USE OF NANOPARTICLES (HPPS) TO CARRY CURCUMIN TO INDUCE HEME
OXYGENASE-1 (HO-1) AS A CYTOPROTECTIVE AGENT IN HUMAN
ENDOTHELIAL CELLS

by

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ABSTRACT

High-density lipoproteins (HDL) are endogenous nanoparticles that maintain physiological levels of low-density lipoprotein (LDL) via reverse cholesterol transport (RCT). HDL is known to reduce atherosclerotic lesions, however, the mechanism of nano-lipoproteins for treating atherosclerosis at the cellular level has not been clearly elucidated. Heme Oxygenase-1 (HO-1) is a stress responsive, cytoprotective and rate-limiting enzyme in heme degradation to produce potent antioxidant effects. Curcumin is a naturally occurring anti-oxidant drug that increases the expression of HO-1. Here, we show synthesis and characterization of HPPS and curcumin-loaded HPPS nanoparticles as a potential nanomedicine candidate for improving endothelial tolerance to stress. Both nanoparticles and nanoparticles loaded with curcumin are physiologically biocompatible in HUVEC. Time and dose-dependent cellular exposure to curcumin was shown to induce HO-1 expression. These results suggest a potential novel therapeutic strategy for atherosclerosis. Confirmation of dose-equivalency and cell specificity of HPPS is still required before we can proceed from *in-vitro* studies to *in-vivo* studies to investigate the efficacy of curcumin-loaded HPPS nanoparticles as a candidate therapy.

LIST OF ABBREVIATIONS USED

5-ALA	5-aminolevulinic acid
ABCA	ATP-binding cassette subfamily B member
ABCG	ATP-binding cassette sub-family G member
ANOVA	Analysis of variance
Ang-II	Angiotensin-II
ATP	Adenosine triphosphate
Apo	Apolipoprotein
BCA	Bicinchonic acid
BH_4	Tetrahydrobiopterine
BSA	Bovine serum albumin
BVE	Biliverdin reductase enzyme
BVR	Biliverdin reductase
BR	Bilirubin
CD	Cluster of differentiation
CE	Cholesteryl ester
CETP	Cholesterol ester transfer protein
CVD	Cardiovascular Disease
cGMP	Cyclic guanosine monophosphate
CO	Carbon monoxide
COX-2	Cycloxygenase-2
Cur	Curcumin
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
d.nm	Deci newton meter
DTT	Dithiothreitol
eNOS	endothelial nitric oxide synthase
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
Egr-1	Early growth response-1 protein
ET-1	Endothelin-1
FACS	Fluorescence-activated cell sorting
FC	Free Cholesterol
GST	Glutathione S-transferase

HDL	High density lipoprotein
HMOX	Heme oxygenase
HMG CoA	Hydroxymethyl glutaryl coenzyme A reductase
HPPS	HDL Mimicking Protein Scaffold
HSP	Heat Shock Protein
HRP	Horseradish peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells
IL	Interleukin
ICAM-1	Intracellular adhesion molecule-1
IFN-γ	Interferon gamma
IkBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
kDa	kiloDalton
LDL	Low density lipoprotein
LCAT	Lecithin:cholesterol acyl transferase
LPS	Lipopolysaccharide
LOX	Lipoxygenase
MCP-1	Monocyte chemoattractant protein-1
MAPKs	Mitogen-activated protein kinases
MMP	Matrix Metalloproteinases
mRNA	Messenger RNA
NOS	Nitric oxide synthase
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
NC	No change
NioCur	Niosome nanoparticle loaded with Curcumin
ND	Not detected
NF-kB	Nuclear factor-kappa B
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NPDDS	Nanoparticle drug delivery system
oxLDL	Oxidized low density lipoprotein
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PGI-2	Prostacycline
PL	Phospholipid
PPARα	Peroxisome proliferator-activated receptor α
PON 1	Paraoxonase 1
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute Medium
SCARB1	Scavenger Receptor Type B1

SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
sGC	Soluble guanylate cyclase
TEM	Transmission Electron Microscopy
TG	Triglyceride
THP1	Human Monocytic-Macrophage Cell Line
TNFα	Tumor necrosis factor-α
TGF-β	Transforming growth factor
VCAM-1	Vascular adhesion molecule
VSMC	Vascular smooth muscle Cell

ACKNOWLEDGEMENTS

Chapter 1: Introduction

Since industrialization, the leading causes of death and disability shifted from nutritional deficiencies and infectious diseases, to chronic diseases, such as cardiovascular disease (CVD), cancer, and diabetes. This change in disease pattern has been termed 'the epidemiologic transition' [1]. Cardiovascular diseases arising from atherosclerosis are a major cause of death globally [2]. Atherosclerosis is the thickening and loss of elasticity of the walls of arteries, which is associated with the formation of atherosclerotic plaques within the arterial intima.

1.1. Atherosclerosis Pathophysiology

Atherosclerosis is a disease whose cause is fundamentally linked with inflammation. It is a multi-factorial disease, associated with risk factors such as hypercholesterolemia, hypertension, diabetes, obesity, smoking, diet, aging, gender, family history, air-pollution-either individually or combined. These risk factors are all associated with oxidative stress at the cellular level causing that can trigger and result in unrelenting inflammation. A healthy artery is composed of three layers: adventitia (outer most layer), media (middle layer) and intima (the inner most layer), the latter consisting of a single layer of endothelial cells (EC). In atherosclerosis, dysfunctional endothelial cells result in an accumulation of macrophages along with oxidized low density lipoproteins (oxLDL) that form a fatty streak which further progresses to unstable plaque. However, the reactions within the injured intima and their severity in the progression of the lesion are more complex than simply oxLDL and macrophages converting into foam cells and thickening of the intima [3].

Endothelial cells are simple squamous cells that functionally play vital role in maintaining homeostasis of blood flow throughout the body [4]. Three important components are released from the endothelium: 1. nitric oxide (NO), 2. prostacycline (PGI-2) and 3. endothelin-1 (ET-1). The former two components act as vasodilators, whereas the latter acts as vasoconstrictor [5]. Nitric oxide (NO) is a gasotransmitter [6], which is synthesized and released by the vascular endothelial cells. NO acts as a vasodilator but also is an anti-inflammatory agent and serves to prevent platelet activation [7]. Inside the endothelium, enzyme nitric oxide synthase (NOS) and

cofactor tetrahydrobiopterine (BH$_4$) convert L-arginine to NO, which then diffuses into the lumen to limit adhesion of platelets [8] and also inhibits monocyte adhesion [9].

Once synthesized by EC, NO also diffuses into vascular smooth muscle cells (VSMC) to activate soluable guanylate cyclase (sGC) to make cGMP as a secondary messenger to mediate vasodilatation [10]. Therefore, excessive ROS (superoxide anion, O$_2^-$) and/or other pro-inflammatory mediators interfere with the NOS pathway, reduces NO synthesis, hence disrupts the balance between vasodilation and vasoconstriction [11]. Lack of NO further triggers the middle layer of the artery (media) to secrete chemokines, cytokines, growth factors and expresses adhesion molecules (e.g. VCAM-1, ICAM-1) [12]. Moreover, reduced NO also makes vascular smooth muscle cells (which were previously in a quiescent and contractile state to enter into an active proliferative, synthetic state) capable of migrating and populating the intima layer. Here they can also turn into foam cells, die and contribute to the necrotic core or attempt to form a fibrotic cap over the necrotic core so as to stabilize the lesion [13]. The advanced lesion with a necrotic core is finally composed of fatty lipids, active-synthetic VSMCs, extracellular matrix, lower pH, less NO, cellular and calcific debris [14]. Based on the degree of physical stability, these plaques can be either stable or unstable and vulnerable. Stable plaques have small lipid cores with more smooth muscle cells, extracellular matrix and a thick fibromuscular cap [14]. This type of plaque tends to reduce the vascular lumen diameter and can block the regular blood flow, but is less likely to rupture, causing infarction. The unstable and vulnerable plaque contains a large portion of lipid core with lots of macrophages and has a thin cap with variable endothelial cell coverage. Inflammatory cytokines, such as TNFα or IL-1, stimulate the up-regulation of matrix metalloproteinases (MMPs) in macrophages. Both the loss of endothelial NO to prevent platelet adhesion and the loss of ECM in the cap can cause the plaque to rupture (see Figure-1 for a summary) [14].

Inflammation can be termed a "double-edged sword" due to its favorable and unfavorable consequences in the body. It is a complex response of the body's immune system to repair and protect the body from injury and infections. An important primary function of healthy endothelial cells is to act as an physiological sensor (to blood shear stress and pressure) to prevent platelet activation and blood clotting through the production of NO. In addition, the endothelium acts as a barrier to the circulating blood and the blood vessel. It does this to keep the blood vessel lumen elastic and responsive to blood flow, by sensing physical stress to release NO

in order to relax the vessel and keep smooth muscle cells in a quiescent/contractile state [15]. However, an activated immune system can create an imbalance between the systemic pro-inflammatory and anti-inflammatory mediators, which may contribute to EC damage and dysfunction, possibly further triggering the formation and progression of atherosclerosis [4]. For example, pro-inflammatory markers in type-1 diabetes patients increase an endogenous inhibitor (asymmetric dimethylarginine) of NO: reducing NO function, causing increased intima-media thickness (with smooth muscle cell proliferation) and leading to the development of atherosclerosis [6]. Inflammation can lead to a release of excess free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) that create imbalance between oxidants and antioxidants causing oxidative stress at the cellular level and impairing mitochondrial function. Cardiovascular risk factors in general, such as mechanical sheer stress from hypertension for example and increased cellular oxidative stress from smoking for example, distress the endothelium. Thus, endothelium either releases chemokines, such as monocyte chemoattractant protein-1 (MCP-1), cytokines (tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1-β), IL-6, interferon γ) or expresses adhesion molecules to attract monocytes, such as vascular adhesion molecule (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), P-selectin, E-selectin, on EC surface [3]. This inflammation leads to increased endothelial permeability, causing LDL to invade the sub-intimal wall; followed by the adhesion and migration of monocytes, lymphocytes, or mast cells into the intima of the artery. Within the inflammatory environment, adherent monocytes convert to macrophages. LDL is ingested by macrophages to become oxidized into oxLDL, which can eventually be toxic to a macrophage. Macrophages engulf either LDL or oxLDL in an attempt clear the damaged area, but the inflammation along with ROS and oxLDL convert macrophages into foam cells in a process of trans-differentiation [6]. Continuous formation of foam cells along with accumulation of lipids, oxLDL and other pro-inflammatory cytokines cause foam cell death, which releases the oxLDL and perpetuates more inflammation. Eventually, a fatty streak is formed in the artery with a hostile microenvironment that can grow to form an unstable necrotic core. Simultaneously, the necrotic and inflamed environment disrupts additional neighboring healthy endothelium, contributing to progressive endothelial dysfunction and continuation of lesion expansion. Therefore, the endothelium is critical to preventing the incipient event of inflammation and ongoing progression of atherosclerosis.
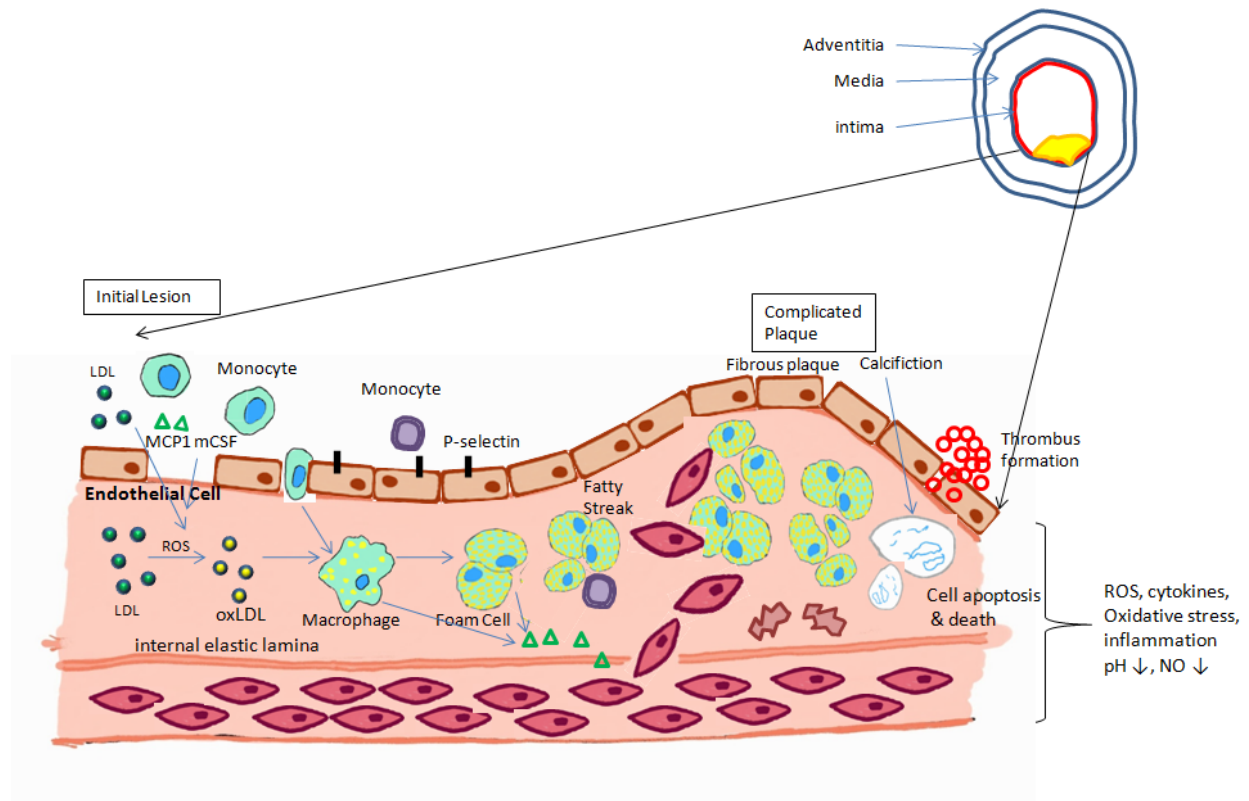
Figure 1:Formation and progression of atherosclerosis.

Blood circulating leukocytes (monocytes, mast cells and lymphocytes) and LDL invade the damaged and inflamed endothelium into the sub-endothelium layer. In the presence of ROS and other pro-inflammatory cytokines, monocytes ingest oxLDL and convert to macrophages. Macrophages generate more ROS and make oxLDL highly reactive, which is further taken up by macrophages to form foam cells. Foam cells accumulate to appear as a fatty streak; as the process continues, foam cells release chemokines and growth factors, which induce smooth muscle cells (SMC) to migrate and proliferate into the sub-endothelial space. Foam cells eventually die and further propagate the inflammatory process. Proliferation and migration of VSMCs along with ingestion of lipids build up the necrotic core with extracellular matrix synthesis and calcification. A protective fibrous cap may form between the fatty deposits and artery lining, but can break down and activate platelets to cause thrombosis and artery occlusion.

1.1.1. Principal Cells Acting in the Progression of Atherosclerosis

Human blood vessels are composed of three basic layers. The inner most layer is known as intima, which is composed of single layer of ECs and an underlying extracellular matrix of elastic lamina. The middle layer is known as media, comprised of vascular smooth muscle cells (VSMC), which is further surrounded by their own basement membrane, a few resident monocytes and fibroblasts. Finally, the outer most layer is called adventitia and consists of connective tissues with nerve fibers, small blood vessels (vasa vasorum, in larger vessels), and adipose tissue in a loose intracellular matrix [16],[17],[18]. These different intact cell layers play essential roles in vascular homeostasis. However, once injured or dysfunctional, they may contribute directly and/or indirectly in the formation and progression of atherosclerosis.

A. Endothelial Cells (EC): Endothelium is an active, single layer of cells comprising the inner most lumen of a blood vessel and separating the vascular wall from the circulating blood [19]. An endothelium acts as, highly selective, semi-permeable barrier and controls the transfer of molecules from the blood circulation [4]. ECs maintain vascular homeostasis by keeping a delicate balance between vasodilation and vasoconstriction [20]. To maintain a balanced vascular tone, factors such as NO, prostacyclin, endothelin-1, angiotensin II, thromboxane A2 and prostaglandin play important roles [21]. There is abundant evidence demonstrating that NO is a vital factor among other endothelial-derived chemicals. NO is a biologically active, volatile gasotransmitter (half-life of 6-30 seconds) in all tissues [22]; the low molecular weight (30.01 g/mol) and lipophilic properties help NO diffuse easily across cell membranes [23]. There are three forms of nitric oxide synthase (NOS) enzymes: 1. endothelial isoform (eNOS) that is being produced in the endothelial cells and is important in managing vascular tone; 2. neuronal isoform (nNOS) that produces NO mainly as a messenger to regulate synaptic neurotransmitter release and 3. inducible isoform (iNOS) expressed in leukocytes and endothelial cells in response to pro-inflammatory cytokines (such as tumor necrosis factor-α (TNF-α) [24]. In general, NO is produced in ECs when constitutive calcium-calmodulin (while other kinases, such as CaM-kinase Akt, AMPK, PKA phosphorylate at S1179, S617, & S635) bind with endothelial NO synthase (eNOS) enzyme to align the oxygenase-reductase domains and stimulate conversion of the amino acid L-arginine to NO [25]. The calcium ion is an important secondary messenger converting extracellular signals into a variety of physiological responses. Calmodulin, the

ubiquitous and multifunctional $Ca2^+$ protein, mediates many of the regulatory effects of $Ca2^+$ including the contractile state of smooth muscle [26]. eNOS is constitutively expressed in certain cells and activated by $Ca2^+$-calmodulin [27]. Calmodulin undergoes phosphorylation (or activated PKC phosphorylates T497) in endothelial cells, which then reduces eNOS activation [28]. Evidence suggests, disturbed, damaged and/or dysfunctional ECs play key role in many disease processes, such as atherosclerosis, hypertension, pulmonary hypertension, sepsis and inflammatory syndromes [4]. Endothelial dysfunction is observed in the early stage of most cardiovascular diseases [29].

