronary artery bypass surgery*. Heart Lung, 2011. **40**(2): p. 130-8.
5. Htay, T. and M.W. Liu, *Drug-eluting stent: a review and update*. Vasc Health Risk Manag, 2005. **1**(4): p. 263-76.
6. Navab, M., et al., *Oral administration of an Apo A-I mimetic peptide synthesized from D-amino acids dramatically reduces atherosclerosis in mice independent of plasma cholesterol*. Circulation, 2001. **105**(3): p. 290-292.
7. Pradhan-Nabzdyk, L., et al., *Current siRNA targets in atherosclerosis and aortic aneurysm*. Discov Med, 2014. **17**(95): p. 233-46.
8. Bi, Y., et al., *M2 Macrophages as a Potential Target for Antiatherosclerosis Treatment*. Neural Plast, 2019. **2019**: p. 6724903.
9. Otvos, L., Jr. and J.D. Wade, *Current challenges in peptide-based drug discovery*. Front Chem, 2014. **2**: p. 1-4.
10. Wang, C.F., et al., *Multifunctional porous silicon nanoparticles for cancer theranostics*. Biomaterials, 2015. **48**: p. 108-18.
11. Kulkarni, N.S., et al., *Exploring potential of quantum dots as dual modality for cancer therapy and diagnosis*. Journal of Drug Delivery Science and Technology, 2019. **49**: p. 352-364.
12. Sanchez-Gaytan, B.L., et al., *HDL-mimetic PLGA nanoparticle to target atherosclerosis plaque macrophages*. Bioconjug Chem, 2015. **26**(3): p. 443-51.
13. Zhao, Y., et al., *Cell-derived biomimetic nanoparticles as a novel drug delivery system for atherosclerosis: predecessors and perspectives*. Regenerative Biomaterials, 2020: p. 1-10.
14. Bhaskar, S. and S. Lim, *Engineering protein nanocages as carriers for biomedical applications*. NPG Asia Materials 2017. **9**: p. e371.

15. Nandwana, V., et al., *Engineered ferritin nanocages as natural contrast agents in magnetic resonance imaging*. RSC Adv 2017. **7**: p. 34892–34900.
16. Terashima, M., et al., *Human ferritin cages for imaging vascular macrophages*. Biomaterials 2011. **32**(5): p. 1430–1437.
17. Tabas, I. and K.E. Bornfeldt, *Macrophage phenotype and function in different stages of atherosclerosis*. Circ Res, 2016. **118**(4): p. 653-667.
18. Libby, P., P.M. Ridker, and G.K. Hansson, *Progress and challenges in translating the biology of atherosclerosis*. Nature, 2011. **473**(7347): p. 317-25.
19. Chistiakov, D.A., et al., *Mechanisms of foam cell formation in atherosclerosis*. J Mol Med (Berl), 2017. **95**(11): p. 1153-1165.
20. Chistiakov, D.A., Y.V. Bobryshev, and A.N. Orekhov, *Macrophage-mediated cholesterol handling in atherosclerosis*. J Cell Mol Med, 2016. **20**(1): p. 17-28.
21. Janoudi, A., et al., *Cholesterol crystal induced arterial inflammation and destabilization of atherosclerotic plaque*. Eur Heart J, 2015. **37**(25): p. 1959-1967.
22. Verhamme, P. and M.F. Hoylaerts, *The pivotal role of the endothelium in haemostasis and thrombosis*. Acta Clin Belg, 2006. **61**(5): p. 213-9.
23. Rajendran, P., et al., *The vascular endothelium and human diseases*. Int J Biol Sci, 2013. **9**(10): p. 1057-69.
24. Ludmer, P.L., et al., *Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries*. N Engl J Med, 1986. **315**(17): p. 1046-51.
25. Tousoulis, D., et al., *The role of nitric oxide on endothelial function*. Curr Vasc Pharmacol, 2012. **10**(1): p. 4-18.
26. Yuyun, M.F., L.L. Ng, and G.A. Ng, *Endothelial dysfunction, endothelial nitric oxide bioavailability, tetrahydrobiopterin, and 5-methyltetrahydrofolate in cardiovascular disease. Where are we with therapy?* Microvasc Res, 2018. **119**: p. 7-12.
27. Matthys, K.E. and H. Bult, *Nitric oxide function in atherosclerosis*. Mediators Inflamm, 1997. **6**(1): p. 3-21.
28. Horton, J.D., J.L. Goldstein, and M.S. Brown, *SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver*. J Clin Invest, 2002. **109**(9): p. 1125-31.
29. Espenshade, P.J. and A.L. Hughes, *Regulation of sterol synthesis in eukaryotes*. Annu Rev Genet, 2007. **41**: p. 401-27.