B. Vascular Smooth Muscle Cells (VSMCs): In healthy blood vessels, the phenotype of VSMCs are quiescent and contractile to regulate blood vessel diameter during vasodilation (relaxing state) and vasoconstriction (contracting state). In response to injury or stress VSMCs convert to synthetic, migratory and proliferative phenotypes [13]. Not only does the active, synthetic state of VSMC migrate and proliferate from media to intima, but they can also degrade extracellular matrix (ECM) proteins until rendered quiescent again to begin reforming ECM [13]. Inflammatory cytokines, such as IL-1, IL-4, and TNF-α, act synergistically with growth factors, such as platelet derived growth factor (PDGF), fibroblast growth factor (FGF)-2 to induce a broad range of MMPs [30]. The mammalian MMPs are a family of at least 25 secreted or surface bound proteases, out of which 14 have been characterized in vascular cells [31]. The MMPs participate in the process of VSMC migration by breaking down matrix to penetrate and migrate towards the chemokine stimulation. MMPs reduce the basement membrane contacts within the interstitial matrix. This could promote a change from quiescent, contractile VSMC to the active, synthetic state cells capable of migrating and proliferating [13]. Braun M et al. states that human smooth muscle cells (SMCs) express VCAM-1 and ICAM-1 to interact with leukocytes to promote inflammation [32]. Migration of VSMCs from the media to the intima is an essential part of the pathologic processes of atherosclerosis. The growth promoters (such as PDGF, ET-1, thrombin, FGF, interferon gamma (IFN-γ), IL-1) and growth inhibitors (including heparin sulfates, NO, transforming growth factor (TGF)-β) regulate the migratory and proliferative activities of VSMCs [33].

C. Monocyte and Macrophages: The pathophysiological roles of phagocytes (e.g. monocyte-macrophages, dendritic cells and neutrophils) are varied in different stages of atherosclerosis. Among the phagocytes, monocytes and macrophages play a major role. In the context of atherosclerosis, these cells become foam cells and initiate formation of fatty streaks [34]. Monocytes do not adhere or migrate into a healthy intimal layer. However, injured or inflammed ECs recruit monocytes by expressing adhesion molecules among like VCAM-1, ICAM-1, or P-selectins [35]. Once these adhesion molecules are expressed on the surface of dysfunctional ECs, monocytes use them to attach, roll, arrest and then transmigrate in the intima. The recruited monocytes differentiate into macrophages or dendritic cells (DCs) and try to clear away lipoproteins (oxLDL, LDL or VLDL) by phagocytosis [35]. The inflammatory environment within the injured intima along with lipoproteins is continuously stimulating macrophage phagocytosis and recruiting additional monocytes. Eventually macrophages become foam cells [36]. As foam cells build up into fatty streaks, macrophage lipid metabolism becomes defective and foam cell death occurs by either apoptosis or necrosis - causing release of modified lipoproteins like oxLDL and ultimately reinforcing the expansion of the plaque [37]. New macrophages may try to clear up the early apoptotic cells by a process known as efferocytosis [38]. Initially, efficient efferocytosis tends to reduce pro-inflammatory mediators and induce anti-inflammatory mediators, such as IL-10 and/or TGFβ [39]. However, with progression of atherosclerosis, efferocytosis deteriorates. This leads to secondary necrosis with the accumulation of more cellular debris, oxidized lipid, pro-inflammatory mediators that magnify the necrosis and speed up the atherosclerosis progression and complexity [39].

D. Platelets: Platelets are small cell-like constituents shed by bone marrow megakaryocytes. They do not have a nucleus, however, they have a few viable mitochondria, and glycogen stores [40]. Platelets play a vital role in hemostasis (clotting), wound healing, and inflammation [41]. Under physiological conditions, platelets remain dormant and flow freely in the blood [42]. Activation of platelets is prevented by intact ECs through NO and prostacyclin [43]. Endothelial dysfunction triggers the activation of platelets, allowing them to interact with the blood vessel and monocytes [44]. Activated platelets tether and eventually adhere to the vascular wall via different types of adhesion molecules, such as VCAM-1 and P-selectin. When the tethering, activation and adhesion phase is complete, platelets discharge a set of chemokines and pro-

inflammatory mediators, such as MCP-1, IL-1β and IL-8, for recruiting more monocytes at the injured site of an endothelium and PDGF possibly attracting VSMC [45].

Understanding the key regulators and involvement of ECs, VSMCs, macrophages and platelets within the different stages of atherosclerosis will facilitate and inform future strategies to modulate the disease process as well as help researchers to come up with various innovative disease prognosis and diagnosis.

1.1.2. Molecular Mechanisms in the Progression of Atherosclerosis

Inflammation and oxidative stress at the cellular level are two major components, acting synergistically and contributing to the progression of atherosclerosis. Oxidative stress and inflammation are the outcome of excess reactive oxygen species (ROS) in the body. ROS are free radicals, oxygen radicals and peroxides - all derived from molecular oxygen (for e.g. superoxide anion $O_2^{\cdot-}$, and hydroxyl radical: $OH^{\cdot}$, H_2O_2, peroxynitrite ($ONOO^-$)). They are formed as necessary intermediates (i.e. H_2O_2) or dangerous waste products and inadvertent reactions (i.e. $OH^{\cdot}$) in a variety of normal biochemical reactions of our cells, mostly from the mitochondria [46]. All cells release a proportion of ROS as a bi-product during regular cellular function [47], which indicates ROS have an important role in cellular homeostasis and cellular communication [46]. Metaphorically, we can also consider ROS as a 'double-edged sword' - because at the lower ranges of concentrations, ROS contribute to cellular homeostasis and can act as secondary messengers; however, in excess, they disrupt homeostasis and can be causative to various diseases. For most tissues, the primary source of O_2^- is situated in the mitochondrial electron transport chain [48]. In vascular pathology, ROS, specifically O_2^-, interfere with endothelial nitric oxide synthase (eNOS) [49]. Tetrahydrobiopterin (BH_4) is required in the catalytic process of L-arginine oxidation as a co-factor and moreover balances nitric oxide (NO) and O_2^- production. Absent or reduced BH_4 causes more O_2^- to be released, rather than NO. As a result, increased O_2^- quickly reacts with available NO to form peroxinitrite ($ONOO^-$). Peroxinitrite oxidizes BH_4 resulting in dysfunctional enzyme [50], [51]. As a result, eNOS contributes to oxidative stress by overproducing superoxides (O_2^-), rather than NO. Such dysfunction is defines as "eNOS uncoupling," [52], [53]. eNOS uncoupling and NO reduction are not the only disturbances made by ROS. Oxidative stress generated by ROS can also affect

various other factors, such as activating MMPs (MMP-1, MMP2, MMP-7, MMP-9) indirectly by activating mitogen-activated protein kinases (MAPKs) or activating NF-κB to increasing inflammatory cytokine production or increase adhesion molecules [54]. Although superoxide is a highly active free radical, the role of hydrogen peroxide (H_2O_2) cannot be overlooked [55]. H_2O_2 belongs to the ROS family but is mostly known as a waste product of mitochondrial electron transfer chain. Maintaining the concentration of H_2O_2 is crucial because high concentrations cause apoptotic cell death, whereas low concentrations are involved in cell signaling and proliferation [55].

1.2. HO-1 in Atherosclerosis

Heme is a prosthetic group that is synthesized by a series of reactions, starting in the mitochondria with the condensation reaction of glycine and succinyl-CoA to form 5-aminolevulenic acid (ALA). 5-ALA moves out from the mitochondria into the cytoplasm for another series of reactions to form coproporhyrinogen III which then re-enters mitochondria to form protoporphyrin IX. As a result, Fe^{2+} (ferrous ion) and ferrochelatase enzyme convert protoporphyrin IX into heme [56]. Heme oxygenase (HO) is a rate-limiting enzyme found in smooth endoplasmic reticulum of almost all tissues. It exists in two isoforms: heme oxygenase-1 (HO-1), a 32kDa inducible enzyme (also known as heat shock protein (HSP)) and heme oxygenase-2 (HO-2), is a 37kD a constitutive enzyme found particularly in brain and testis [57]. The major factors inducing HO-1 expression are heme, cellular ROS, UV-light, NO, pro-inflammatory cytokines, hypoxia and/or electrophiles, including polyphenolic compounds [57]. Inflammation, free radicals and oxidative stress are interconnected elements, contributing to the pathogenesis of atherosclerosis. Yet SF et al. (2003) showed that the knock-out of HO-1 in *in vivo* models (both HO-1 and apolipoprotein E knockout mice models) increased atherosclerotic lesion formation and vascular remodeling [58]. Therefore, considerable amounts of evidence suggest HO-1 has anti-atherogenic properties, as it shields against ROS and inflammatory cells by breaking down heme into three by-products that individually and/or in combination have cytoprotective and anti-atherogenic properties. HO-1 metabolizes heme into three important by-products; 1). free iron (Fe^{2+}), 2). carbon monoxide (CO) and 3) biliverdin, which is converted into bilirubin via biliverdin reductase. [56]. Figure 2 demonstrates the benefits of heme

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catabolism by HO-1 along with the anti-apoptotic, anti-oxidant, vasodilatory and anti-inflammatory properties of the by-products, which individually and cumulatively exhibit anti-atherogenic actions.

To begin with, biliverdin (BVR), an immediate by-product of heme degradation, can be quickly converted into bilirubin (BR) by biliverdin reductase enzyme (BVR). This reversible conversion of BVR to BR creates a cellular antioxidant cycle, where BR acts a scavenger for free radicals and attenuates lipid peroxidation as an antioxidant. Furthermore, the by-product of heme break down is carbon monoxide (CO) [59]. CO is a gasotransmitter with properties that closely mimic those of NO as a vasodilator, however, it is less potent than NO and has a longer half-life. CO acts on soluble guanylate cyclase (sGC) and converts GTP to cGMP, to elicit vasodilatory action on SMCs [60]. CO also reduces pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α and increases anti-inflammatory cytokine IL-10. It also decreases VCAM-1 secretion and attenuates endothelium-leukocyte adhesion [56]. The final by-product of heme degradation is free iron (Fe^{2+}). Although, free iron (Fe^{2+}) exhibits pro-oxidant effects, it also participates in the Fenton reaction to generate hydroxyl ion (OH^-), however, it stimulates the iron-storage protein, ferritin which is believed to reduce this secondary reaction and shows anti-oxidant properties [57].
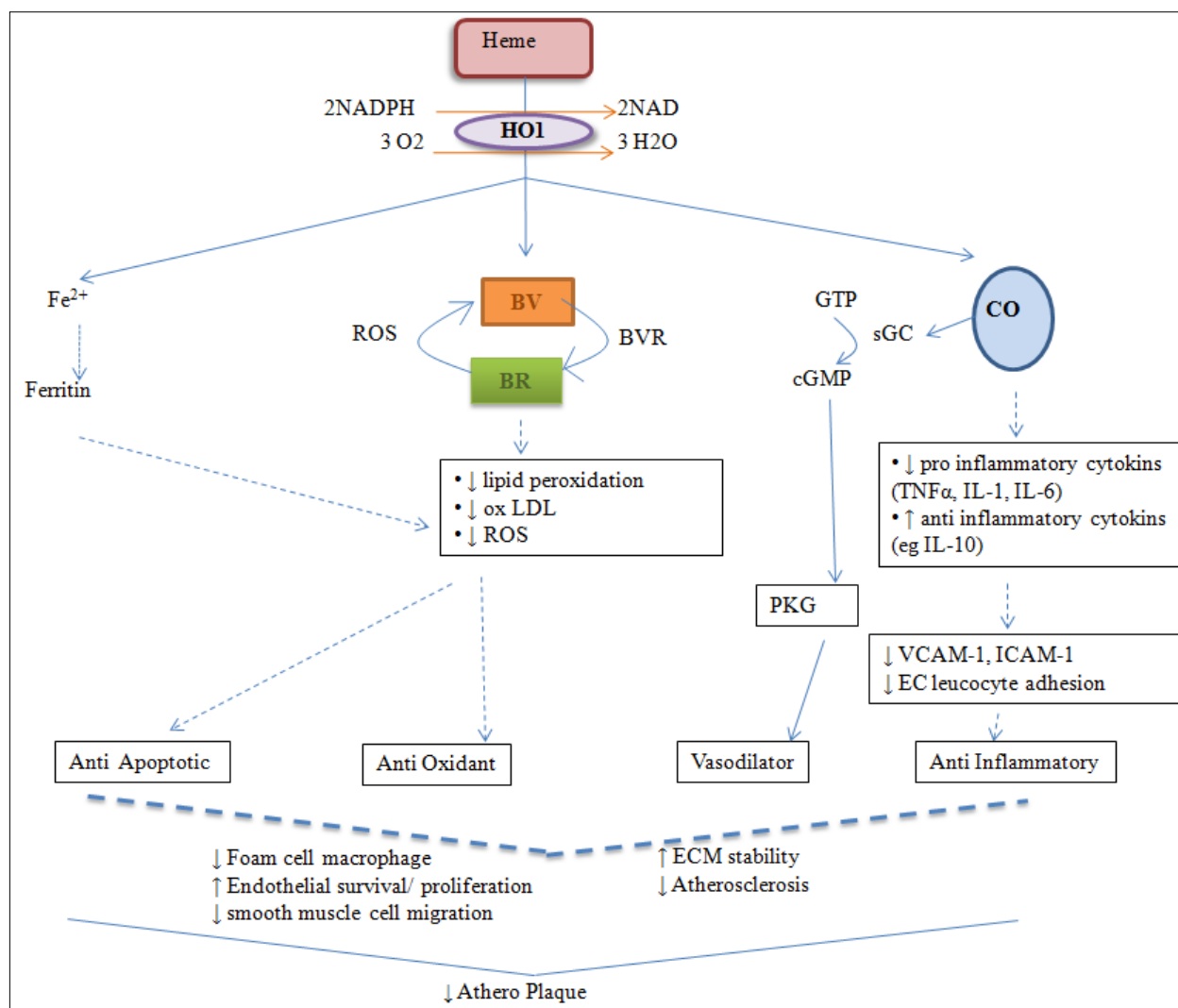
Figure 2 Benefits of heme oxygenase-1 (HO-1) mediated heme catabolism.

Heme is degraded by HO-1 to generate iron (Fe2+), biliverdin (BV) and carbon monoxide (CO). BV is immediately converted to bilirubin (BR) via biliverdin reductase (BVR). Iron induces and is sequestered by anti-oxidant ferritin. Carbon monoxide (CO) functions as a gasotransmitter for vasodilatory and cytoprotective actions. These by-products possess anti-apoptotic, anti-oxidant, vasodilatory and anti-inflammatory properties, which individually and or cumulatively have anti-atherogenic actions.

Within the vascular cells, HO-1 is a significant anti-oxidant gene controlled by the transcription factor Nrf2 (NFE2 related factor 2) and NF-kB. HO-1 is significantly expressed in all cell types (ECs, macrophages, and SMCs) present in mouse and human atherosclerotic lesions [56].

There is cumulative evidence that suggests HO-1 is a pharmacologically potential target for anti-atherogenic therapy. Kruger et al. (2005), showed apoA-1 mimetic peptides up-regulate HO-1 expression in rat models of diabetes with improved vascular reactivity [61]. Taha et al.(2010) showed expression of HO-1 protein in EC slower pro-inflammatory cytokine and adhesion molecules, such as MCP-1, VCAM-1, IL-1β, IL-6, ICAM-1 [62]. It is also found that, HO-1 is elevated in macrophages preventing foam cell formation and death in atherosclerotic lesions, therefore exhibiting its protective anti-atherogenic roles [56]. Juan SH et al. (2001) showed adenovirus carrying the human HO-1 gene resulted in overexpression of HO-1 protein in arteries, which attenuated iron overload and prevented lesion formation in apolipoprotein E (apoE)-deficient mice [63]. However, J. Andrew Pospisilik et al. (2014) described HO-1 to be one of the dominant forecaster of metabolic disease in humans. Their findings from both hepatocytes and macrophages of human obese liver indicated that HO-1 is also essential for development of metainflammation and metabolic diseases. Therefore, Pospisilik et al. findings suggest inhibition of this stress-responsive enzyme (HO-1) to be a potential therapeutic strategy for metabolic disease [64]. Taken together, early HO-1 can usually be beneficial to prevent disease but may also be a contributing factor in chronic inflammation with metabolic dysfunction.

1.3. Cholesterol in Atherosclerosis

Hypercholesterolemia is one of the major risk factors for the formation of atherosclerosis. Cholesterol is a sterol (a lipid molecule) compound majorly synthesized by the liver and a minor portion coming from the intestine via food or reabsorbed from bile. Biosynthesis of cholesterol is a complex process beginning with the mevalonate pathway and ending with a 19-step conversion of lanosterol to cholesterol [65]. Cholesterol is required for the formation and maintenance of cell membranes and its fluidity; it is a precursor of many biochemical synthetic pathways, such as vitamin D, hormones and other cell signaling pathways [65]. However, excess or uncontrolled cholesterol production contributing to disease can occur by lifestyle or genetic reasons. Cholesterol and fatty acid in combination contribute to building up of plaque in atherosclerosis.

It can form plaque (a thick, hard deposit that clog arteries and make them less flexible) between the layers of an artery and is responsible for disease, such as atherosclerosis. If the plaque ruptures, cholesterols exposed to platelets can cause blood clots or thrombosis to take place. Blood clots are considered to be very dangerous because a clot blocking an artery that supplies oxygenated blood to the brain could possibly cause a stroke and/or to the heart, cause a myocardial infarction [66].

1.3.1. High-Density Lipoprotein (HDL) and Low-Density Lipoprotein (LDL)

As cholesterol is a lipid it is water insoluble by nature, it cannot travel effectively by itself in the blood and thus requires a transporter or a carrier. Lipoproteins are the carriers for cholesterol and fatty acids around the body [65]. Formation of lipoproteins take place mostly in the liver and intestine, allowing transportation of lipids from the intestine to liver as well as between liver and peripheral cells. Mature lipoprotein contains lipids (triglyceride (TG), cholesteryl ester (CE) and cholesterol) in the core, surrounded by a surface monomolecular film of phospholipids (PL), cholesterol and apolipoproteins (apo) [65]. Common classification of lipoproteins is based on their densities and they are: chylomicron, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). LDL and HDL are more elaborately described as they are significantly correlated with the term atherosclerosis and are monitored clinically.

Both high LDL and low HDL are risk factors for the development of atherosclerosis [65]. HDL has anti-atherogenic properties and might be a therapeutic target for treating atherosclerosis. ApoB100 is the primary protein component of LDL, which acts as a ligand for the LDL receptor to deliver triglyceride (TG) and cholesterol to the cells.

There is a contrary relationship between HDL and triglycerides. HDL is significantly involved in reverse cholesterol transport (RCT). HDL is known to protect LDL from oxidation by paraoxonase-1 (PON-1). PON-1 is a protein of 354 amino acids with a molecular weight of 43kDa, which is capable of reducing lipid peroxidation, suggesting that PON-1 may be a therapeutic target to treat atherosclerosis. Both low levels of HDL and dysfunctional HDL could be considered as a substantial marker for atherosclerosis [66]. Lipid poor protein ApoA1 is released from the intestine, which collects phospholipids from ABCA1 in the liver and forms a

discoidal disk pre-β-HDL (it is also commonly known as ndHDL i.e. nascent discoidal HDL), that circulates in the plasma to collect phospholipids (PL), free cholesterol (FC) and mature to spherical HDL. Maturation of HDL obtains diversified functions and is able to interact with few different proteins, including ATP-binding cassette (ABC)-G1, G5 (ABCG1, ABCG5), lecithin:cholesterol acyl transferase (LCAT) and scavenger receptor class B type 1 (SCARB-1), found mainly in liver, endothelium and macrophages [67]. Figure-3 demonstrated how HDL mimetic nanoparticles (HPPS) initially targets SCARB-1 receptor to get attached to the cell membrane and secondly via lipid-raft or caveola-like pathway may enter into the cell cytoplasm. Lipid-rafts are a special part of the cell membrane composed of shingolipid and cholesterol that remain very intact and tight, yet allow selected components to pass across the cell membrane as required.
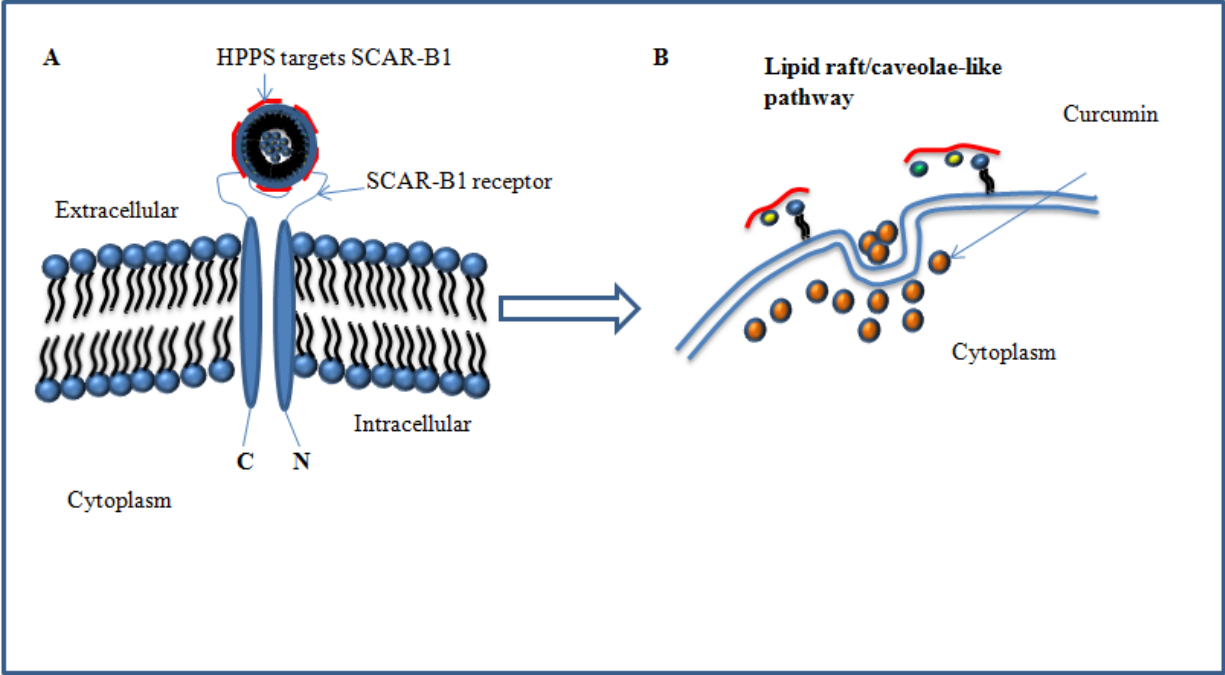
Figure 3: Uptake of nanoparticles can occur via the SCAR-B1 receptor [68].

1.3.2. Reverse Cholesterol Transport (RCT) in Atherosclerosis

Plasma cholesterol levels can be a biomarker in atherosclerosis [69]. Regulation of cholesterol is a multi-step complex process involving cholesterol synthesis, uptake, transport, metabolism, and excretion. Reverse cholesterol transport (RCT) is a pathway that removes cholesterol from peripheral cells and tissues to the liver and intestine for excretion. There are two elements in RCT. They are: 1). Cholesterol efflux and 2) HDL mediated transport [69].

Cholesterol efflux is an important process where macrophages externalize cholesterol outside the cells by both active and passive processes [65]. Apolipoprotein A-I (apoA-I) is the principal apolipoprotein in HDL composition. It is comprised of 243 amino acid residues with the molecular weight of 28kDa. To begin with, ApoA-I is produced by the intestine and released into the plasma. Next, it travels to the liver and via cholesterol efflux pathway, ATP-binding membrane cassette transport protein A1 (ABCA1) transfers cholesterol to ApoA-1, which then forms nascent discoidal HDL (ndHDL) [70]. Indeed, Tangiers disease is a genetic disorder associated with severe HDL deficiency caused by mutations in the ATP-binding cassette transporter A1 (ABCA1), indicating the importance of ABCA1 in HDL production [70]. Apart from ABCA1, there are four other factors that are also involved in cholesterol efflux. They are: 1) SCARB-1, 2) ABCG1, 3) Caveolins and 4) Sterol 27 hydroxylase (CYP27A1). Efflux of cellular free cholesterol (FC) from macrophages to HDL can be mediated by SCARB-1. SCARB-1 is a membrane bound glycoprotein (mol. wt: 80kDa) expressed on the surface of ECs, macrophages and hepatic cells but not expressed in smooth muscle cells (Figure 4).
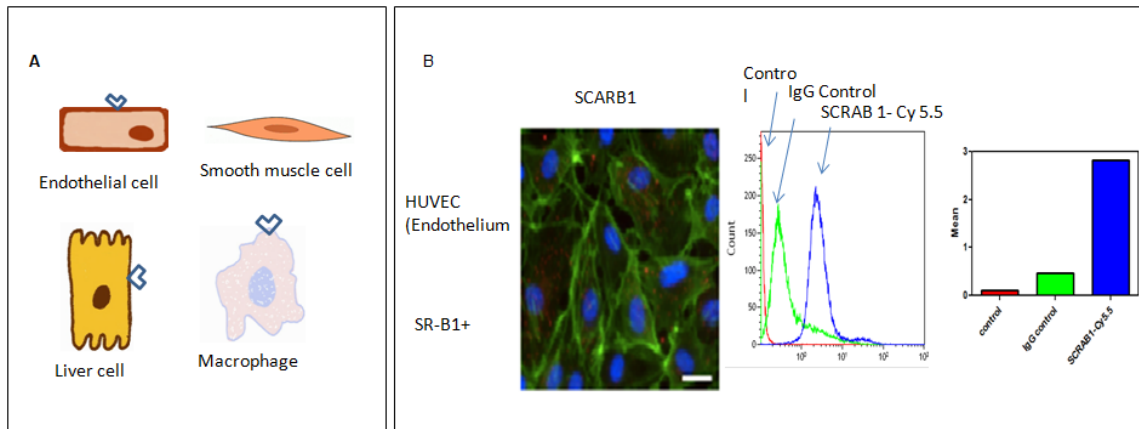
Figure 4: Presence of SCARB-1 receptors in various cell types.

(A). SCARB-1 is a membrane bound glycoprotein (mol. wt: 80kDa) expressed on the surface of endothelial cells, macrophages and hepatic cells but not in smooth muscle cells. (B) The image representing endothelial cell (HUVEC) where the cell nucleus is in blue stain (DAPI) and conjugation of secondary antibody (anti-rabbit) with Cy 5.5 is making all the SCARB-1 present in HUVEC visible in red dots and green (phalloidin) indicating presence of actins in the cytoplasm of the cell.

Libby et. al. reported a subpopulation of intimal SMCs in rabbit atherosclerotic lesions that expressed the scavenger receptors *in vivo*, whereas, SMCs in the adjacent arterial media did not do so [71]. SCARB-1 is abundantly expressed in the liver, where it facilitates selective uptake of free cholesterol (FC) and cholesteryl ester (CE) from HDL. Once ndHDL is generated, it triggers cholesterol efflux in macrophages and peripheral cells into the sub-endothelial space. Externalized cholesterol is absorbed by ndHDL, and immediate esterification is performed by lecithin:cholesterol acyltransferase (LCAT). Maturation of HDL particles with cholesteryl ester makes HDL larger and spherical in shape. Cholesterol ester transfer protein (CETP) facilitates the equimolar exchange of cholesteryl esters from HDL for triglycerides in apoB100-containing lipoproteins [69]. These cholesteryl esters are then delivered back to the liver by two pathways: 1) mature HDL attached to SCARB-1 receptor to unload cholesteryl esters or 2) via low density-lipoprotein receptor (LDL-R). Once delivered in the liver, cholesterol is converted into bile salts and eliminated through the gastrointestinal tract. The HDL particles that are present must be able to maintain the appropriate flux of cholesterol through the RCT pathway. Removal of cholesterol from macrophages in the walls of blood vessels via RCT pathway (Figure 5) is critical for preventing the development of atherosclerotic plaque. In addition, enhancement of RCT from plaque to liver may be considered a new and effective therapeutic approach to reduce lesion of atherosclerosis (atheroma).
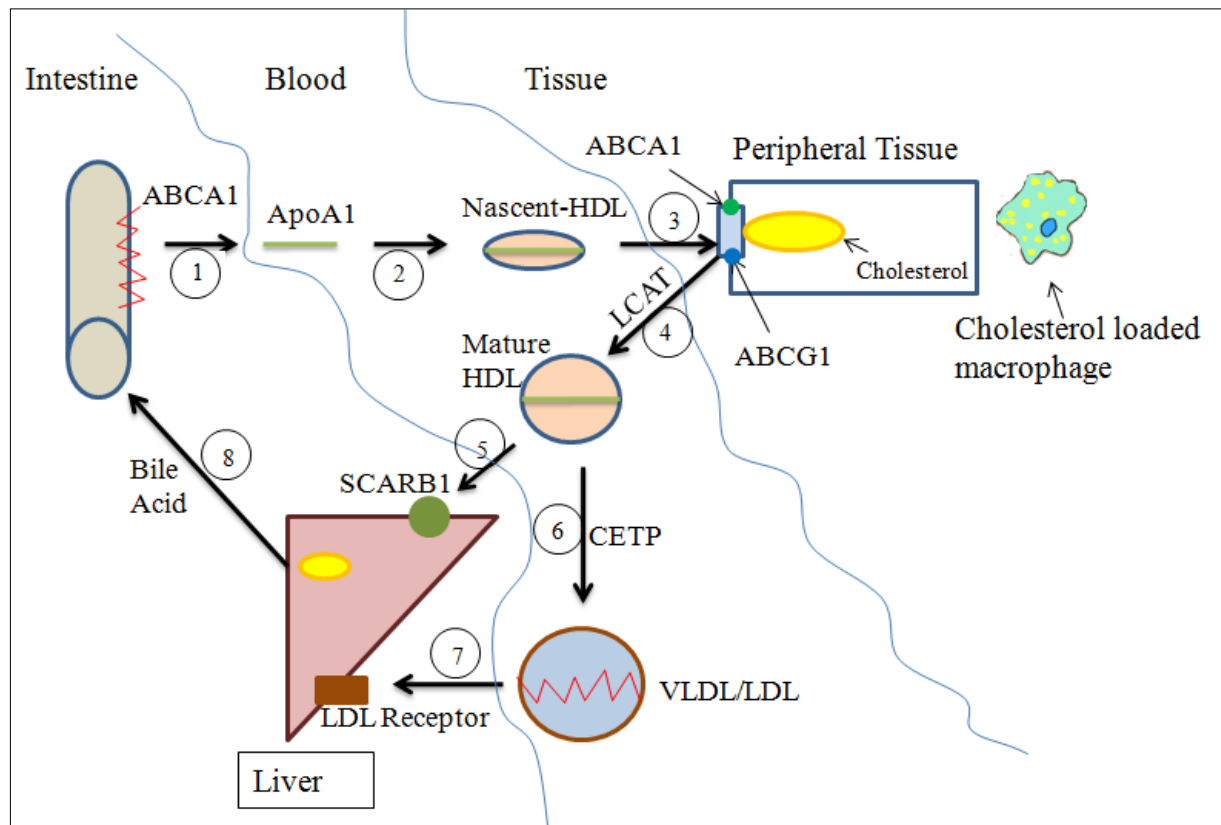
Figure 5: Reverse Cholesterol Transport Pathway.

1) ATP-binding membrane cassette transport protein A1 (ABCA1) is located in intestine and transports cholesterol from the intestine into the blood plasma 2) ApoA1 combines with cholesterol to form nascent-discoidal HDL (ndHDL). 3) ndHDL via ABCA1and/or ABCG1 receptor collects cholesterol from both peripheral tissues and or cholesterol loaded macrophages. 4) Externalized cholesterol is absorbed by ndHDL, and immediate esterification is performed by lecithin:cholesterol acyltransferase (LCAT). ndHDL converts into mature HDL, which is spherical in shape. Then cholesteryl esters are delivered back to the liver by two pathways: 5) mature HDL via SCARB-1 receptor (the only receptor for HDL) unload cholesteryl esters into the liver or 6) Cholesterol ester transfer protein (CETP) facilitates the equimolar exchange of cholesteryl esters from HDL for triglycerides in apoB100-containing lipoproteins; 7) via low density- lipoprotein receptor (LDL-R) cholesteryl esters are unloaded into the liver. 8) Finally, cholesterol is converted to bile salts, and eliminated through the gastrointestinal tract.

1.3.3. Pharmacology Affecting RCT

HDL is the primary component in the RCT pathway; abundant evidence from *in-vitro* and *in-vivo* studies demonstrate how therapeutic agents directly or indirectly target HDL, apoA-I, HDL receptors and/or enzymes to enhance the RCT pathway for anti-atherogenic therapy [69]. Anti-hypertensive agents, hypolipidemic drugs, hormones and microsomal enzyme inducers and supplements, such as fish oil have all been clinically shown to elevate plasma-HDL levels in patients with atherosclerosis [72]. Statins (e.g. Atorvastatin) are commonly used in patients with elevated cholesterol. Statins are the inhibitors of hydroxymethylglutaryl coenzyme A (HMG CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis. Statins decrease intracellular hepatic cholesterol and up-regulate LDL receptors to reduce plasma LDL and triglyceride levels [69]. Clinical evidence established that statins elevate apoA-I levels (possibly increasing the amount of HDL too) and decrease CETP-mediated transfer of cholesterol from HDL. CETP is directly associated with HDL and known to aid transfer of cholesteryl ester (CE) from HDL to apo-B containing lipoproteins. Therefore, inhibiting CETP could be one possible target to up-regulating HDL and down-regulating LDL/VLDL-cholesterol [69]. Peroxisome proliferator-activated receptor, (PPAR)-α is a nuclear hormone receptor involved in lipid metabolism. Targeting PPAR-α could indirectly make RCT more efficient. Fibric acid derivatives (fibrates) are agonists of (PPAR)-α, which tend to lower triglyceride levels effectively and slightly increases HDL levels. According to Fricket al., fibrate (Gemfibrozil) therapy significantly reduced cardiovascular disease with a modest increase in HDL levels [73]. Niacin or nicotinic acid is another therapeutic agent that exhibited similar mechanisms of action to that of fibrates and tends to raise HDL by ~35% [69]. HDL contains exchangeable apolipoproteins (apo) of A, C and E, but apoA-I is the principal constituent of HDL [65]. Eriksson et al. reported that infusing the precursor of apoA-I into patients with familial hypercholesterolemia speeds up net cholesterol excretion from the body. Therefore, targeting apoA-I to enhance cholesterol efflux and RCT could be another potential mechanism that might be beneficial to the treatment of atherosclerosis [69].

1.4. Curcumin as a Pharmacological Agent

Turmeric is a globally recognized, popular spice, which for thousands of years has been consumed for medicinal purposes. Curcumin is an active ingredient of the dietary spice turmeric, but the chemical name of curcumin is diferuloylmethane with its chemical structure shown in Figure 6. This natural yellow colored solid polyphenolic chemical, has been isolated from the rhizome of the plant *curcuma longa* and is hydrophobic in nature [74]. Curcumin is commonly used as an ingredient in foods, traditional medicines and cosmetics. Scientific investigations over the years have established the potential for curcumin's ability to modulate signaling pathways directly or indirectly [75]. Curcumin has antioxidant, anti-inflammatory, wound healing, anti-carcinogenic and anti-tumor properties. Being fluorescent in nature, it has been used for scientific purposes as a fluorescent agent, both *in vivo* and *in vitro* as a tracking tool and also criticized for generating false positive results in numerous high-content and high-throughput pharmacology assays [76].