30. Lee, S.D. and P. Tontonoz, *Liver X receptors at the intersection of lipid metabolism and atherogenesis*. *Atherosclerosis*, 2015. **242**(1): p. 29-36.
31. Repa, J.J., et al., *Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta*. *J Biol Chem*, 2002. **277**(21): p. 18793-800.
32. Ou, J., et al., *Unsaturated fatty acids inhibit transcription of the sterol regulatory element-binding protein-1c (SREBP-1c) gene by antagonizing ligand-dependent activation of the LXR*. *Proc Natl Acad Sci U S A*, 2001. **98**(11): p. 6027-32.
33. Schulman, I.G., *Liver X receptors link lipid metabolism and inflammation*. *FEBS Lett*, 2017. **591**(19): p. 2978-2991.
34. Joseph, S.B., et al., *Reciprocal regulation of inflammation and lipid metabolism by liver X receptors*. *Nat Med*, 2003. **9**(2): p. 213-9.
35. Hong, C. and P. Tontonoz, *Liver X receptors in lipid metabolism: opportunities for drug discovery*. *Nat Rev Drug Discov*, 2014. **13**(6): p. 433-44.
36. Joseph, S.B., et al., *Synthetic LXR ligand inhibits the development of atherosclerosis in mice*. *Proc Natl Acad Sci U S A*, 2002. **99**(11): p. 7604-9.
37. Joseph, S.B., et al., *Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors*. *J Biol Chem*, 2002. **277**(13): p. 11019-25.
38. Schultz, J.R., et al., *Role of LXRs in control of lipogenesis*. *Genes Dev*, 2000. **14**(22): p. 2831-8.
39. Abbaspour, N., R. Hurrell, and R. Kelishadi, *Review on iron and its importance for human health*. *J Res Med Sci*, 2014. **19**(2): p. 164-74.
40. Wallace, D.F., *The Regulation of Iron Absorption and Homeostasis*. *Clin Biochem Rev*, 2016. **37**(2): p. 51-62.
41. Bystrom, L.M., M.L. Guzman, and S. Rivella, *Iron and reactive oxygen species: friends or foes of cancer cells?* *Antioxid Redox Signal*, 2014. **20**(12): p. 1917-24.
42. Emerit, J., C. Beaumont, and F. Trivin, *Iron metabolism, free radicals, and oxidative injury*. *Biomedicine & Pharmacotherapy*, 2001. **55**(6): p. 333-339.
43. Sukhbaatar, N. and T. Weichhart, *Iron Regulation: Macrophages in Control*. *Pharmaceuticals (Basel)*, 2018. **11**(4): p. 137.
44. Gammella, E., et al., *Macrophages: central regulators of iron balance*. *Metallomics*, 2014. **6**(8): p. 1336-1345.

45. Anderson, G.J. and D.M. Frazer, *Current understanding of iron homeostasis*. Am J Clin Nutr, 2017. **106**(6): p. 1559S-1566S.
46. Harrison, P.M., *The structure and function of ferritin*. Biochemical Education, 1986. **14**(4): p. 154-162.
47. Theil, E.C., *Ferritin: the protein nanocage and iron biomineral in health and in disease*. Inorg Chem, 2013. **52**(21): p. 12223-33.
48. Fan, Y., et al., *The effect of anti-inflammatory properties of ferritin light chain on lipopolysaccharide-induced inflammatory response in murine macrophages*. Biochim Biophys Acta, 2014. **1843**(11): p. 2775-83.
49. Cohen, L.A., et al., *Serum ferritin is derived primarily from macrophages through a nonclassical secretory pathway*. Blood, 2010. **116**(9): p. 1574-1584.
50. Munro, H.N., *Iron regulation of ferritin gene expression*. J Cell Biochem, 1990. **44**(2): p. 107-15.
51. Schalinske, K.L., et al., *Iron regulatory protein 1 is not required for the modulation of ferritin and transferrin receptor expression by iron in a murine pro-B lymphocyte cell line*. Proc Natl Acad Sci U S A, 1997. **94**(20): p. 10681-6.
52. Cairo, G. and S. Recalcati, *Iron-regulatory proteins: molecular biology and pathophysiological implications*. Expert Rev Mol Med, 2007. **9**(33): p. 1-13.
53. Wang, W., et al., *Serum ferritin: Past, present and future*. Biochim Biophys Acta, 2010. **1800**(8): p. 760-9.
54. Jezequel, P., et al., *Validation of tumor-associated macrophage ferritin light chain as a prognostic biomarker in node-negative breast cancer tumors: A multicentric 2004 national PHRC study*. Int J Cancer, 2012. **131**(2): p. 426-37.
55. Vinchi, F., et al., *Atherogenesis and iron: from epidemiology to cellular level*. Front Pharmacol, 2014. **5**(94): p. 1-20.
56. Wang, J. and K. Pantopoulos, *Regulation of cellular iron metabolism*. Biochem J, 2011. **434**(3): p. 365-381.
57. Parrow, N.L., R.E. Fleming, and M.F. Minnick, *Sequestration and scavenging of iron in infection*. Infection and Immunity, 2013. **81**(10): p. 3503-3514.
58. F. Vinchi, et al., *Dissecting the role of systemic versus macrophage iron levels for the pathogenesis of atherosclerosis*. Abstracts Atherosclerosis, 2015. **241**: p. e10.
59. Rice, A.E., et al., *Investigation of the biophysical and cell biological properties of ferroportin, a multipass integral*

- membrane protein iron exporter*. J Mol Biol, 2009. **386**(3): p. 717-32.
60. Drakesmith, H., E. Nemeth, and T. Ganz, *Ironing out Ferroportin*. Cell Metab, 2015. **22**(5): p. 777-87.
 61. Nemeth, E., et al., *Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization*. Science, 2004. **306**(5704): p. 2090-3.
 62. Endo, A., M. Kuroda, and K. Tanzawa, *Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme a reductase by ML-236A and ML-236B fungal metabolites, having hypocholesterolemic activity*. FEBS LETTERS, 1976. **72**(2): p. 323-326.
 63. Sirtori, C.R., *The pharmacology of statins*. Pharmacol Res, 2014. **88**: p. 3-11.
 64. Bäck, M. and G.K. Hansson, *Anti-inflammatory therapies for atherosclerosis*. Nature Reviews Cardiology, 2015. **12**: p. 199-211.
 65. DeBakey, M.E., *Surgical treatment of atherosclerotic heart disease*. The American Journal of Cardiology, 1989. **63**(16): p. 9-11.
 66. Huang, Y., et al., *Drug-eluting biostable and erodible stents*. J Control Release, 2014. **193**: p. 188-201.
 67. Rikhtegar, F., et al., *Drug deposition in coronary arteries with overlapping drug-eluting stents*. J Control Release, 2016. **238**: p. 1-9.
 68. Charo, I.F. and R. Taub, *Anti-inflammatory therapeutics for the treatment of atherosclerosis*. Nat Rev Drug Discov, 2011. **10**(5): p. 365-76.
 69. Mullard, A., *Anti-inflammatory cardiovascular therapies take another hit*. Nat Rev Drug Discov, 2018. **17**(12): p. 853.
 70. Bogiatzi, C. and J.D. Spence, *Ezetimibe and regression of carotid atherosclerosis importance of measuring plaque burden*. Stroke, 2012. **43**(4): p. 1153-1155.
 71. Bittencourt, M.S. and R.J. Cerci, *Statin effects on atherosclerotic plaques: regression or healing?* BMC Med, 2015. **13**(260).
 72. Pankajakshan, D. and D.K. Agrawal, *Clinical and Translational Challenges in Gene Therapy of Cardiovascular Diseases*, in *Gene Therapy - Tools and Potential Applications*, F.M. Molina, Editor. 2013, INTECH: USA. p. 651-683.
 73. Chyu, K.-Y. and P.K. Shah, *HDL/ApoA-I infusion and ApoA-I gene therapy in atherosclerosis*. Front Pharmacol, 2015. **6**(187).