Various animal models and human studies have reported that curcumin is extremely safe even at very high doses. For example, a clinical trial indicated that curcumin, when taken at doses as high as 12 g per day, is still well tolerated, though no plasma concentration of curcumin were detected in the subjects [77]. In spite of being pharmacologically safe at high doses, curcumin struggles to be considered as an 'active therapeutic agent' due to its hydrophobicity, poor absorption, rapid elimination and low bioavailability [78]. To overcome the barrier of poor systemic bioavailability as well as to address the hydrophobic characteristic, incorporating curcumin into nanoparticles could increase drug penetration, membrane permeability and therapeutic efficacy. For example, a combination of curcumin with phospholipids, liposomes and micelles are some of the latest strategies of nanomedicine, which has proven to elevate the therapeutic efficacy of curcumin to some extent [79],[80].
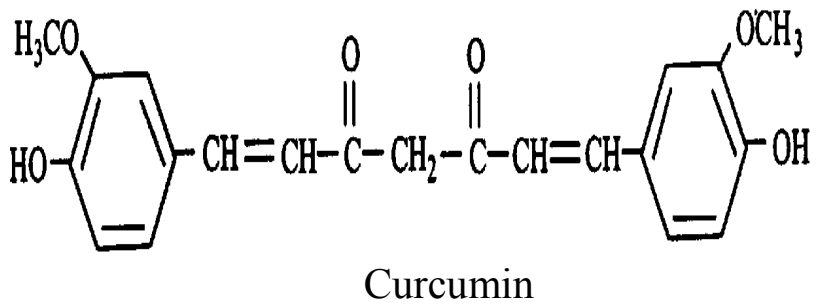
Curcumin

Figure 6: Structure of Curcumin [81].

1.4.1. Curcumin in Atherosclerosis

Cardiovascular Diseases (CVDs), including heart disease, vascular disease and atherosclerosis, are currently the most critical global health threat. It is widely appreciated that mechanisms, such as inflammation and oxidative stress, individually or in combination, play a key role in the development of CVDs [82]. Evidence suggests that curcumin mediates anti-atherosclerotic action via anti-inflammatory and anti-oxidant properties [82]. Moreover, curcumin also has the potential to alter the cholesterol homeostasis and reduce platelet aggregation [83]. It may also be useful in increasing HDL and reducing LDL [84]. Several *in vivo* studies indicate curcumin's ability to inhibit LDL oxidation, reduce cholesterol and triglyceride levels, lower oxidative stress along with reducing lipid peroxidation [80],[85]. Evidence also suggests, the antioxidant property of curcumin alone can exhibit a protective role against the formation and progression of atherosclerosis [86]. Anomalous proliferation of VSMCs and monocytes are key factors in the progression of atherosclerosis. One potential role of curcumin is the suppression of VSMCs migration [80]. This prevention is associated with lowering of ROS production, inhibition of matrix metallopeptidase-9 (MMP-9) activation and protein expression via down-regulation of NF-kB [87]. *In vivo* studies suggest that curcumin controls the platelet-derived growth factor (PDGF) dependent abnormal proliferation of VSMC along with reducing neutrophil activation [88]. Curcumin's anti-oxidant and anti-inflammatory properties may combine to have anti-atherogenic effects in prohibiting proliferative and migratory signaling pathways of VSMCs.

1.4.2. Molecular Mechanism Affected by Curcumin

Curcumin exhibits anti-inflammatory properties by targeting transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) [89], early growth response-1 (Egr-1) protein [90], enzymes such as cycloxygenase-2 (COX-2), lipoxygenase (LOX) [91] as well as pathways such as mitogen-activated protein kinases (MAPK) [92]. NF-kB plays an important role in the onset of many pro-inflammatory mediators involved in various cancers, chronic and acute inflammatory diseases and. Curcumin restrains NF-kB dependent gene products that initiate proliferation, invasion and angiogenesis and also attenuates pro-inflammatory cytokines expressions, such as TNF-α, IL-1 and IL-6 [89],[93].

Egr-1 is another transcription factor in control of the transcription of several other genes involved in inflammation, differentiation, growth development and wound healing [90]. Suppression of Egr-1 induction via curcumin in endothelial cells, fibroblasts and vascular smooth muscle cells (VSMCs) has demonstrated curcumin's anti-inflammatory effects [94]. Curcumin has also been shown to inhibit enzymes such as cyclooxygenase-2 (COX-2) and lipooxygenase (LOX), which are involved in producing lipid mediators that could enhance inflammation via arachidonic acid metabolism [95],[91]. Animal studies show lipopolysaccharide greatly enhanced expression of inflammatory cytokines (MCP-1, TNF-α, TLR4 and iNOS) and NO production, resulting in an increased phosphorylation of IkBα, nuclear translocation of NF-kB (p65) and phosphorylation of MAPKs in VSMCs [80]. *In vivo* experimental results indicate curcumin tends to inhibit NADPH-mediated intracellular ROS production in a rat model, resulting in suppression of lipopolysaccharide-induced over-expression of inflammatory mediators in the rat VSMCs via blockade of TLR-4-MAPK/NF-kB pathways [96]. Curcumin is also a potential direct scavenger of various ROS (e.g. O_2, OH, nitrogen oxide radical, H_2O_2) [97]. In addition to being a strong scavenger, curcumin can also amplify the activities of natural anti-oxidants, such as superoxide dismutase (SOD), glutathione S-transferase (GST) [98] and HO-1, in our body [99]. Structural features of curcumin i.e. presence of two methoxy groups in the ortho position on the phenolic ring, is responsible for enhanced HO-1 expression [99]. In contrast to curcumin's antioxidant effect, its pro-oxidant activity should also be considered. The pro-oxidant activity appears to be associated with the generation of phenoxyl radical of curcumin by the peroxidase–H_2O_2 system, that co-oxidizes cellular glutathione or NADH, accompanied by O_2 uptake to form ROS [100]. However, it has also been reported that, curcumin not only failed to protect cells from single strand DNA damage caused by H_2O_2 but also caused further DNA damage [101].

The beneficial effects of curcumin have been discussed in a numerous articles. Abnormal levels of oxidative stress and increased inflammatory response are considered to be key factors responsible for several cardiovascular mechanisms such as atherosclerosis, cardiac hypertrophy, hypertension and ischemia. Curcumin is a potential scavenger that eliminates ROS, and reduces oxidative stress, which in turn could restore NO bioavailability and improve endothelial functions. As previously discussed, curcumin is capable of suppressing NF-kB activation and can

down regulate other   inflammatory cytokines (TNFα, IL-1, IL-6) along with other signaling pathways to prevent inflammation and further endothelial dysfunction (Figure 7).
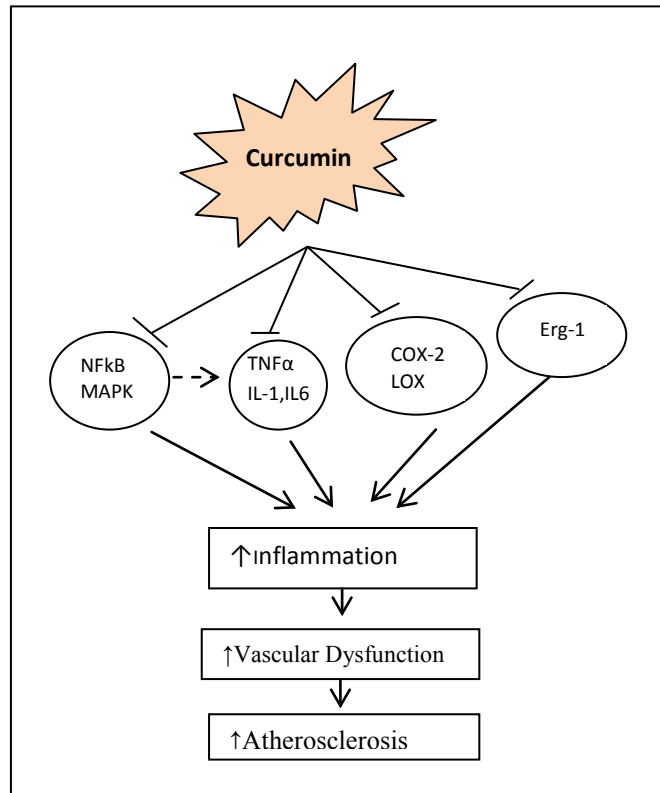
Figure 7: Schematic diagram of curcumin's anti-atherogenic effect.

By attenuating and/or inhibiting transcriptional factors, cytokines, pro-inflammatory cytokines and other pathways, curcumin is potent in reducing oxidative stress, inflammation and vascular dysfunctions, which are responsible for the formation and progression of atherosclerosis [80].

1.5. Nano-Pharmacology:

Nano-pharmacology is an emerging field of science. Integrating nanotechnology with medicine as a part of medical science is known as nanomedicine. Nanoscale endogenous entities are already present in our biological systems, such as proteins, antibodies, lipoproteins etc. Through nanomedicine, we are able to overcome limitations, such as selective delivery of therapeutic moieties with hydrophobic properties in aqueous media, reducing side-effects and increasing therapeutic efficacy, while maximizing therapeutic index.

Nano-pharmacology investigates the interaction of a nanomedicine within living systems [102]. Nano-pharmacology is concerned with the methods to deliver drugs and are designed to operate on a nanometer size range to carry drugs to sites of disease or injury; referred generally as nanoparticle drug delivery systems (NPDDS) [103]. NPDDS can be used to improve pharmacokinetics of drugs, such as by increasing the surface-to-volume ratio with nanoparticles or improving the solubility of the drug. Similarly, bioavailability has been improved by protecting the drug from excessive metabolism, including nucleic acids. NPDDS display positive effects on pharmacodynamic parameters too, such as selective drug delivery in the target tissue and controlled dosage with significantly reduced side effects of drug toxicity [104]. Lewis et al. identified several advantages of nanotechnology and designed synthetic nano-assemblies for advanced atherosclerosis therapies. They aimed for nanoparticles that could 1) increase systemic circulation time of the carrier, 2) lower drug cytotoxicity, 3) increase drug solubility, 4) reduce the required dose, 5) incorporate imaging and therapeutic agents for inspection of disease progression, 6) raise specific tissue accumulation through active or passive targeting [105].

As curcumin is considerably hydrophobic in nature, NPDDS have the potential for masking hydrophobic agents like curcumin and make it dispersible in aqueous media to reach the target tissue and enhance the bioavailability of curcumin. HDL are endogenous nanoparticles in our system and ApoA-1 is considered as a primary component of HDL. HDL acts as a cholesterol scavenger, picking up excess cholesterol from our blood and tissue and returning them back to liver via RCT pathway. Therefore, by applying the knowledge of nanotechnology and nano-pharmacology, we tried to formulate synthetic HDL, i.e. HDL mimetic peptide-lipoprotein nanoparticles surface (HPPS), that would allow us to incorporate the hydrophobic therapeutic

agent curcumin into HPPS, which in turn could significantly increase HO-1 protein expression as a strategy for atherosclerosis regression. Cholesterol lowering drugs, such as statins, PPARγ agonists, anti-oxidants and/or anti-platelet therapies, are a few conventional therapeutic approaches that could treat atherosclerosis. However, these therapeutic approaches have primary drawbacks that show poor efficacy in treating atherosclerosis. Therefore, developing alternative potential strategies and designing novel approaches via nano-pharmacology is very much needed [105].

1.6. Rational and Hypothesis:

Rationale: Atherosclerosis is a chronic disease where the artery wall thickens and becomes hard due to accumulation of lipids and/or inflammatory cells. This is a consequence of risk factors, such as hypertension, hypercholesterolemia, diabetes, smoking, age, gender and other genetic factors that leads to endothelial cell dysfunction. In this thesis, Human Umbilical Vein Endothelial Cells (HUVEC) were used to conduct most of the experiments. This cell line is much easier to obtain and highly proliferative. Inflammation and oxidative stress (pre-conditions for accumulation of reactive oxygen species (ROS)) play a major role in atherosclerosis formation and progression. HO-1 is a transcriptionally regulated cytoprotective protein and/or stress-responsive enzyme that metabolizes heme into bilirubin, iron, CO and shows potential anti-inflammatory and anti-oxidant properties. HO-1 is regulated by transcription factors such as Nrf2 (ROS), NFkB (inflammation) or Bach-1 (heme). Curcumin (a polyphenolic natural compound) is an bioactive chemical ingredient of turmeric that has been used as a traditional medicinal agent to treat a wide range of diseases for centuries. Recent studies have suggested the natural anti-oxidant properties of curcumin are a result of potent induction of HO-1. Thus, a plausible mechanism responsible for anti-atherogenic actions of curcumin could depend on the induction of HO-1. However, the hydrophobic nature and low systemic bioavailability of oral curcumin do not allow it to reach an efficacious concentration to exert beneficial effects on most tissues *in vivo*. Cholesterol in particular, LDL plays a role in atherosclerotic plaque formation. RCT is a multi-step process, which regulates cholesterol levels in the body. Cholesteryl esters(CE) are delivered to the liver via HDL through scavenger receptor B1 (SCAR-B1), converted to bile salts and finally eliminated through the gastrointestinal tract. HDL are endogenous nanoparticles with apoA1 as a primary component. HDL acts as a cholesterol scavenger, picking up excess

cholesterol in our blood and peripheral tissues and returning them to the liver via RCT. Increased HDL is known to reduce atherosclerotic lesions, but the mechanism of HPPS, for treating atherosclerosis at the cellular level has not been elucidated yet. Hence, HPPS could be used to deliver therapeutic agents, such as curcumin, to significantly increase HO-1 protein expression as a strategy to reduce atherosclerosis.

We hypothesize that HPPS carrying the therapeutic agent curcumin to endothelial cells will increase HO-1 activity, reduce ROS, inflammation and protect endothelial cells from death and/or dysfunction.

My objectives are:

1. To synthesize and characterize HPPS-curcumin.

2. To compare HPPS-curcumin to curcumin for dose equivalent of HO-1 induction in HUVEC.

3. To ensure that SCARB-1, the principle HDL-receptor, is not down-regulated by HPPS or HPPS-curcumin.

4. To evaluate HPPS-curcumin mediated protection of HUVEC from oxidative stress (H_2O_2) induced cell death.

CHAPTER 2: Materials and Methods

2.1 Synthesis of HPPS

HPPS were synthesized as shown in Figure 9. Reagents used for HPPS synthesis were cholesteryl oleate (Sigma, catalog # C 9253), chloroform (OmniSolv), 1,2-Dimyristoyl-sn-glycero-3-phosphorylcholine (DMPC) (Echolon Bioscience, catalog # L-1114), oleylamine (Aldrich, catalog # C-112-90-3) and dye Cy 5.5 (GE Healthcare, catalog # Q15408). To begin with, oleylamine ($14.21\mu M$) and Cy5.5 (100% W/W) of $9.84\mu L$, were taken in an aluminum wrapped eppendorf tube and placed in a rotator at 4°C for 30 minutes , as per protocol. Addition of dye Cy 5.5 was optional. Next, DMPC (6.184mM) was dissolved in $250\mu L$ of chloroform and mixed with cholesteryl oleate (16.27mM) that was kept on ice. After 30 minutes, the oleylamine-dye mixture along with DMPC and cholesteryl oleate were mixed in a round-bottom glass flask and swirled gently for 2-3 minutes. Afterwards, the mixture was subjected to a rotavaporizer for 40 minutes followed by incubation at 50°C for 60 minutes. The resultant solution was then placed in a water-bath sonication at 50°C for 60 minutes. After performing sonication, $1000\mu L$ 1x PBS (sterile) was added in the flask and this step was performed in a hood. Then the flask was placed in an ultra-sonication bath for 20 minutes. Finally, the solution was transferred into an aluminum-wrapped eppendorf tube (this step was also performed in a sterile hood) and centrifuged at 1500 x rcf for 15 minutes at 4°C followed by filtration with a sterile $0.22\mu m$ filter. The prepared product was finally labeled as HPPS and stored at 4°C fridge for further use (Figure 8). Prior to any experiment, an addition of synthetic apoA1 peptide (ApoA-1 peptide Ac-FAEKFKEAVKDYFAKFWD-NH2; molecular weight, 2310.64 g/mol; China Peptides Inc.) mimetic would be added to the HPPS (at a ratio of 1:1) along with 5 minutes water-bath sonication. As some of the ingredients are light sensitive, throughout the synthesis process, the flask and/or eppendorf tube must be protected from the light, using aluminum foil.