74. Zarzycki, P.K., B.e. Fenert, and B.K. Głód, *Cyclodextrins-based nanocomplexes for encapsulation of bioactive compounds in food, cosmetics, and pharmaceutical products: Principles of supramolecular complexes formation, their influence on the antioxidative properties of target chemicals, and recent advances in selected industrial applications*, in *Principles of supramolecular complexes formation*, A.M. Grumezescu, Editor. 2016, Elsevier p. 717-767.
75. Stefánsson, E. and T. Loftsson, *Microspheres and nanotechnology for drug delivery*, in *Retinal Pharmacotherapy*, R. Gabbedy, Editor. 2010, Saunders Elsevier p. 86-90.
76. Cagno, M.P.d., *The potential of cyclodextrins as novel active pharmaceutical ingredients: A short overview*. *Molecules* 2017. **22**(1): p. 1-14.
77. Zidovetzki, R. and I. Levitan, *Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies*. *Biochim Biophys Acta*, 2007. **1768**(6): p. 1311–1324.
78. Rosenbaum, A.I., et al., *Endocytosis of beta-cyclodextrins is responsible for cholesterol reduction in Niemann-Pick type C mutant cells*. *Proc Natl Acad Sci U S A*, 2010. **107**(12): p. 5477-5482.
79. Zimmer, S., et al., *Cyclodextrin promotes atherosclerosis regression via macrophage reprogramming*. *Sci Transl Med*, 2016. **8**(333): p. 333ra50.
80. Mark A. Crumling, Kelly A. King, and R.K. Duncan, *Cyclodextrins and Iatrogenic Hearing Loss: New Drugs with Significant Risk*. *Frontiers in Cellular Neuroscience*, 2017. **11**(355): p. 1-14.
81. Loftsson, T. and M.E. Brewster, *Pharmaceutical applications of cyclodextrins: basic science and product development*. *J Pharm Pharmacol*, 2010. **62**(11): p. 1607–1621.
82. Bhatt, K.N., et al., *High-density lipoprotein therapy: Is there hope?* *Curr Treat Options Cardiovasc Med*, 2010. **12**(4): p. 315-328.
83. Shaish, A., et al., *Imaging of aortic atherosclerotic lesions by ¹²⁵I-Oxidized-LDL, ¹²⁵I-HDL, ¹²⁵I-LDL and ¹²⁵I-BSA*. *Pathobiology*, 2001. **69**(4): p. 225-229.
84. Marrachea, S. and S. Dhar, *Biodegradable synthetic high-density lipoprotein nanoparticles for atherosclerosis*. *Proc Natl Acad Sci U S A*, 2013. **110**(23): p. 9445–9450.

85. Sanchez-Gaytan, B.L., et al., *HDL-mimetic PLGA nanoparticle to target atherosclerosis plaque macrophages*. Bioconjug Chem, 2015. **26**(3): p. 443-451.
86. Skajaa, T., et al., *High-density lipoprotein-based contrast agents for multimodal imaging of atherosclerosis*. Arterioscler Thromb Vasc Biol, 2009. **30**(2): p. 160-176.
87. Sviridov, D. and A.T. Remaley, *High-Density Lipoprotein mimetics: promises and challenges*. Biochem J, 2015. **472**(3): p. 249-259.
88. Tardif, J.-C., et al., *Effects of the high-density lipoprotein mimetic agent CER-001 on coronary atherosclerosis in patients with acute coronary syndromes: a randomized trial*. European Heart Journal 2014. **35**(46): p. 3277–3286.
89. Undurti, A., et al., *Modification of high density lipoprotein by myeloperoxidase generates a pro-inflammatory particle*. J Biol Chem, 2009. **284**(45): p. 30825–30835.
90. Khalid, K., et al., *Advanced in developmental organic and inorganic nanomaterial: a review*. Bioengineered, 2020. **11**(1): p. 328-355.
91. Blanco, E., H. Shen, and M. Ferrari, *Principles of nanoparticle design for overcoming biological barriers to drug delivery*. Nat Biotechnol, 2015. **33**(9): p. 941-951.
92. Jain, V., S. Jain, and S.C. Mahajan, *Nanomedicines Based Drug Delivery Systems for Anti-Cancer Targeting and Treatment*. Curr Drug Deliv, 2015. **12**(2): p. 177-191.
93. Lobatto, M.E., et al., *Atherosclerotic plaque targeting mechanism of long-circulating nanoparticles established by multimodal imaging*. ACS Nano, 2015. **9**(2): p. 1837–1847.
94. Matoba, T., et al., *Nanoparticle-mediated drug delivery system for atherosclerotic cardiovascular disease*. J Cardiol, 2017. **70**(3): p. 206-211.
95. Katsuki, S., et al., *Nanoparticle-mediated delivery of pitavastatin inhibits atherosclerotic plaque destabilization/rupture in mice by regulating the recruitment of inflammatory monocytes*. Circulation, 2014. **129**(8): p. 896-906.
96. Akbarzadeh, A., et al., *Liposome: classification, preparation, and applications*. Nanoscale Res Lett, 2013. **8**(102): p. 1-9.
97. Deng, W., et al., *Controlled gene and drug release from a liposomal delivery platform triggered by X-ray radiation*. Nat Commun, 2018. **9**(2713): p. 1-11.

98. Almer, G., et al., *Interleukin-10: an anti-inflammatory marker to target atherosclerotic lesions via PEGylated liposomes*. Mol Pharm, 2013. **10**(1): p. 175-86.
99. Li, X., et al., *Synergistic effects of liposomes encapsulating atorvastatin calcium and curcumin and targeting dysfunctional endothelial cells in reducing atherosclerosis*. Int J Nanomedicine, 2019. **14**: p. 649-665.
100. Inglut, C.T., et al., *Immunological and Toxicological Considerations for the Design of Liposomes*. Nanomaterials (Basel), 2020. **10**(190): p. 1-24.
101. Huang, C., et al., *Formation and size distribution of self-assembled vesicles*. Proc Natl Acad Sci U S A, 2017. **114**(11): p. 2910-2915.
102. Ornes, S., *Core Concept: Quantum dots*. Proc Natl Acad Sci U S A, 2016. **113**(11): p. 2796-7.
103. Jayagopal, A., et al., *Quantum dot mediated imaging of atherosclerosis*. Nanotechnology, 2009. **20**(16): p. 165102.
104. Sun, X., et al., *In Vivo Targeting and Imaging of Atherosclerosis Using Multifunctional Virus-Like Particles of Simian Virus 40*. Nano Lett, 2016. **16**(10): p. 6164-6171.
105. Spicer, C.D., C. Jumeaux, and M.M. Stevens, *Peptide and protein nanoparticle conjugates: versatile platforms for biomedical applications*. Chem. Soc. Rev., 2018. **47**: p. 3574-3620.
106. Recalcati, S., et al., *New functions for an iron storage protein: The role of ferritin in immunity and autoimmunity*. J Autoimmun, 2008. **30**(1-2): p. 84-89.
107. Theil, E.C., *Ferritin protein nanocages-the story*. Nanotechnol Percept, 2012. **8**(1): p. 7-16.
108. Behera, R.K. and E.C. Theil, *Moving Fe²⁺ from ferritin ion channels to catalytic OH centers depends on conserved protein cage carboxylates*. Proc Natl Acad Sci U S A, 2014. **111**(22): p. 7925-30.
109. Wang, Z., et al., *Functional ferritin nanoparticles for biomedical applications*. Front Chem Sci Eng, 2017. **11**(4): p. 633-646.
110. Uchida, M., et al., *Targeting of cancer cells with ferrimagnetic ferritin cage nanoparticles*. J Am Chem Soc, 2006. **128**(51): p. 16626-33.
111. Kang, H.J., et al., *Developing an antibody-binding protein cage as a molecular recognition drug modular nanoplatfrom*. Biomaterials, 2012. **33**(21): p. 5423-30.

112. Fan, K., L. Gao, and X. Yan, *Human ferritin for tumor detection and therapy*. Wiley Interdiscip Rev Nanomed Nanobiotechnol, 2013. **5**(4): p. 287-98.
113. Johnson, E., et al., *Crystal structures of a tetrahedral open pore ferritin from the hyperthermophilic archaeon Archaeoglobus fulgidus*. Structure, 2005. **13**(4): p. 637-48.
114. Sana, B., et al., *The role of nonconserved residues of Archaeoglobus fulgidus ferritin on its unique structure and biophysical properties*. J Biol Chem, 2013. **288**(45): p. 32663–32672.
115. Swift, J., et al., *Efficient self-assembly of Archaeoglobus fulgidus ferritin around metallic cores*. Langmuir, 2009. **25**(9): p. 5219-25.
116. Sana, B., E. Johnson, and S. Lim, *The unique self-assembly/disassembly property of Archaeoglobus fulgidus ferritin and its implications on molecular release from the protein cage*. Biochim Biophys Acta, 2015. **1850**(12): p. 2544–2551.
117. Barquera, S., et al., *Global Overview of the Epidemiology of Atherosclerotic Cardiovascular Disease*. Arch Med Res, 2015. **46**(5): p. 328-38.
118. Angelovich, T.A., A.C. Hearps, and A. Jaworowski, *Inflammation-induced foam cell formation in chronic inflammatory disease*. Immunol Cell Biol, 2015. **93**(8): p. 683–693.
119. Llodra', J., et al., *Emigration of monocyte-derived cells from atherosclerotic lesions characterizes regressive, but not progressive, plaques*. Proc Natl Acad Sci U S A, 2004. **101**(32): p. 11779-11784.
120. Niyonzima, N., et al., *Reconstituted high-density lipoprotein attenuates cholesterol crystal-induced inflammatory responses by reducing complement activation*. J Immunol, 2015. **195**(1): p. 257-264.
121. Gu, X., et al., *Preparation and characterization of a lovastatin-loaded protein-free nanostructured lipid carrier resembling high-density lipoprotein and evaluation of its targeting to foam cells*. AAPS PharmSciTech, 2011. **12**(4): p. 1200-8.
122. Kitagawa, T., et al., *RGD-conjugated human ferritin nanoparticles for imaging vascular inflammation and angiogenesis in experimental carotid and aortic disease*. Mol Imaging Biol, 2012. **14**(3): p. 315-24.
123. Sana, B., et al., *Iron-based ferritin nanocore as a contrast agent*. Biointerphases, 2010. **5**(3): p. 48-52.