2.1.1. Loading of curcumin in HPPS

The procedure for preparing curcumin loaded HPPS is similar to the method described above. In terms of loading curcumin into the nanoparticles, we first dissolved curcumin (16.1mM) (Sigma, catalog # C1386) in 1000 μL chloroform in a round-bottom glass flask (curcumin is
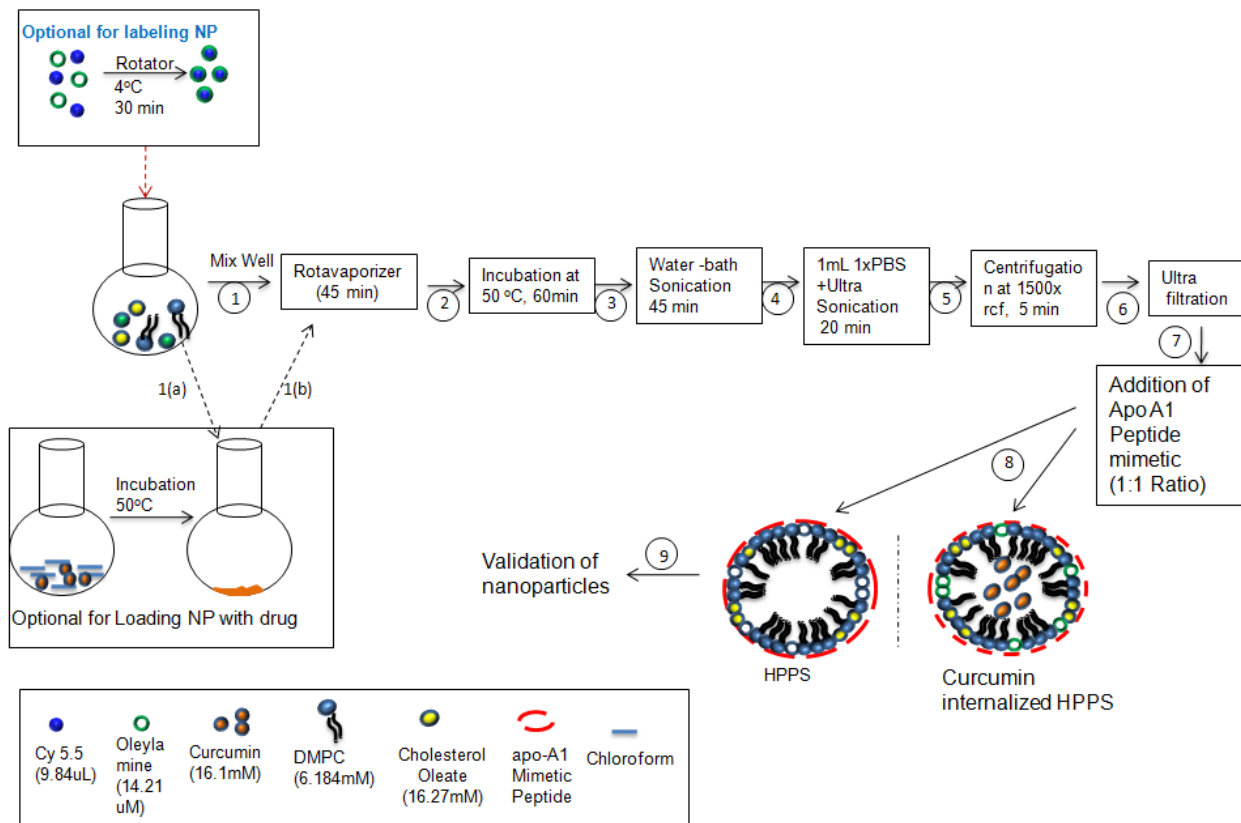
Figure 8 Schematic representation of HPPS formulation

photosensitive, so the flask was wrapped with aluminum foil) and then placed it in an incubator at 50°C until a thin dry film of curcumin was formed. Then, as per the protocol above, the mixture of chloroform dissolved DMPC along with cholesteryl oleate, followed by oleylamine-dye mixture was added to the flask containing the dry-film curcumin. Following this, the HPPS-curcumin preparation was carried out as identical to the one outlined above. A slight variation in the protocol, where the curcumin is added to the nanoparticles (i.e. direct addition of curcumin dissolved in chloroform (without drying to a curcumin film) onto HPPS (at step 7), would cause the formed nanoparticles to be decorated (not incorporated) with curcumin, or surface-coated HPPS-curcumin. (Figure 9)
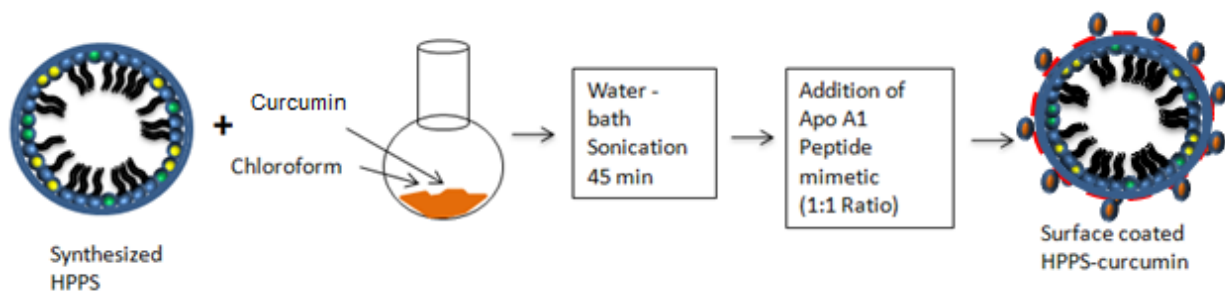
Figure 9. Schematic representation of surface coated HPPS-curcumin formulation

2.1.2. Calculating loading efficiency of curcumin on nanoparticles by UV-Spectroscopy

The loading efficacy of curcumin within HPPS was determined, by measuring the curcumin concentration through UV absorption spectrum. Curcumin encapsulation efficiency in HPPS was determined in triplicate, and the values reported by means of SD.

The percentage of the drug entrapped in nanoparticles was calculated by the following equation [106],[107]:

EE(Entrapment efficiency) = [Amount of Curcumin in HPPS/

Total amount of Curcumin] x 100%

Significance of encapsulation or entrapment efficacy of curcumin inside the nanoparticles is not only to refer the loading efficacy of the therapeutic agents, but also to determined in concert with dose equivalency. We define dose equivalency as the determination of molar equivalency between dissolved drug and nanoparticle loaded drug, that elicits the equivalent biological effect when applied similarly to a biological system (cells or animals). Dose equivalency is the molar amount of a nano-drug that achieves the same biological effect an established dose effect used as reference, which in our case was 5μM curcumin dissolved in DMSO.

2.2. Synthesis of Niosome and Niosome-curcumin (Nio-curcumin)

Synthesis of niosomes: A mixture of tween 80 (Amresco; Cat # M126-IL), PEG-6000 (0.2 g in 10 ml PBS; EMD Cat # PX1286L-4; stored @ 25°C) and water were mixed according to optimum weight ratio (i.e. 0.500:0.060:0.500) then vortexed for 5 min, followed by sonication of 30 min. In addition, the resultant solution was diluted with 2 (w/v %) PEG-6000, followed by sonication for 30 min, resulting in formation of niosomes (Nio) [76].

Synthesis of curcumin encapsulated niosomes: 2 mg of curcumin (Sigma, catalog # C1386) was dissolved in 1 ml of chloroform and solvent was evaporated under vacuum resulting to form a dry film. Then, a mixture of tween 80 (0.500 g), PEG-6000 (0.016 g) and water (0.500 g) were added. Next, the mixture solution containing Nio-curcumin was subjected to centrifugation at 1100 x rcf for 3 min to separate the curcumin encapsulated niosome in the form of supernatant containing any residual native curcumin. At this point, synthesis of niosome-curcumin (Nio-curcumin) was completed and could be stored at 4°C. The loading efficiency of curcumin within

a niosome was determined by measuring the curcumin concentration in the pellet through UV absorption spectrum and this value was subtracted from a value of the initial concentration of curcumin, to determine loading efficiency of the niosome which was found to be 86% [76].

2.2. Characterization of HPPS and HPPS-curcumin

The shapes of nanoparticles HPPS and HPPS-curcumin were determined by Transmission Electron Microscopy (TEM). The absorption spectra of HPPS and HPPS-curcumin were measured using a UV-vis spectrophotometer. By using ZetasizerNano (Malvern instruments, USA), both particle size and zeta potential of the nanoparticles were measured by Dynamic Light Scattering (DLS). Prior to the measurement of particle size and zeta potential, the nanoparticles were dispersed in PBS at pH 7.4.

2.2.1. Transmission Electron Microscopy (TEM):

Specimen preparation was done by taking an aliquot amount (50μL) of sample HPPS and/or HPPS-curcumin in a test-tube and adding 200μL of PBS to it. Sonication of 20 minutes followed by centrifugation at 1000 x rcf for 5 minutes were done. Then the sample was transferred to the core facility in Dalhousie University where the remaining steps were completed. An amount of the specimen, within the range of 10-20μL, was taken for fixation, embedding and negative staining on a copper-coated carbon grilled base and dried. TEM reading was taken at 80kV in JOEL-1230 with a 4 mega pixel camera.

2.2.2. UV-vis spectrophotometer:

Samples were prepared by taking an equal volume of HPPS and/or HPPS-curcumin in sterile eppendorf tubes, then sonication was performed for 20 minutes. Sonication of the samples should be done on ice in a dark room as both peptide and liposome are temperature sensitive, while curcumin is photosensitive. Curcumin (16.1mM), dissolved in 1000 μL chloroform in a around-bottom glass flask, was dried at 50°C and then re-dissolved in 1000μL PBS. For taking UV-vis spectrophotometer reading, samples were added to a 96 well plate. Consecutively, 30μL of HPPS, HPPS-curcumin each as well as curcumin alone, were added in 3 wells respectively.

2.2.3. Dynamic Light Scattering (DLS):

Sample preparation for DLS was similar to that of UV-vis spectrophotometer. Besides the sample, 0.22 μm filters, a syringe and a cell (also known as cuvettes) were required. At first, 2ml PBS was taken up by syringe and 0.5ml PBS discarded for rinsing purpose. Next, 10μL of the sample (HPPS and/or HPPS-curcumin) was added into the syringe via a micropipette. A 0.22

μm filter was attached to the tip of the syringe and very slowly, the sample was transferred into the cell with great caution take to avoid formation of bubbles in the sample solution. DLS is a useful technique to measure the particle size, size distribution, and the zeta potential of the nanoparticles in solution. The zeta potential is determined by applying an electric field to give direction to the random Brownian motion detected by the DLS instrument (sometimes called electrophoretic light scattering or ELS). The zeta potential in ELS evaluates the nanoparticle surface in a given buffer to determine surface charge relative to buffer composition (ionic strength/type, pH, additives etc). DLS reports size distribution data in intensity, volume and number. Although, almost all the articles on nanoparticles address DLS to characterize nanoparticles, they do not consistently mention whether the size distribution is based on intensity, volume, or number. Therefore, in general for a DLS user, it is important to know that the intensity-based diameter (the z-average) is the actual value obtained directly from the correlation function [108]. Intensity measures are most useful to detect with the greatest sensitivity even the smallest amount of aggregation. Whereas volume (mass amount) or number (concentration of particles, ie counts relative to volume detected by laser) will inform as to the degree of conformity of size within the majority of the measured particles. The volume or the number distribution size could be used for comparing DLS data with other techniques for more detailed characterization of the nanoparticles only when the data quality is met [109].

2.3. Cellular viability curcumin, HPPS and HPPS-curcumin by endothelial cells:

To investigate the effects of curcumin, nanoparticles (HPPS) and HPPS-curcumin on cellular viability, we performed fluorescence-activated cell sorting (FACS). Endothelial cells (HUVEC) were cultured in 60 mm dishes at a density of 0.5 million cells / per dish, in MV 2 medium (endothelial cell growth medium) at 37°C for 24 hours. Then the cells were treated with 5μM curcumin, HPPS of (5μM)Equ and HPPS-curcumin of (5μM)Equ respectively for 16 hours. For positive control of cell death, 500μM H_2O_2 was provided. After that the cells in each of the dishes were treated with 4μL JC1 dye. After a 30 minute incubation period, the culture media was carefully discarded and each dish was washed three times with 1X PBS. This was followed by 1ml of trypsin being added to each dish. After 5 mins of incubation time, each dish was rinsed with 2ml MV 2 media and the cells along with media were collected in 15ml falcon tubes. After centrifugation at 500 x rcf for 5 min at 4°C, the supernatant was carefully aspirated and the cells

re-suspended in 1ml media in FACS tubes. Finally, fluorescence-activated cell sorting (FACS) was performed by using a Gallios 10-color flow-cytometer (Beckman Coulter). To assess the curcumin we used the excitation 405nm violet laser with an emission wavelength of 550/40nm curcumin (FL10 channel) and the Cy5.5 on nanoparticles we used the 635nm excitation laser with an emission wavelength of 725/20nm (FL7) for the nanoparticles. To determine at the same time the viability, we measured the JC-1 dye using a 488nm excitation, to generate spectra in two colors uncondensed JC-1 in emission spectra 525nm (FL1) and condensed JC-1 in emission spectra 575nm (FL2). Viable cells condense JC-1 in the mitochondria to maintain a high intensity red channel (FL2) signal. This was done to determine, whether cells remain viable when treated with curcumin, nanoparticles (HPPS) and HPPS-curcumin

2.4. Uptake of curcumin, HPPS and HPPS-curcumin in endothelial cells:

To independently verify the dose response effect of curcumin, HPPS and HPPS-curcumin by HUVEC, FACS analysis was performed. HUVEC were cultured in 60mm dishes at a density of 0.5 million cells / per dish, in MV 2 medium (endothelial cell growth medium) at 37°C for 24 hours. The cells were then treated with curcumin, HPPS and HPPS-curcumin with various concentrations of 1μM, 2.5μM, 5μM and 10μM respectively for 16 hours. The culture medium was aspirated and each dish was washed three times with 1X PBS. Afterwards, 1ml trypsin was added in each dish and kept in the incubator for 5 minutes. Each dish was rinsed with 2ml MV2 media and cells along with media were collected in 15ml falcon tubes. After centrifugation at 500 x rcf for 5 min at 4°C, the supernatant was carefully aspirated and cells re-suspended in 1ml media in FACS tubes. Finally, fluorescence-activated cell sorting (FACS) was performed by using a Gallios 10-color flow cytometer (Beckman Coulter) To assess the curcumin we used the excitation 405nm violet laser with an emission wavelength of 550/40nm curcumin (FL10 channel) and the Cy5.5 on nanoparticles we used the 635nm excitation laser with an emission wavelength of 725/20nm (FL7) for the nanoparticles. This was done to determine the dose-response uptake of curcumin, HPPS and HPPS-curcumin on endothelial cells.

2.5. Cell Culture:

For experiments, 0.5 million cells were seeded per 60mm plate and cultured to 80% confluence at $37^{\circ}C$ in a humidified atmosphere composed of 95% air, 5% CO_2. All experiments were conducted on cells that were passaged at least once following recovery from cryogenic storage and did not exceed 7 passages. Cells were cultured for a minimum of 24h after passaging prior to experimental treatment. Initially, THP1 cells were cultured in RPMI-1640 media in 75ml flask. However, for experimental purpose, 0.65million THP1 cells per 60mm dish were seeded with 100nM phorbol 12-myristate 13-acetare (PMA). PMA helps differentiate THP1 cells monocytes into macrophages. Both HUVEC and THP-1 cell lines were purchased from ATCC.

2.5.1. Cell Viability Assay

Cell viability was determined by using the resazurin method. HUVEC were seeded at 10×10^3 cells/well in a 96-well plate with 100µl media/well. Following cell treatment, media was carefully aspirated and wells were rinsed twice with 100ul sterile 1X PBS. Fresh media supplemented with 10% PrestoBlue™ Cell Viability Reagent assay according to manufacturers (Invitrogen) was added to each well and cells were incubated for 3 hours. After the incubation period, the optical density in the medium of each well was measured using a plate-reader (Synergy H4 Hybrid Reader (Biotek) with excitation and emission wavelengths of 560nm and 590nm respectively. The assay is based on the detection of metabolic activity of living cells using a redox indicator, which changes from oxidized (blue) to reduced (red) form. Intensity of red color is proportional to the viability of cells, which is calculated as difference in absorbance between 560nm and 590nm and expressed as percentage of control.

2.6. Immunoblotting

2.6.1. Cell Collection and Lysate Preparation

Cells were harvested on ice by aspirating the media, gently rinsing cells with 1X PBS and scraping the cells in freshly prepared lysis buffer (30µl/60mm plate) that contained sodium orthovanadate, protease and phosphatase inhibitor cocktails. Cell lysates were transferred to fresh 1.5ml tubes and were left on ice for 30 minutes before sonicating the samples on ice for 10 seconds at 20kHz, using the QSonicator. Total protein concentrations were quantified by BCA.

Protein samples (8-20µg) were boiled at 99°C for 5min in 4X Laemmli Buffer with dithiothreitol (DTT).

2.6.2. Western Blotting

Boiled samples (8-20µg) were separated (3.5% stacking gel) and resolved via 10% Mini-Protean sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Mini-Protean self-cast gels were run at 90V at room temperature in 1X Tris/Glycine/SDS Electrophoresis Buffer (BioRad); The samples were wet-transferred to nitrocellulose (0.2µm; BioRad) at 100V for 1 hour 30 minutes at 4ºC. Membranes were briefly rinsed in ddH$_2$O and equal protein transfer was confirmed by incubating the membranes in Pierce® Reversible Memcode Stain for 5 min. The stained blot was labeled and imaged using a ChemiDoc™ MP Imaging System before removing the stain using Pierce® Stain Eraser. Membranes were then blocked in 5% skim-milk in 1X Tris-Buffered-Saline-Tween 20 (TBS-T) for 45 minutes. Membranes were subsequently incubated at 4ºC overnight in primary antibody (1% skim-milk in TBS-T with sodium azide) targeting HMOX1 (1:2000, #ab82585, Abcam), SCARB1 (1:1000, #sab3500048, Sigma). Then the blots were incubated with secondary horseradish peroxidase (HRP)-conjugated anti-rabbit (1:2000-5000, #sc-2054, Santa Cruz) IgG for 2h in 5% milk at room temperature. Immunoreactivity was detected using Clarity™ Western Enhanced Chemiluminescence Substrate (BioRad) or SuperSignal® West Dura Extended Duration Substrate with a ChemiDoc™ MP Imaging System. Membranes were then stripped by incubation in 25ml 0.5M Tris-HCl/SDS buffer supplemented with 125µl β-mercaptoethanol (OmniPur®) for 1 hour and probed again.

2.6.3. Densitometry

The densitometric measurements were completed using ImageLab™ Software v5.0 (BioRad). The relative integrated density was calculated as the density of the target protein normalized to the total protein density of the respective memcode-stained lane. The rearrangement of bands within an individual blot was denoted by a vertical dashed line to aid in presentation and interpretation.

2.7. Statistical Analyses

Thw results are expressed as mean ± standard deviation (SD) unless otherwise stated. The statistical significance between treatments was determined in GraphPad Prism 5 (GraphPad Software Inc.) using either the Student's two-tailed t-Test (comparisons involving two groups) or the One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (P-value <0.05 was considered significant).

CHAPTER 3: Results

3.1. Formulation of HPPS and nanoencapsulation (NC):

Formulation of HDL-mimicking peptide-phospholipid scaffold (HPPS) nanocarrier to take up lipophilic molecules or lipophilic siRNA (via cholesterol-conjugation) *in vitro* and *in vivo* was first established by Zheng et. al. as a form of nanopharmacology [110]. HPPS-nanoparticles is illustrated in Figure-9. The first step in HPPS formulation was the combination of DMPC, chloroform, cholesterol oleate and oleylamine with an optional step of labeling the nanoparticles with dye Cy5.5. The addition of a hydrophobic therapeutic agent, curcumin, was another optional step in the formulation of HPPS. Therefore, in this optional step our therapeutic cargo payload (i.e. curcumin) was first dissolved in chloroform, completely dried and then combined into the constituent ingredients as mentioned above. After a series of steps, such as degassing, drying, water-bath sonication, centrifugation, ultra-sonication and filtration processes, the final end result was the formation of lipid film i.e. HPPS and or HPPS-curcumin. The addition of apoA1 peptide was performed freshly as it was determined empirically by DLS to be a limiting feature of nanoparticle shelf-life. The addition of apoA-1 peptide kept HPPS stable for a week, whereas, without the peptide, HPPS was stable for one-year at 4°C. DLS was used to assess the shelf-life of nanoparticles (storage condition 4°C), on weekly and monthly basis and the data were compared empirically.

3.1.1. Characterization of HPPS and HPPS-curcumin:

Morphology and shape of HPPS and HPPS-Curcumin were recorded using Transmission Electron Microscopy (TEM). TEM analysis of nanoparticles is a semi quantitative technique, which provides approximate information on morphology and the size of nanoparticles [111]. Similar to nascent HDL, empty HPPS are slightly disk shaped in nature, however, addition of curcumin as a payload makes HPPS-curcumin appear more spherical in shape (Figure 10.A). Particle size distribution of nanoparticles were measured by DLS in PBS at pH 7.4. (Figure 10.C). Results of DLS were slightly different from the TEM result. The possible explanation for such variation is because DLS measures the hydrodynamic size of nanoparticles, however, TEM measures the size of nanoparticles in fixed and dried form. The absorption spectra of HPPS and HPPS-Curcumin were measured using a UV-vis spectrophotometer to confirm drug loading.

Although the resultant spectra showed an intense absorption band range from 270 nm - 800 nm in PBS at pH 7.4 for curcumin, however, the appearance of a shoulder peak at around 430nm confirms the presence of curcumin in HPPS (Figure 10.B).
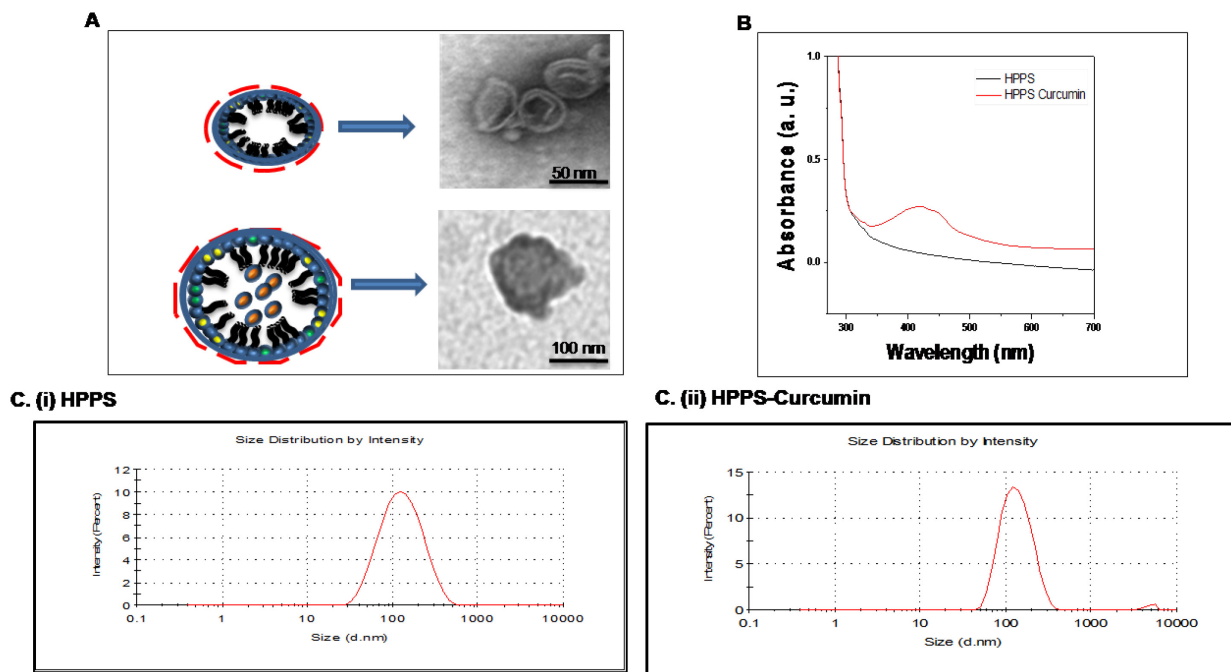
Figure 10 Characterization of HPPS and HPPS-Curcumin.

(A) TEM image of both HPPS and curcumin loaded HPPS (B) the absorption spectra of HPPS, HPPS-curcumin and curcumin were measured using UV-Vis spectrophotometer. (C) the size of the nanoparticles and nanoparticles containing drug payload were measured by DLS. (D) the Z-potential of both HPPS and HPPS-curcumin were measured by DLS.

3.1.2. Validation of HPPS and HPPS-curcumin:

To validate the particle size distribution we measured the DLS of the nanoparticles and to validate the size of nanoparticles, we dispersed 10µL, 20µL and 30µL of HPPS separately in 1mL PBS at pH 7.4 and performed water-bath sonication for 5 min, 10 min and 30min respectively. We did the same for HPPS-curcumin and Figure-11 shows with increasing time of sonication, the nanoparticles mean size distribution reduced. For HPPS the Z-Avg size reduced from 255.9 (d.nm) to 209.2 (d.nm) and for HPPS-curcumin size decreased from 336.6 (d.nm) to 175.9 (d.nm). A possible explanation was that sonication for longer periods of time improved the dissolution rate of compounds (which are lipophilic in nature) in the liquid medium.
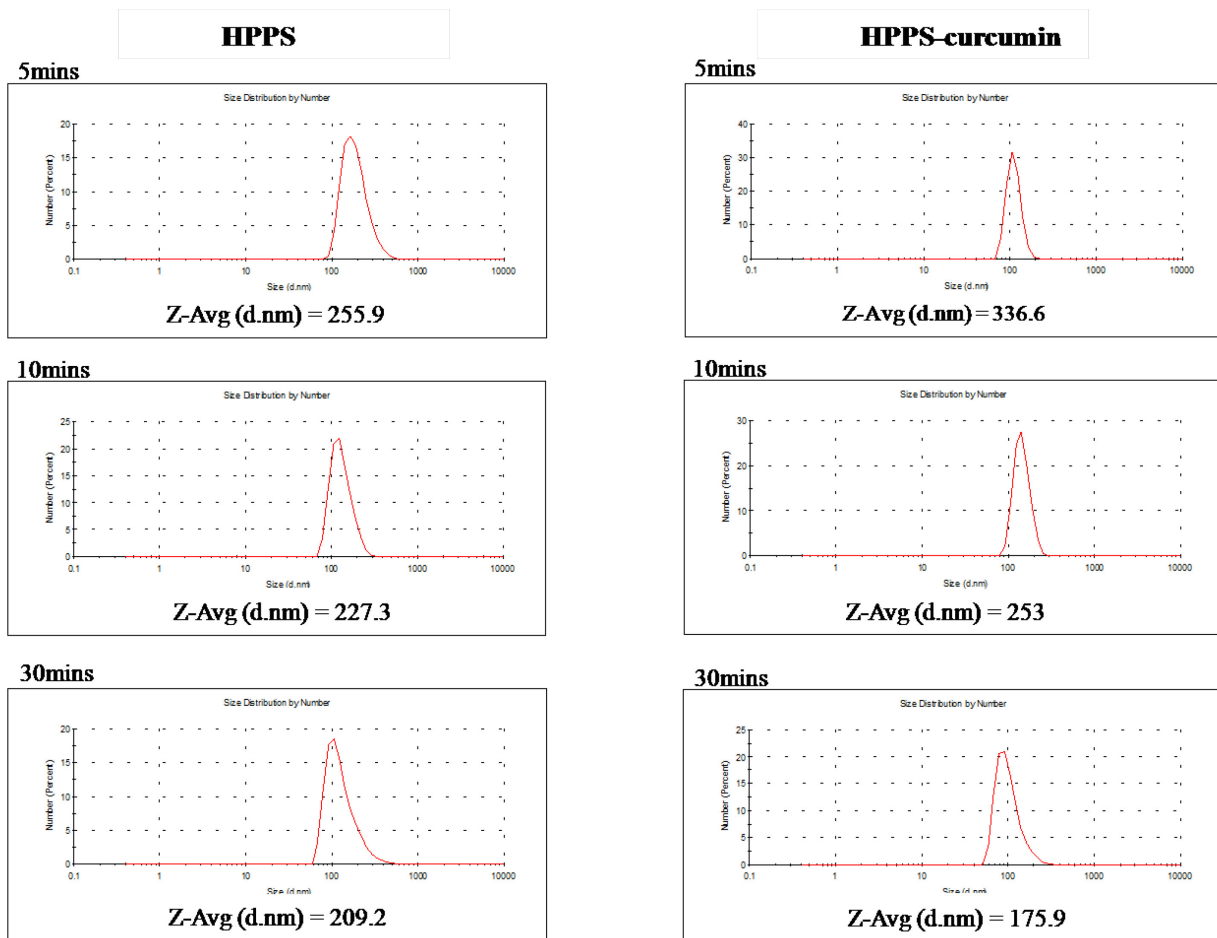
Figure 11. Validation of HPPS and HPPS-curcumin by DLS.

HPPS of 10μL, 20μL and 30μL were dispersed separately in 1mL PBS at pH 7.4 and performed water-bath sonication for 5 min, 10 min and 30min respectively. Similarly, HPPS-curcumin of 10μL, 20μL and 30μL were dispersed separately in 1mL PBS at pH 7.4 and performed water-bath sonication for 5 min, 10 min and 30min respectively. In both HPPS and HPPS-curcumin with increasing the time of sonication the Z-Avg (d.nm) decreased.

3.2. Effect of curcumin, HPPS and HPPS-curcumin on cell viability:

In order to investigate the cytotoxicity/cytocompatibiliy of our therapeutic compounds: curcumin, HPPS and HPPS-curcumin respectively in endothelial cells, a colorimetric resazurin cell viability assay was performed. Figure 12 shows endothelial cell viability measured 16 hours after exposure to various concentrations of our test compounds. Figure 12(A) and Figure 12(C) show concentrations ranging from 0.1μM to 10μM of either curcumin or HPPS alone did not change cell viability compared to the untreated cells. However, the higher concentration (25μM and 50 μM) of curcumin or HPPS alone produced a significant decrease in cell viability. Nevertheless, Figure 12(B) shows a slightly different pattern in the result. Unlike curcumin and HPPS, HPPS-curcumin did not show a significant change in cell viability at 25μM, but did at 50μM. The probable explanation was that unlike curcumin or HPPS alone, a combination of HPPS-curcumin of 25μM compensated for cytotoxic effects to some extent.
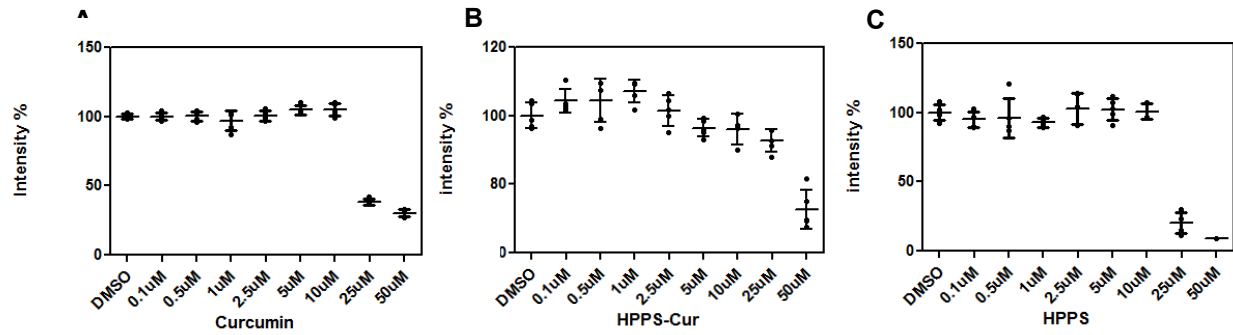
Figure 12 Effect of curcumin, HPPS and HPPS-curcumin on cell viability.

HUVEC were seeded in 96 well plate and treated with: (A) curcumin, (B) HPPS and (C) HPPS-curcumin at concentrations from 0.1μM to 50μM on HUVEC for 16hours. Percentage of viable cells were determined. The intact cell viability level was taken as 100%. Independent assay performed in triplicates

3.2.1. Effect of curcumin, HPPS and HPPS-curcumin on cell viability by fluorescence-activated cell sorting (FACS):

To independently confirm whether cells remain viable or die when treated with curcumin or HPPS-curcumin, we analyzed the curcumin and/or nanoparticles treated cells with JC-1 mitochondrial stability assay by FACS. JC-1 is a mitochondrial membrane potential dependent, fluorescent dye, which accumulate in mitochondria and condense where there is viable membrane integrity. Figure-13 shows: (A) HUVEC without any treatment represented as negative control; B) HUVEC treated with H_2O_2 of 500µM represents as positive control, showing the percentage of dead cells were higher than the viable cells; HUVEC were treated with (C) curcumin; (D) HPPS and (E) HPPS-curcumin of 5µM concentrations respectively - all showed higher percentage of viable cells than dead cells. Taken together, neither curcumin nor HPPS-curcumin were toxic to HUVEC when at a reasonable dose of 5µM.

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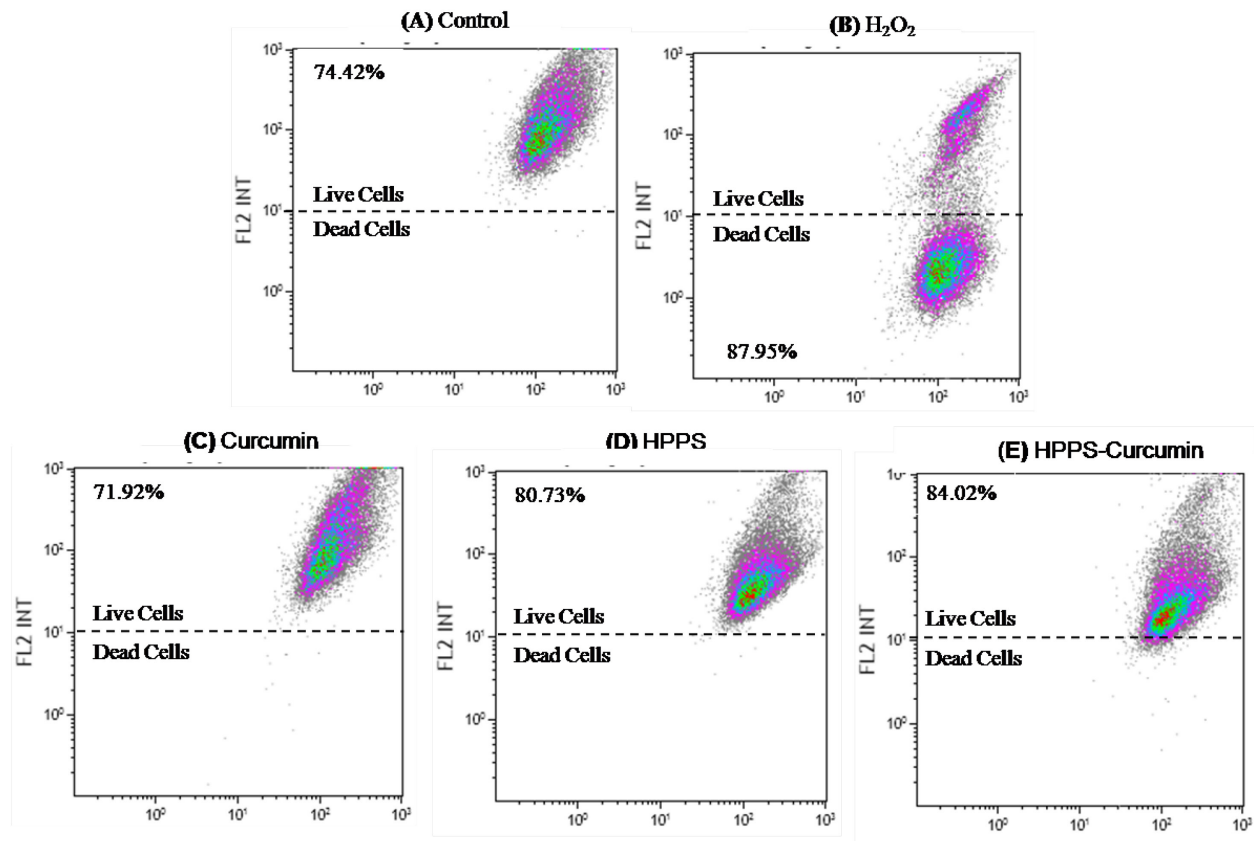
Figure 13 Effect of curcumin, HPPS and HPPS-curcumin on cells viability by fluorescence-activated cell sorting FACS) JC-1 mitochondrial integrity assay.

(A) HUVEC without any treatment represented as negative control; (B) HUVEC treated with H_2O_2 of 500μM represented as positive control, showing percentage of dead cells were higher than the viable cells; HUVEC treated with (C) curcumin; (D) HPPS and (E) HPPS-curcumin of 5μM concentrations - all showed higher percentage of viable cells than dead cells. JC-1 is a mitochondrial membrane potential dependent, fluorescent monomer green dye, which accumulate in mitochondria and condensed into red dimer dye where there is viable membrane integrity.