124. Sana, B., C.L. Poh, and S. Lim, *A manganese–ferritin nanocomposite as an ultrasensitive T2 contrast agent* Chem Commun, 2012. **48**(6): p. 862-864.
125. Liu, S.M., et al., *Cyclodextrins differentially mobilize free and esterified cholesterol from primary human foam cell macrophages*. J Lipid Res, 2003. **44**(6): p. 1156-1166.
126. Atger, V.M., et al., *Cyclodextrins as catalysts for the removal of cholesterol from macrophage foam cells*. J Clin Invest, 1997. **99**(4): p. 773-780.
127. Loftsson, T., et al., *Cyclodextrins in drug delivery*. Expert Opin Drug Deliv, 2005. **2**(2): p. 335-351.
128. Davis, M.E. and M.E. Brewster, *Cyclodextrin-based pharmaceuticals: Past, present and future*. Nat Rev Drug Discov, 2004. **3**: p. 1023-1035.
129. Bachman, J., *Site-directed mutagenesis*. Methods Enzymol, 2013. **529**: p. 241-8.
130. Kwon, C., et al., *Development of protein-cage-based delivery nanoplatfoms by polyvalently displaying β -cyclodextrins on the surface of ferritins through copper(I)-catalyzed azide/alkyne cycloaddition*. Macromol Biosci, 2012. **12**(11): p. 1452–1458.
131. Besenicar, M.P., et al., *Kinetics of cholesterol extraction from lipid membranes by methyl-beta-cyclodextrin--a surface plasmon resonance approach*. Biochim Biophys Acta, 2008. **1778**(1): p. 175-84.
132. Tobias, R. and S. Kumaraswamy, *Biomolecular binding kinetics assays on the octet platform*. ForteBio Application Note, 2014. **14**: p. 1-21.
133. Buecheler, J.W., et al., *Development of a protein nanoparticle platform for targeting EGFR expressing cancer cells*. J Chem Technol Biotechnol, 2015. **90**(7): p. 1230–1236.
134. Li, L., et al., *Binding and uptake of H-ferritin are mediated by human transferrin receptor-1*. Proc Natl Acad Sci U S A, 2010. **107**(8): p. 3505–3510.
135. Li, W., et al., *Overexpression of transferrin receptor and ferritin related to clinical symptoms and destabilization of human carotid plaques*. Exp Biol Med (Maywood), 2008. **233**(7): p. 818-826.
136. Fenyvesi, F., et al., *Fluorescently labeled methyl-beta-cyclodextrin enters intestinal epithelial Caco-2 cells by fluid-phase endocytosis*. PLoS One, 2014. **9**(1): p. e84856.

137. Leman, L.J., B.E. Maryanoff, and M.R. Ghadiri, *Molecules That Mimic Apolipoprotein A-I: Potential Agents for Treating Atherosclerosis*. J. Med. Chem, 2013. **57**(6): p. 2169-2196.
138. Hafiane, A. and J. Genest, *HDL, Atherosclerosis, and Emerging Therapies*. Cholesterol, 2013. **2013**: p. 891403.
139. Rosenson, R.S. and A.M. Gotto, Jr., *When clinical trials fail to address treatment gaps: the failure of niacin-laropiprant to reduce cardiovascular events*. Curr Atheroscler Rep, 2013. **15**(332): p. 1-6.
140. Murphy, A.J., J. Chin-Dusting, and D. Sviridov, *Reconstituted HDL: a therapy for atherosclerosis and beyond*. Clinical Lipidology, 2009. **4**(6): p. 731-739.
141. Kuai, R., et al., *High-Density Lipoproteins: Nature's Multifunctional Nanoparticles*. ACS Nano, 2016. **10**(3): p. 3015-3041.
142. Ditiatkovski, M., et al., *Apolipoprotein A-I Mimetic Peptides: Discordance Between In Vitro and In Vivo Properties-Brief Report*. Arterioscler Thromb Vasc Biol, 2017. **37**(7): p. 1301-1306.
143. Sana, B., C.L. Poh, and S. Lim, *A manganese-ferritin nanocomposite as an ultrasensitive T2 contrast agent*. Chem Commun (Camb), 2012. **48**(6): p. 862-4.
144. Lee, E.J., et al., *Engineered Proteinticles for Targeted Delivery of siRNA to Cancer Cells*. Advanced Functional Materials, 2015. **25**(8): p. 1279-1286.
145. Uchida, M., et al., *Intracellular Distribution of Macrophage Targeting Ferritin-Iron Oxide Nanocomposite*. Advanced Materials, 2009. **21**(4): p. 458-462.
146. Tatur, J., W.R. Hagen, and P.M. Matias, *Crystal structure of the ferritin from the hyperthermophilic archaeal anaerobe Pyrococcus furiosus*. J Biol Inorg Chem, 2007. **12**(5): p. 615-30.
147. de Turrís, V., et al., *Humanized archaeal ferritin as a tool for cell targeted delivery*. Nanoscale, 2017. **9**(2): p. 647-655.
148. Lu, S.C., et al., *An apoA-I mimetic peptibody generates HDL-like particles and increases alpha-I HDL subfraction in mice*. J Lipid Res, 2012. **53**(4): p. 643-52.
149. Behzadi, S., et al., *Cellular uptake of nanoparticles: journey inside the cell*. Chem Soc Rev, 2017. **46**(14): p. 4218-4244.
150. Gustafson, H.H., et al., *Nanoparticle Uptake: The Phagocyte Problem*. Nano Today, 2015. **10**(4): p. 487-510.

151. Kettler, K., et al., *Uptake of silver nanoparticles by monocytic THP-1 cells depends on particle size and presence of serum proteins*. J Nanopart Res, 2016. **18**(9): p. 286.
152. Liu, Y., et al., *Effects of engineered nanoparticles on the innate immune system*. Semin Immunol, 2017. **34**: p. 25-32.
153. Cieslewicz, M., et al., *Targeted delivery of proapoptotic peptides to tumor-associated macrophages improves survival*. Proc Natl Acad Sci U S A, 2013. **110**(40): p. 15919-24.
154. Sharma, G., et al., *Targeting of Macrophage Foam Cells in Atherosclerotic Plaque Using Oligonucleotide-Functionalized Nanoparticles*. Nano Life, 2010. **1**(3-4): p. 207-214.
155. Gu, J., et al., *The internalization pathway, metabolic fate and biological effect of superparamagnetic iron oxide nanoparticles in the macrophage-like RAW264.7 cell*. Sci China Life Sci, 2011. **54**(9): p. 793-805.
156. Chithrani, B.D., A.A. Ghazani, and W.C. Chan, *Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells*. Nano Lett, 2006. **6**(4): p. 662-8.
157. Chithrani, B.D. and W.C. Chan, *Elucidating the mechanism of cellular uptake and removal of protein-coated gold nanoparticles of different sizes and shapes*. Nano Lett, 2007. **7**(6): p. 1542-50.
158. Su, Y.L. and S.H. Hu, *Functional Nanoparticles for Tumor Penetration of Therapeutics*. Pharmaceutics, 2018. **10**(4): p. 193.
159. Greish, K., *Enhanced permeability and retention (EPR) effect for anticancer nanomedicine drug targeting*. Methods Mol Biol, 2010. **624**: p. 25-37.
160. Skajaa, T., et al., *High-density lipoprotein-based contrast agents for multimodal imaging of atherosclerosis*. Arterioscler Thromb Vasc Biol, 2010. **30**(2): p. 169-76.
161. Truffi, M., et al., *Ferritin nanocages: A biological platform for drug delivery, imaging and theranostics in cancer*. Pharmacol Res, 2016. **107**: p. 57-65.
162. Zhao, Y., et al., *Bioengineered Magnetoferritin Nanoprobes for Single-Dose Nuclear-Magnetic Resonance Tumor Imaging*. ACS Nano, 2016. **10**(4): p. 4184-91.
163. Ramm, G.A., R.G. Ruddell, and V.N. Subramaniam, *Identification of ferritin receptors: their role in iron homeostasis, hepatic injury, and inflammation*. Gastroenterology, 2009. **137**(5): p. 1849-51.

164. Truman-Rosentsvit, M., et al., *Ferritin is secreted via 2 distinct nonclassical vesicular pathways*. Blood, 2018. **131**(3): p. 342-352.
165. Brix, K., P. Lemansky, and V. Herzog, *Evidence for extracellularly acting cathepsins mediating thyroid hormone liberation in thyroid epithelial cells*. Endocrinology, 1996. **137**(5): p. 1963-74.
166. Ravishankar, S. and S. Lim, *Cyclodextrin conjugated ferritin nanocages reduce intracellular cholesterol level in foam cells*. Nano Research, 2019. **12**(12): p. 2925-2932.
167. Kumari, S., S. Mg, and S. Mayor, *Endocytosis unplugged: multiple ways to enter the cell*. Cell Res, 2010. **20**(3): p. 256-75.
168. Hu, Y.B., et al., *The endosomal-lysosomal system: from acidification and cargo sorting to neurodegeneration*. Transl Neurodegener, 2015. **4**(18): p. 1-10.
169. McGlinchey, R.P. and J.C. Lee, *Cysteine cathepsins are essential in lysosomal degradation of alpha-synuclein*. Proc Natl Acad Sci U S A, 2015. **112**(30): p. 9322-7.
170. Stoka, V., B. Turk, and V. Turk, *Lysosomal cysteine proteases: structural features and their role in apoptosis*. IUBMB Life, 2005. **57**(4-5): p. 347-53.
171. Brzicova, T., et al., *Molecular Responses in THP-1 Macrophage-Like Cells Exposed to Diverse Nanoparticles*. Nanomaterials (Basel), 2019. **9**(5).
172. Dai, J., et al., *Autophagy Inhibition Contributes to ROS-Producing NLRP3-Dependent Inflammasome Activation and Cytokine Secretion in High Glucose-Induced Macrophages*. Cell Physiol Biochem, 2017. **43**(1): p. 247-256.
173. Yang, Y., et al., *Recent advances in the mechanisms of NLRP3 inflammasome activation and its inhibitors*. Cell Death Dis, 2019. **10**(128): p. 1-11.
174. Tai, W., et al., *A ribonucleoprotein octamer for targeted siRNA delivery*. Nat Biomed Eng, 2018. **2**(5): p. 326-337.
175. Kim, J.A., et al., *Role of cell cycle on the cellular uptake and dilution of nanoparticles in a cell population*. Nat Nanotechnol, 2011. **7**(1): p. 62-8.
176. La, A., et al., *Mobilization of iron from ferritin: new steps and details*. Metallomics, 2018. **10**(1): p. 154-168.
177. Sabelli, M., et al., *Human macrophage ferroportin biology and the basis for the ferroportin disease*. Hepatology, 2017. **65**(5): p. 1512-1525.

178. Ward, D.M. and J. Kaplan, *Ferroportin-mediated iron transport: expression and regulation*. Biochim Biophys Acta, 2012. **1823**(9): p. 1426-33.
179. Arosio, P. and S. Levi, *Ferritin, iron homeostasis, and oxidative damage*^{1,2} ¹Guest Editor: Mario Comporti ²This article is part of a series of reviews on "Iron and Cellular Redox Status." The full list of papers may be found on the homepage of the journal. Free Radical Biology and Medicine, 2002. **33**(4): p. 457-463.
180. Bakos, E., et al., *CCR2 Regulates the Immune Response by Modulating the Interconversion and Function of Effector and Regulatory T Cells*. J Immunol, 2017. **198**(12): p. 4659-4671.
181. Grome, H.N., et al., *Association of T Cell and Macrophage Activation with Arterial Vascular Health in HIV*. AIDS Res Hum Retroviruses, 2017. **33**(2): p. 181-186.
182. Bhaskar, S. and S. Lim, *Engineering protein nanocages as carriers for biomedical applications*. NPG Asia Materials 2017. **9**.
183. Parodi, A., et al., *Enabling cytoplasmic delivery and organelle targeting by surface modification of nanocarriers*. Nanomedicine (Lond), 2015. **10**(12): p. 1923-40.
184. Bus, T., A. Traeger, and U.S. Schubert, *The great escape: how cationic polyplexes overcome the endosomal barrier*. Journal of Materials Chemistry B, 2018. **6**(43): p. 6904-6918.
185. Boussif, O., et al., *A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine*. Proc Natl Acad Sci U S A, 1995. **92**(16): p. 7297-301.
186. Vaidyanathan, S., B.G. Orr, and M.M. Banaszak Holl, *Role of Cell Membrane-Vector Interactions in Successful Gene Delivery*. Acc Chem Res, 2016. **49**(8): p. 1486-93.
187. Sun, P., et al., *siRNA-loaded poly(histidine-arginine)6-modified chitosan nanoparticle with enhanced cell-penetrating and endosomal escape capacities for suppressing breast tumor metastasis*. Int J Nanomedicine, 2017. **12**: p. 3221-3234.
188. Plaza-Ga, I., et al., *pH-triggered endosomal escape of pore-forming Listeriolysin O toxin-coated gold nanoparticles*. J Nanobiotechnology, 2019. **17**(1): p. 108.
189. Suk, J.S., et al., *PEGylation as a strategy for improving nanoparticle-based drug and gene delivery*. Adv Drug Deliv Rev, 2016. **99**(Pt A): p. 28-51.
190. Khodabandehlou, K., et al., *Targeting cell adhesion molecules with nanoparticles using in vivo and flow-based in vitro models*