3.3. Effect of curcumin on endothelial cells HMOX-1 expression:

In order to evaluate the effect of curcumin on HMOX-1 expression, HUVEC were treated with curcumin, both dose and time dependently. Exposure of HUVEC to variable non-toxic doses of curcumin (1µM, 2.5µM, 5µM and 10µM) for 16hrs resulted in a concentration dependent increase in heme oxygenase-1 (HMOX-1) activity (Figure-14A). Sub-maximal induction in endothelial heme-oxygenase-1 of 5µM curcumin (which was also shown to be non-toxic with a two-fold safety consideration) was chosen to determine the time response effect of curcumin in HMOX-1 expression. As shown in Figure-14B, treatment of HUVEC with 5µM curcumin resulted in a time-dependent increase in heme-oxygenase expression. After 16hrs post-exposure to curcumin, expression of HMOX-1 gradually decreased with increasing time and returned to baseline by 48hrs.
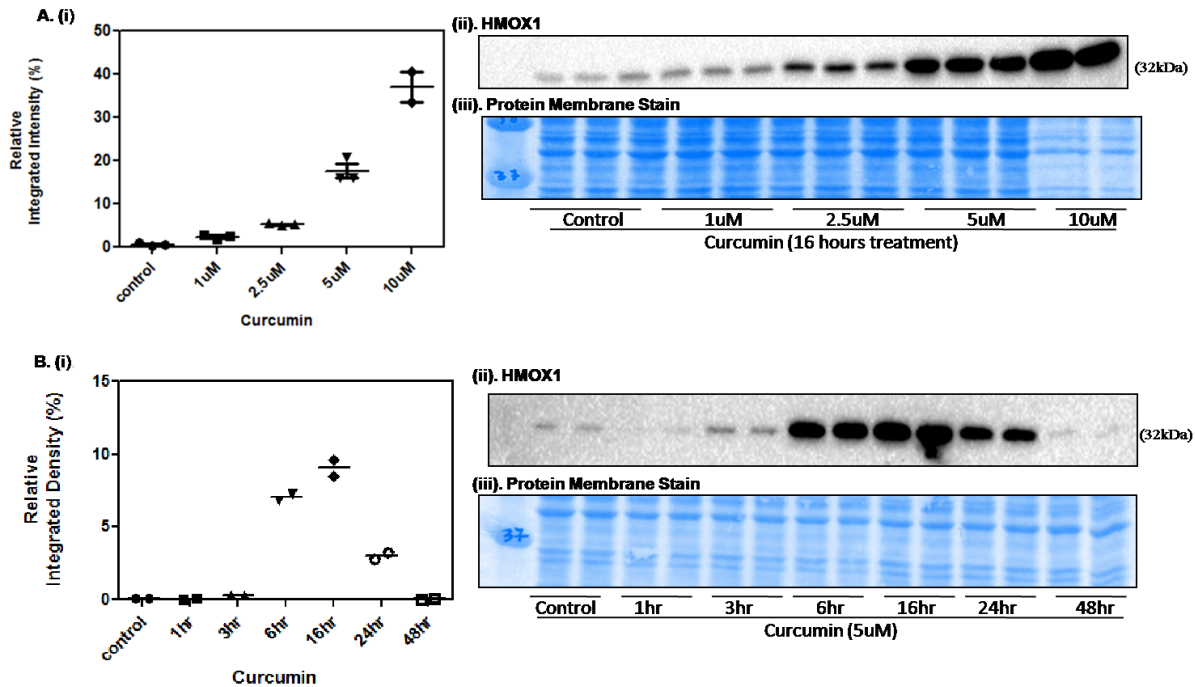
Figure 14 Dose response and time response effect of curcumin on heme oxygenase 1 (HMOX-1) expression.

To detect the effect of curcumin on HMOX1 expression, HUVEC were treated for 16 hours for: (A) dose dependency and (B) time dependency. A(i). There was a gradual increase in HMOX1 expression with increased concentration of curcumin. A(ii) Representative HMOX-1 probed membrane displayed specific prominent HMOX-1 band at 32 kDa. A(iii) Pierce® Memcode staining demonstrated uniform protein loading on the membrane prior to antibody probe (except for 10uM which was underloaded in total protein to avoid signal saturation). Representative blot of concentration dependent in two identical triplicates B (i). At 16 hours treatment the expression of HMOX1 were maximum in comparison to other time frames. B (ii). Representative HMOX-1 probed membrane displayed specific prominent HMOX-1 band at 32 kDa. B(iii) Pierce® Memcode staining demonstrated uniform protein loading on the membrane prior to antibody probe.; Representative blot is empirical data of time dependent studies performed in duplicate of one independent trial.

3.4. Relative uptake with variable doses of nanocarriers:

We next evaluated the relative uptake of various concentrations of curcumin and HPPS with or without curcumin by HUVEC using FACS. Curcumin is auto-fluorescent in nature, hence FL10 (emission of 550/40nm) channel was used to detect the dose dependent uptake of curcumin alone (Figure-15A). Having added the dye Cy 5.5 during the synthesis of HPPS and HPPS-curcumin, we were able to use FL7 (emission of 725nm) for monitoring HPPS uptake (Figure-15B and Figure 15C). These data show that either curcumin or Cy5.5 conjugated HPPS can effectively be used for uptake analysis tracking in detached HUVEC cells by FACS and confirm single-cell dose-dependency in uptake.
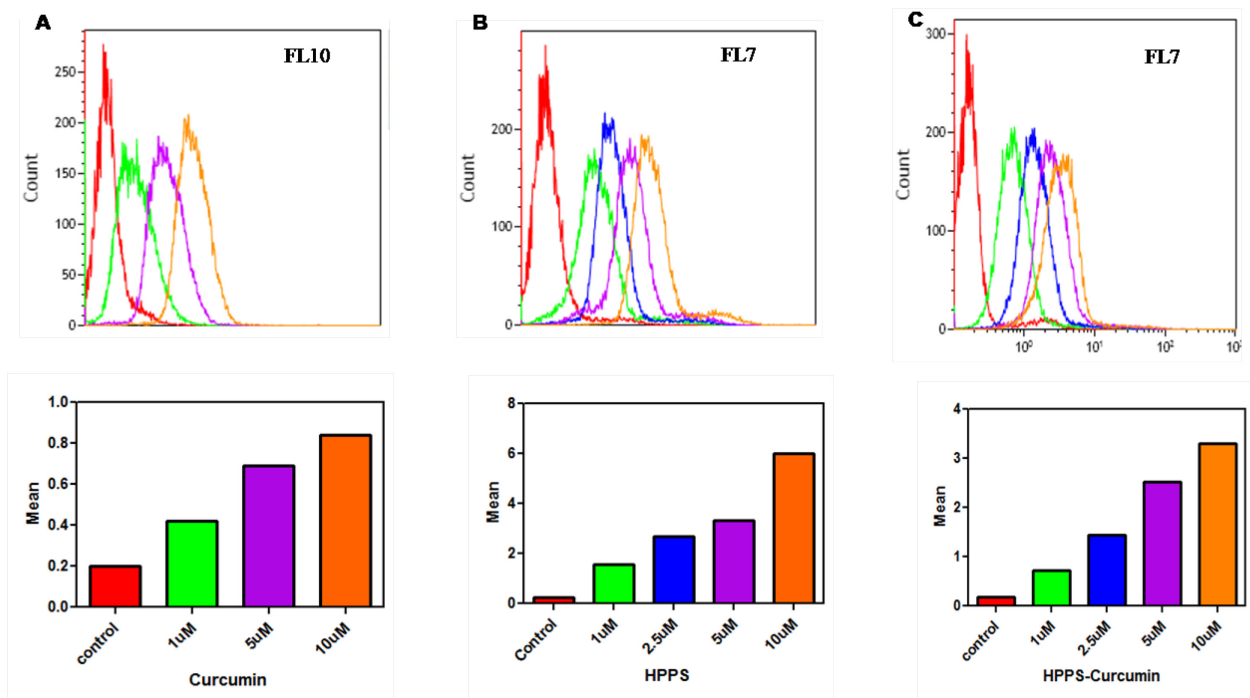
Figure 15 Comparing the effect of curcumin, HPPS and HPPS-curcumin on cellular uptake by Fluorescence Activated Cell Sorting (FACS).

(A) curcumin of (1μM), (5μM) and (10μM) concentrations, **(B)** HPPS and **(C)** HPPS-curcumin of (1μM)Equ, (2.5μM)Equ, (5μM)Equ and (10μM)Equ concentrations were used to treat HUVEC for 16hours. All three histograms show a gradual increase in cellular uptake with increasing concentration.

3.5. Effect of HPPS and HPPS-curcumin on endothelial cells HMOX-1 expression:

After analyzing the effect of curcumin on HUVEC, we next evaluated the bio-equivalency of nanoparticles HPPS and HPPS-curcumin on HUVEC. Therefore, Figure 16 represents Western Blot analysis of effect of HPPS and HPPS-curcumin in heme oxygenase-1 (HMOX-1) activity. Figure 16(A) shows exposure of HUVEC to HPPS for 16hrs resulted in concentration dependent gradual increase in heme oxygenase-1 (HMOX-1) activity similar to that of curcumin in Figure 14(A). Western blot analysis revealed that enhanced heme oxygenase activity by HPPS treatment directly correlated with HMOX-1 protein level. Interestingly, HPPS-curcumin did not follow the similar pattern as curcumin and/or HPPS. As shown in Figure 16(B), unlike curcumin and/or HPPS, exposure of HUVEC with a gradual increment in HPPS-curcumin concentration did not enhance the heme oxygenase-1 protein expression. These data may suggest, a combination of nanoparticles with curcumin may not have the similar effect as individual curcumin and or HPPS in inducing HMOX-1 protein in endothelial cell line. Collectively, based on cell viability, western blot and FACS analysis, we chose the concentration (5µM) and the time exposure (16 hrs) of further experiments as a potentially therapeutically relevant dose.
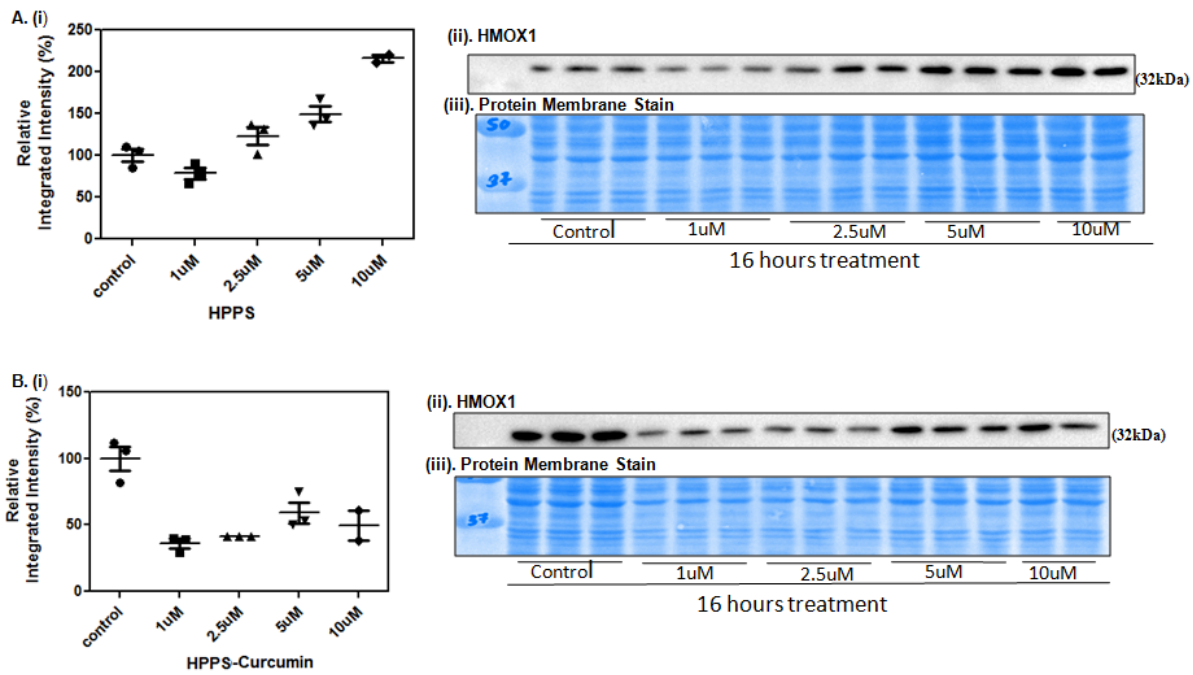
Figure 16 Comparison of effect of various concentrations of HPPS and HPPS-curcumin on the expression of heme oxygenase-1 (HO-1) protein.

To detect the dose-dependent effect, HUVEC were treated with (A) HPPS and (B) HPPS-curcumin of (1µM), (2.5µM), (5µM) and (10µM) equivalent concentrations, respectively for 16 hours. A(i) HMOX1 expression showed there is a gradual increase in expression with increasing the concentration of HPPS alone. A(ii) Representative HMOX-1 probed membrane displayed specific prominent HMOX-1 band at 32 kDa. A(iii) Pierce® Memcode staining demonstrated uniform protein loading on the membrane prior to antibody probe. B(i) HMOX1 expression showed there was no significant change in HO-1 expression with increasing HPPS-curcumin concentration. B(ii) Representative HMOX-1 probed membrane displayed specific prominent HMOX-1 band at 32 kDa. B(iii) Pierce® Memcode staining demonstrated uniform protein loading on the membrane prior to antibody probe. Representative blot is the duplicate of one independent trial.

3.6. Dose equivalency effect of HPPS-curcumin to curcumin in HMOX-1 protein expression:

After getting data regarding the safety and efficacy of inducing HO-1 of our therapeutic agent curcumin, HPPS and/or HPPS-curcumin on HUVEC and their individual dose-response effect on the expression of HMOX-1 protein, it was vital to find out the dose equivalency between HPPS-curcumin and curcumin to build a dose-efficacy relationship. Therefore, to evaluate the dose-equivalency, HUVEC were treated with 5µM curcumin and (5µM)Equ HPPS-curcumin and HPPS respectively for 16 hours in order to express HMOX-1 protein. Figure 17(A) shows that there was no dose-equivalency between 5µM curcumin and (5µM)Equ HPPS-curcumin and HPPS. Effect of 5µM curcumin in expressing HMOX-1 protein was significantly higher than those of encapsulated curcumin and or of empty nanoparticles. Moreover, our nanoparticles are HDL-mimetic nanoparticles so we had to analyze the impact of curcumin or HPPS-curcumin on our target receptor i.e. SCARB-1 and Figure 17(B) shows expression of SCARB-1 receptor did not change much with either curcumin or with HPPS-curcumin.
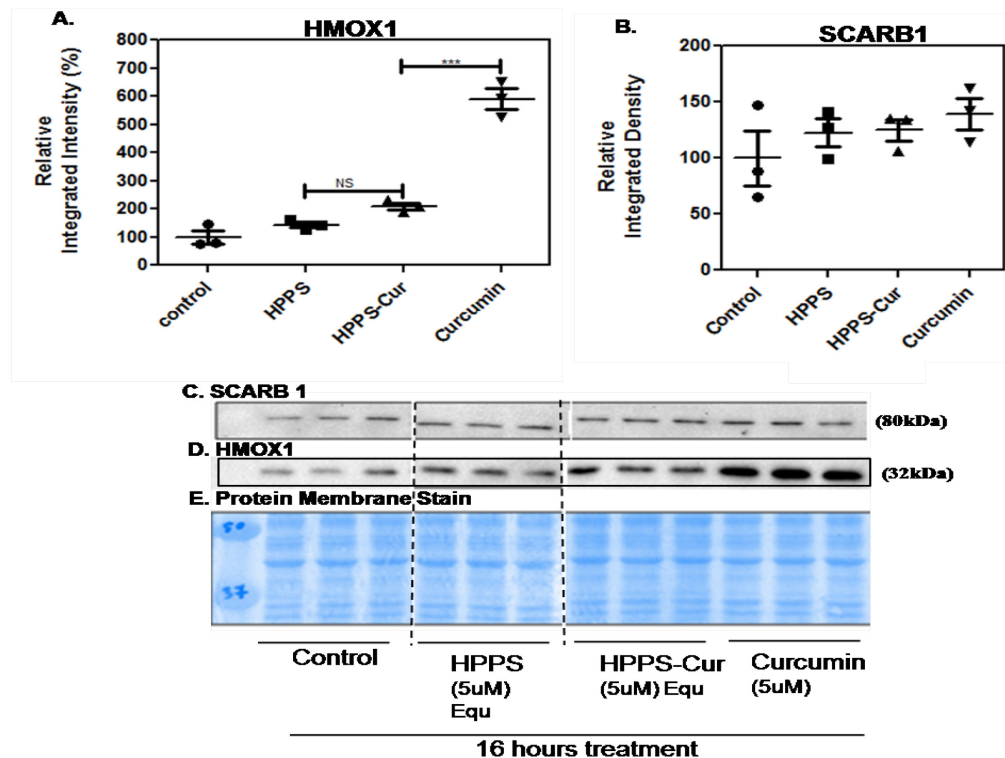
Figure 17 Dose equivalency effect of HPPS-curcumin to curcumin in HMOX-1 protein expression

 To detect the dose equivalency of HPPS-curcumin to curcumin of 5μM concentration, on HMOX1 expression, HUVEC were treated with 5μM curcumin and (5μM)Equ, HPPS and HPPS-Curcumin respectively for 16 hours . (A) with equivalent concentrations of curcumin and HPPS-curcumin, HMOX1 expression was not found to be at the same level. Hence, no dose equivalency was found between curcumin and HPPS-curcumin. (B) Expression of SCARB1 did not vary much with curcumin, HPPS and or HPPS-curcumin (C) Representative SCARB-1 probed membrane displayed specific prominent SCARB-1 band at 80 kDa. (D) Representative HMOX-1 probed membrane displayed specific prominent HMOX-1 band at 32 kDa. (E). Pierce® Memcode staining demonstrated uniform protein loading on the membrane prior to antibody probe. Representative blot is the triplicate of one independent trial.

3.6.1. Dose response effect of HPPS-curcumin to curcumin on HMOX-1 expression:

Even though any dose equivalency between 5µM curcumin and (5µM)Equ HPPS-curcumin could not be established from previous data, the dose equivalency between curcumin and the nanoparticles needed to be explored. Therefore, to investigate the dose equivalency, HUVEC was treated with 5µM curcumin and HPPS-curcumin of (5µM)Equ, (25µM)Equ, (40µM)Equ, (80µM)Equ and (160µM)Equ respectively for 16 hours. Surprisingly, even with HPPS-curcumin of 32 times higher concentration than that of curcumin (5µM), dose-equivalency could not be determined (Figure 18). This piece of data might suggest that, to achieve the desired dose-equivalency in HUVEC, we needed to reconsider either (a) the nanoparticles (HPPS-curcumin) or (b) the target for cell type uptake and processing.
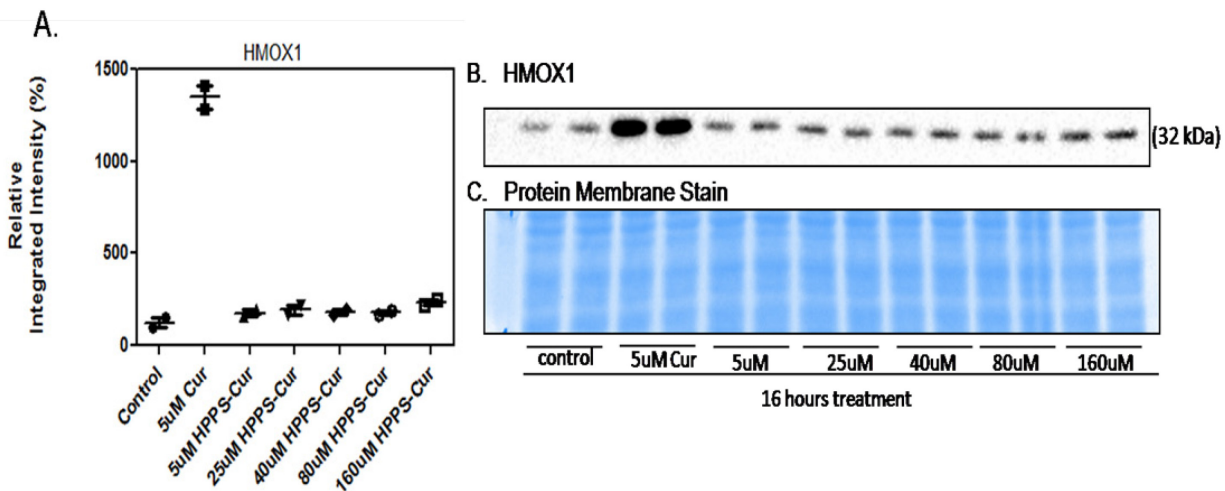
Figure 18 Dose response effect of HPPS-curcumin on heme oxygenase 1 (HMOX-1) expression.