- of atherosclerosis*. Exp Biol Med (Maywood), 2017. **242**(8): p. 799-812.
191. Rodriguez, P.L., et al., *Minimal "Self" peptides that inhibit phagocytic clearance and enhance delivery of nanoparticles*. Science, 2013. **339**(6122): p. 971-5.
 192. Tang, Y., et al., *Overcoming the Reticuloendothelial System Barrier to Drug Delivery with a "Don't-Eat-Us" Strategy*. ACS Nano, 2019. **13**(11): p. 13015-13026.
 193. Chandrasekar, I., et al., *Nonmuscle myosin II is a critical regulator of clathrin-mediated endocytosis*. Traffic, 2014. **15**(4): p. 418-32.
 194. Lee, S. and N. Murthy, *Targeted delivery of catalase and superoxide dismutase to macrophages using folate*. Biochem Biophys Res Commun, 2007. **360**(1): p. 275-9.
 195. Wang, Y., et al., *Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling*. J Cell Biol, 2018. **217**(6): p. 1915-1928.
 196. Younus, H., *Therapeutic potentials of superoxide dismutase*. Int J Health Sci (Qassim), 2018. **12**(3): p. 88-93.

Chapter 7 : Appendix

Figure 7-1: Plasmid sequence and plasmid map of AfFtnAA sequence with a cysteine modification introduced at the 94th position in a pET11a vector.

Figure 7-2: Macrophage differentiation to form foam cells A. Visualized using oil red O and filipin staining and B. increase in intracellular cholesterol levels in foam cells vs. macrophage cells measured using cholesterol enzymatic assay.

Figure 7-3: A) Iron content per cell as quantified by using ICP-MS $\pm$ SD ($n = 3$, where n refers to the total number of wells). Endogenous iron present in AfFtnAA and Transferrin were obtained to be 1706 and 9 atoms respectively using the same method. Correlation between iron content per cell and endogenous iron content in individual protein molecules was used to quantify the

number of protein molecules localized per cell. B) Dye (Alexa Fluor 488) conjugation of AfFtnAA/M54C using thiol-maleimide reaction chemistry. Peak integration of mass spectrometry (MADLI-TOF) peaks indicate degree of labelling of 5 dye molecules conjugated per ferritin cage. C) Competitive uptake studies using dye conjugated ferritin nanocages (AfFtnAA-AF488) incubated with RAW 264.7 macrophage cells in the absence and presence of 5 times excess (by mass) transferrin. Reduced fluorescence intensity is observed for cells co-incubated with both ferritin and transferrin indicating decrease in AfFtnAA uptake due to competitive inhibition.

Figure 7-4: Quantification of intracellular cholesterol accumulation in foam cells treated with 5 μ M simvastatin and 5 μ M M β CD for 24h using Amplex Red Cholesterol assay. Cholesterol amounts per ug of cell protein has been normalized with untreated cells to obtain the % of accumulated intracellular cholesterol. Data is represented as a percentage of the average value of control (untreated) for each experiment $\pm$ SEM (n = 6, where n is the total number of wells).

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1  MGSSHHHHHHSSGLVPRGSMDFKAFYDKVAEKFKEAFGGGSHMMASISE 50
  ||||||||||||||||||||||||||||||||||||||||||||||||
1  MGSSHHHHHHSSGLVPRGSMDFKAFYDKVAEKFKEAFGGGSH-MASISE 49

51  KMVEALNRQINAEIYSAYLYLSMASYFDSIGLKGFSNWMRVQWQEELMHA 100
  ||||||||||||||||||||||||||||||||||||||||||||||||
50  KMVEALNRQINAEIYSAYLYLSMASYFDSIGLKGFSNWMRVQWQEELMHA 99

101 MKMDFVSEGRGGRVKLYAVEEPPSEWDSPLAAFEHVYEHEVNVTKRIHEL 150
  ||||||||||||||||||||||||||||||||||||||||||||||||
100 MKMDFVSEGRGGRVKLYAVEEPPSEWDSPLAAFEHVYEHEVNVTKRIHEL 149

151 VEMAMQEKDFATYNFLQWYVAEQVEEEASALDIVEKLRLIGEDAAALLFL 200
  ||||||||||||||||||||||||||||||||||||||||||||||||
150 VEMAMQEKDFATYNFLQWYVAEQVEEEASALDIVEKLRLIGEDAAALLFL 199

201 DKELSLRQFTPPAEEEEK--- 217 >> Sequenced
  ||||||||||||||||
200 DKELSLRQFTPPAEEEEK*GS 219 >> Template

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Figure 7-5: Sequence alignment 4F-AA amino acid sequence

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1  MGSSHHHHHHSSGLVPRGSMDFKAFYDKVAEKFKEAFKVEPLRADWFKA 50
  ||||||||||||||||||||||||||||||||||||||||||||||||
1  MGSSHHHHHHSSGLVPRGSMDFKAFYDKVAEKFKEAFKVEPLRADWFKA 50

51  FYDKVAEKFKEAFGGGSH-MASISEKMVEALNRQINAEIYSAYLYLSMAS 99
  ||||||||||||||||||||||||||||||||||||||||||||||||
51  FYDKVAEKFKEAFGGGSHMMASISEKMVEALNRQINAEIYSAYLYLSMAS 100

100 YFDSIGLKGFSNWMRVQWQEELMHAMKMDFVSEGRGGRVKLYAVEEPPSE 149
  ||||||||||||||||||||||||||||||||||||||||||||||||
101 YFDSIGLKGFSNWMRVQWQEELMHAMKMDFVSEGRGGRVKLYAVEEPPSE 150

150 WDSPLAAFEHVYEHEVNVTKRIHELVEAMQEKDFATYNFLQWYVAEQVE 199
  ||||||||||||||||||||||||||||||||||||||||||||||||
151 WDSPLAAFEHVYEHEVNVTKRIHELVEAMQEKDFATYNFLQWYVAEQVE 200

200 EEASALDIVEKLRLIGEDAAALLFLDKELSLRQFTPPAEEEEK*GS >> Template
  ||||||||||||||||||||||||||||||||||||||||||||||||
201 EEASALDIVEKLRLIGEDAAALLFLDKELSLRQFTPPAEEEEK--- >> Sequenced

```

Figure 7-6: Sequence alignment 2X4F-AA amino acid sequence

Figure 7-7: Size exclusion chromatograms of A) Apo 4F-AA and B) Apo 2X4F-AA.

Figure 7-8: Apo protein structural prediction of A) 4F-AA and B) 2X4F-AA predicted using Rosetta Software.

Figure 7-9: SDS-PAGE analysis of solubility of 4F-HsFtn (Red) and 2X4F-HsFtn (Blue) resuspended in 25mM HEPES (pH 7.4), 50mM Tris (pH 8.5), 20mM Tris 50mM NaCl (pH 9), 10mM Tris 0.25M sucrose (pH 7.4), DI water, Glycine (pH 2.0), 2M urea 20mM Tris and 8M urea conditions yielding supernatant (S) and pellet fractions (P) after centrifugation at 100,000xg.

Figure 7-10: Relative fold change of ABCA1 mRNA expression levels in foam cells treated with 200nM AfFtnAA and 4F-AfFtnAA with respect to untreated foam cells.

Table 7-1: Primers used for RT-PCR of different genes.

Gene	Primers
ABCA1	F: 5'-GGTTTGGAGATGGTTATACAATAGTTGT-3' R: 5'-CCCGGAAACGCAAGTCC-3'
ABCG1	F: 5'-TTCCCCTGGAGATGAGTGTC-3' R: 5'-CAGTAGGCCACAGGGAACAT-3'
SR-BI	F: 5'-ACCCTAACCCAAAGGAGCAT-3' R: 5'-CACAGCAACGGCAGAACTAC-3'
Ferroportin	F: 5'-TCATTGCTCTAGAATCGGTCTT-3' R: 5'-CACACCATTGATAATGCCTCTTTC-3'
L-Ferritin	F: 5'-TTCCAGGATGTGCAGAAGCC-3' R: 5'-AAGAGGGCCTGATTCAAGTTTC-3'
H-Ferritin	F: 5'-CTAACCTCCTCGCCAATAGTTC-3' R: 5'-CGCACTCACCATAGACAGATAG-3'
β -Actin	F: 5'-TCAAGATCATTGCTCCTCCTGAG-3' R: 5'-CTGCTTGCTGATCCACATCTG-3'
VCAM1	F: 5'-AGTTGGGGATTTCGGTTGTTC-3' R: 5'-CATTCTTACCACATTG-3'
CCR2	F: 5'-CCTGCAAAGACCAGAAGAGG-3' R: 5'-GTGAGCAGGAAGAGCAGGTC-3'
TNF α	F: 5'-ATAGCTCCCAGAAAAGCAAGC-3' R: 5'-CACCCCGAAGTTCAGTAGACA-3'
IL1 β	F: 5'-GCCTTGGGCCTCAAAGGAAAGAATC-3' R: 5'-GGAAGACACAGATTCCATGGTGAAG-3'
NLRP3	F: 5'-TGCTCTTCACTGCTATCAAGCCCT-3' R: 5'-ACAAGCCTTTGCTCCAGACCCTAT-3'

Figure 7-11: Fluorescence imaging of cells treated with 200 nM FITC conjugated AfFtnAA for 1 h at 4°C.

Figure 7-12: Average cell numbers for ferritin treated macrophages for varying time-points indicated mitosis (cell proliferation over time).

Figure 7-13: Amount of Fe accumulated per cell in cells incubated with 200nM of AfFtnWT (open pore Af ferritin), AfFtnAA and Humanized Af ferritin (HuAftn) for 24 h. Negative control correspond to untreated cells in the absence of any ferritin treatment.

Figure 7-14: mRNA expression changes A) IL1 β expression in cells treated with Apo AA and (Fe2400) AA for 24 h normalized with respect to untreated cells to obtain degree of fold change (n = 2). B) NLRP3 expression in cells treated with AA for 48 h normalized with respect to untreated cells to obtain degree of fold change.

Figure 7-15: A) Schematic representation of insert plasmid sequence of HHEN-AA and HHEC-AA with the alternatively spaced linkers highlighted. The insert plasmid is ligated in a pET11-a vector at the indicated restriction sites. B)

Temperature stability of HHEN-AA and AA-HHEC at 65°C, 75°C and 85°C visualized using SDS-PAGE indicating soluble fractions (S) and insoluble pellet fractions (P). The expected molecular weight of both constructs is at 25kDa. C) Hydrodynamic diameter of HHEN-AA and HHEC-AA measured using dynamic light scattering.

Figure 7-16: Transmission electron micrographs of AA, HHEN-AA and HHEC-AA. Stained images (First row), Unstained images (Middle row) and Particle size distribution estimated using ImageJ (Last row). (1200Fe)₆₀₄AA indicates that 1200 Fe atoms added during protein assembly and 604 atoms were loaded within the core as measured using ICP-MS after desalting. The same notation for the Fe atoms added verses that loaded is followed for HHEN-AA and AA-HHEC.

Figure 7-17: Ex-vivo fluorescence image obtained from IVIS for liver, kidney, heart, spleen, lungs, stomach and intestine of mice injected with Cy5 conjugated AfFtnAA after 1 h.