To detect the dose equivalency of HPPS-curcumin to curcumin of 5µM concentration, on HMOX1 expression, HUVEC were treated with 5µM curcumin and (5µM)Equ, (25µM)Equ, (40µM)Equ, (80µM)Equ and (160µM)Equ of HPPS-curcumin for 16 hours . (A) There was no change in HMOX1 expression with increasing HPPS-curcumin concentrations. Therefore, there was no dose equivalency between curcumin and HPPS-curcumin. (B). Representative HMOX-1 probed membrane displayed specific prominent HMOX-1 band at 32 kDa. (C). Pierce® Memcode staining demonstrated uniform protein loading on the membrane prior to antibody probe. Representative blot is the duplicate of one independent trial.

3.6.2. Dose response effect of surface-coated HPPS-curcumin on HMOX-1 expression:

In this thesis, not only did we aim to synthesize HPPS, but also to formulate nanoparticles with internalized cargo (curcumin), which have dose-equivalency. However, previous data indicated a failure to establish dose-equivalency or any dose-response effect even with increased concentrations of HPPS-curcumin. Therefore, in the following experiment curcumin of various concentrations were directly incorporated onto HPPS (termed as surface-coated HPPS-curcumin) to evaluate dose-equivalency. Figure 19 shows HUVEC treated with 5µM curcumin and (5µM)Equ, (25µM)Equ, (40µM)Equ, (80µM)Equ and (160µM)Equ of surface-coated HPPS-curcumin for 16 hours provided a gradual rise in HMOX1 expression. There was a dose equivalency between 5uM curcumin and surface-coated HPPS-curcumin of (25µM)Equ. In comparison to curcumin alone, this decorated nanoparticles play no advantage over curcumin as the therapeutic agent was tagged on the surface of the nanoparticles; hence, chance of instability and rapid oxidation remain same as curcumin itself.

Figure 19 Dose response effect of HPPS decorated "curcumin" on heme oxygenase-1 (HMOX-1) expression.

To detect the dose equivalency of HPPS decorated curcumin on HMOX1 expression, HUVEC were treated with 5μM curcumin and (5μM) Equ, (25μM)Equ, (40μM)Equ, (80μM)Equ and (160μM)Equ of HPPS decorated curcumin for 16 hours . (A) There was a gradual increase in HMOX1 expression with increasing the concentration of HPPS decorated curcumin. There was a dose equivalency between 5μM curcumin and HPPS decorated curcumin of (25μM)Equ. (B). Representative HMOX-1 probed membrane displayed specific prominent HMOX-1 band at 32 kDa. (C). Pierce® Memcode staining demonstrated uniform protein loading on the membrane prior to antibody probe. Representative blot is the duplicate of one independent trial

3.7. Dose-Equivalency effect of Nio-curcumin to curcumin for HMOX-1 expression:

Since we could not establish the dose equivalency between curcumin and HPPS-curcumin and we observed a five-fold difference in dose equivalency in decorated-HPPS-curcumin; we postulated that it might be cell/receptor dependent. We therefore repeated the dose-equivalency experiment with different nanoparticles containing curcumin i.e. Nio-curcumin, these niosome nanoparticles are taken up by cells in a receptor independent fashion (or by non-specific scavenger receptors). HUVEC were treated with 5μM curcumin, (5μM)Equ HPPS-curcumin and (5μM)Equ Nio-curcumin respectively, for 16 hours in order to analyze HMOX-1 protein expression. Interestingly, Figure 20(A) shows no dose-equivalency between 5μM curcumin and (5μM)Equ Nio-curcumin. Similar to that of Figure 17, here we found the effect of 5μM curcumin in expressing HMOX-1 protein was significantly higher than Nio-curcumin. Figure 20(B) shows expression of SCARB-1 receptor did not change much with either curcumin, Nio-curcumin or HPPS-curcumin.
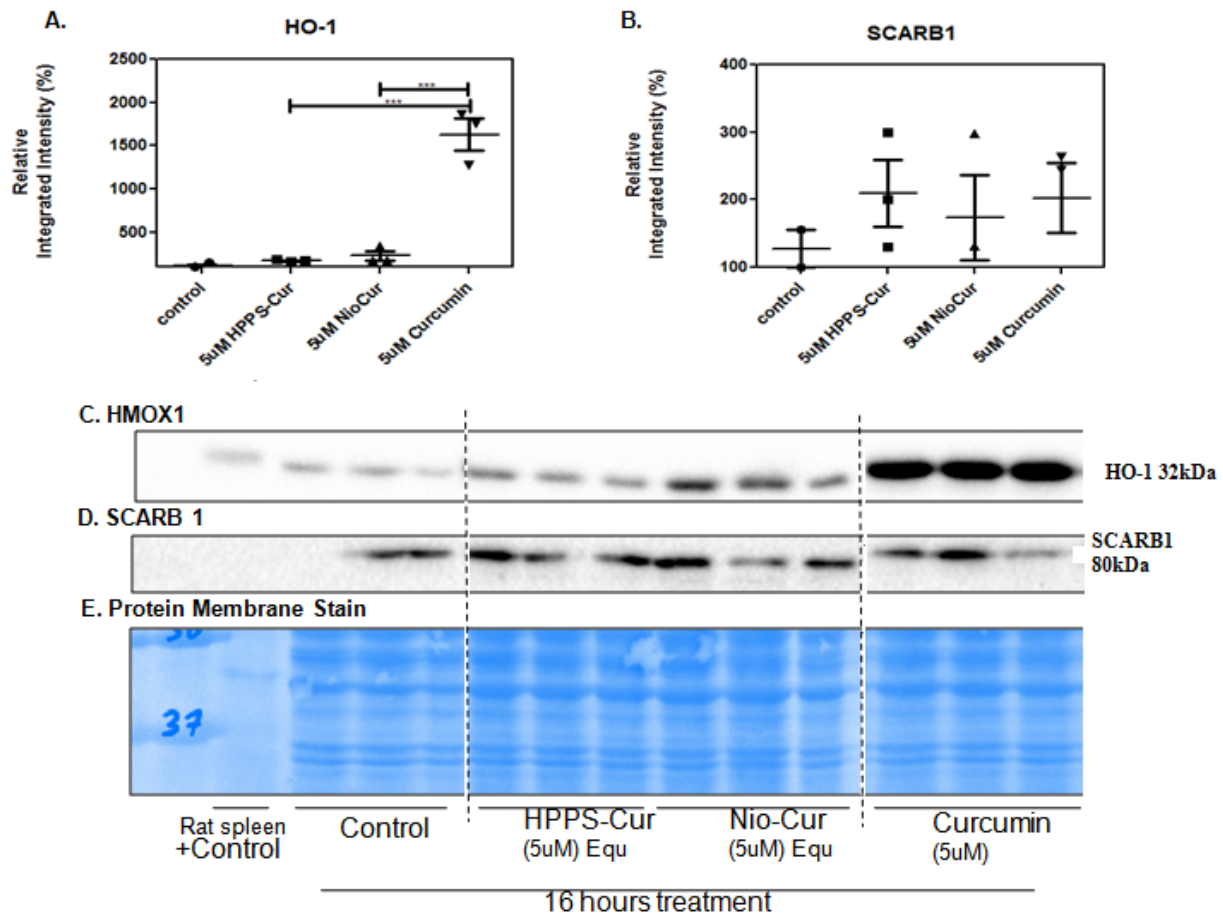
Figure 20 Comparison of dose equivalency of HPPS-curcumin, Nio-curcumin and curcumin of 5μM equivalent concentration for the expression of HO-1 in endothelial cells.

To detect the dose equivalence, HUVEC were treated with HPPS-curcumin, Nio-curcumin and curcumin of 5μM concentration for 16 hours. (A) HMOX1 expression showed curcumin of 5μM was not dose equivalent to either HPPS-curcumin or Nio-curcumin of 5μM (B) Expression of SCARB1 did not vary with HPPS-curcumin, Nio-curcumin or curcumin (C) Representative HMOX-1 probed membrane displayed specific prominent HMOX-1 band at 32 kDa. (D) Representative SCARB-1 probed membrane displayed specific prominent SCARB-1 band at 80 kDa. (E). Pierce® Memcode staining demonstrated uniform protein loading on the membrane prior to antibody probe. Representative blot is the triplicate of one independent trial.

3.7.1. Dose-response effect of Nio-curcumin for HMOX-1 protein expression:

We did not find dose equivalency between 5µM curcumin and (5µM)Equ Nio-curcumin from though there was an indication for Nio-curcumin to induce HMOX-1 and so we were curious to find out if there was a dose response effect of various concentrations of Nio-curcumin on HUVEC. Hence, HUVEC were treated with 5µM curcumin and Nio-curcumin of (5µM)Equ, (10µM)Equ, and (15µM)Equ respectively, for 16 hours. Interestingly, with 5µM curcumin and (15µM)Equ Nio-curcumin expression of HMOX-1 protein were similar (Figure 21). These data suggest that uptake of curcumin varies by nanoparticle type and determines the bioavailability of curcumin to induce HMOX-1 in endothelial cells. We observe no change in SCARB1 but unlike receptor specific HPPS, receptor independent/non-specific nanoparticles (niosomes) were able to achieve dose equivalency, though at a relative higher concentration (three fold).
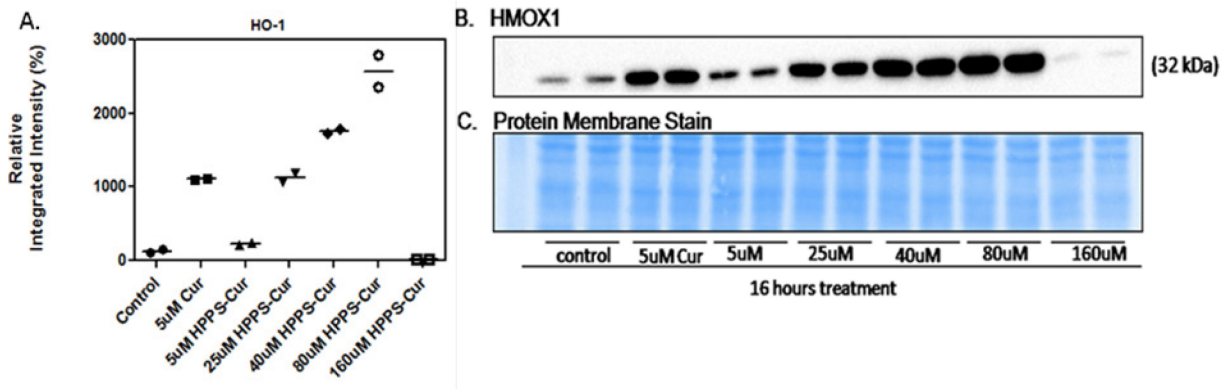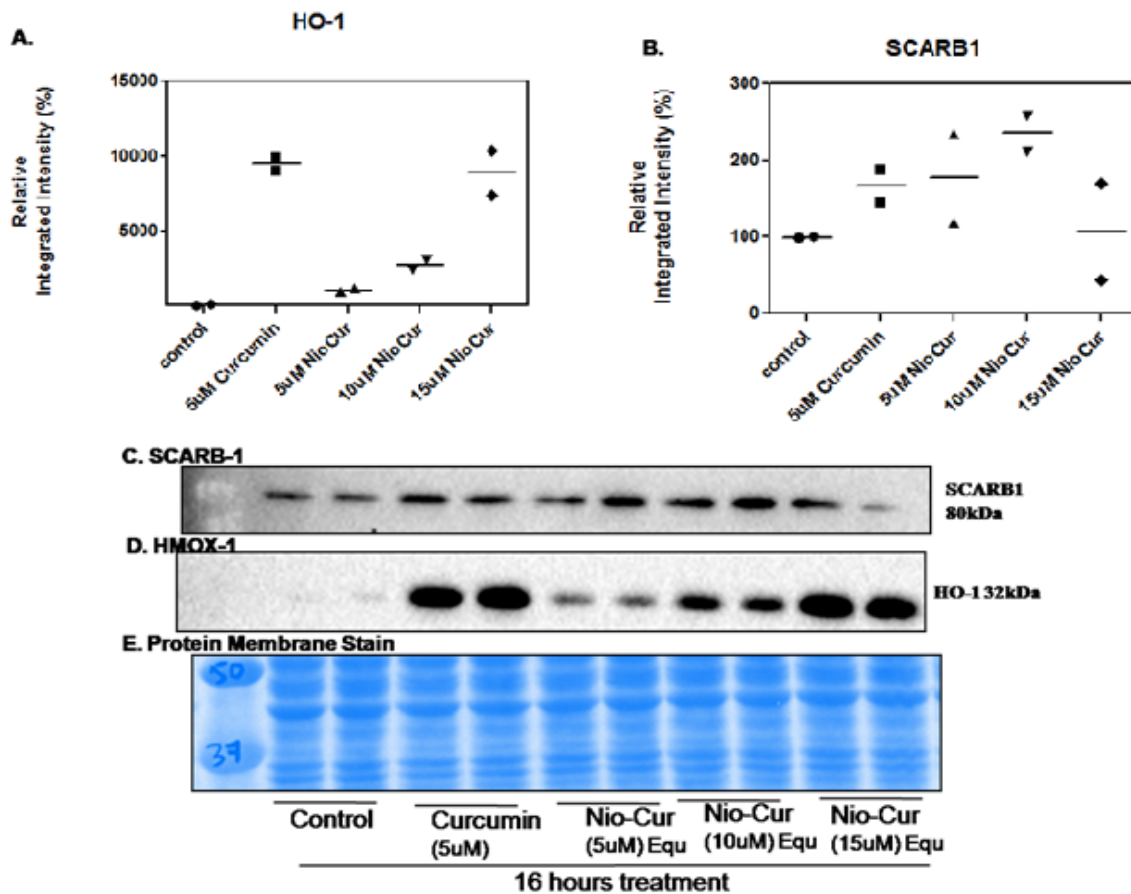
Figure 21 Dose response effect of Nio-curcumin on heme oxygenase 1 (HMOX-1) expression.

To detect the dose equivalency of Nio-curcumin to curcumin of 5μM concentration, on HMOX1 expression, HUVEC were treated with 5μM curcumin and (5μM)Equ, (10μM)Equ, and (15μM)Equ of Nio-curcumin for 16 hours . (A) with 5μM curcumin and (15μM)Equ Nio-curcumin expression of HMOX-1 protein is very close. Therefore, there is a dose equivalency between 5μM curcumin and (15μM)Equ Nio-curcumin. (B) Expression of SCARB1 did not vary much with curcumin and various concentrations of Nio-curcumin. (C) Representative SCARB-1 probed membrane displayed specific prominent SCARB-1 band at 80 kDa. (D). Representative HMOX-1 probed membrane displayed specific prominent HMOX-1 band at 32 kDa. (E). Pierce® Memcode staining demonstrated uniform protein loading on the membrane prior to antibody probe. Representative blot is the duplicate of one independent trial.

3.8. Dose response effect of Nio-curcumin on macrophages (THP1 cells) for HMOX-1 expression:

To investigate whether the challenge of obtaining dose-equivalency is cell dependent or not, the dose response effect of Nio-curcumin for HMOX-1 expression were performed with macrophages. Macrophages (THP1 cells) were treated with 5μM curcumin, HPPS-curcumin and Nio-curcumin of (5uM)Equ concentrations, respectively, for 16 hours. Figure 22(A) shows there was a dose-equivalency between 5μM curcumin and (5μM)Equ Nio-curcumin in macrophages; Figure 22(B) shows expression of SCARB-1 receptor did not change much with curcumin, HPPS-curcumin or Nio-curcumin in either cell type. All our western blot data on SCARB-1 suggest, there is a possibility of nanoparticles variably entering/interacting with different cell types (both in endothelial cells and macrophages) via either different receptors or different pathways of internalization of the nanoparticles. These data suggest, cell type and variable scavenger receptors may be essential to achieving accurate dose-equivalency between free curcumin and nanoparticles loaded curcumin. Our data shows, HPPS-curcumin is not able to achieve dose equivalency to curcumin in HUVEC if that curcumin is internalized. However, that surface-coated HPPS-curcumin achieves five-fold less dose equivalency to curcumin in HUVEC. Furthermore, macrophages demonstrated only 40% dose equivalence to curcumin with HPPS-curcumin and yet dose equivalence with niosome-curcumin to curcumin. This indicates that drug distribution in nanoparticles affects the bioavailability of the drug in certain cell types. These data taken together indicate that HPPS is not an effective carrier to target HUVEC and is less effective than other nanocarriers to target macrophages at dose equivalence. Future experiments will be required to determine whether macrophage-specific targeting by HPPS could promote reverse cholesterol transport and to determine what the mechanisms for the variable nano-pharmacokinetics are in macrophages in comparison to that of HUVEC. Tracking the drug distribution inside or on the surface of cells may be necessary. The therapeutic utility of HPPS may be limited to cells with additional mechanisms of nanoparticle uptake (other scavenger receptors) and could be independent of SCARB1 mechanisms. HPPS do not appear to be effective targeting nanocarriers for the endothelium. Additional experiments exploring contextual nano-pharmacokinetics concomitant with inflammation or oxidative stress are warranted.
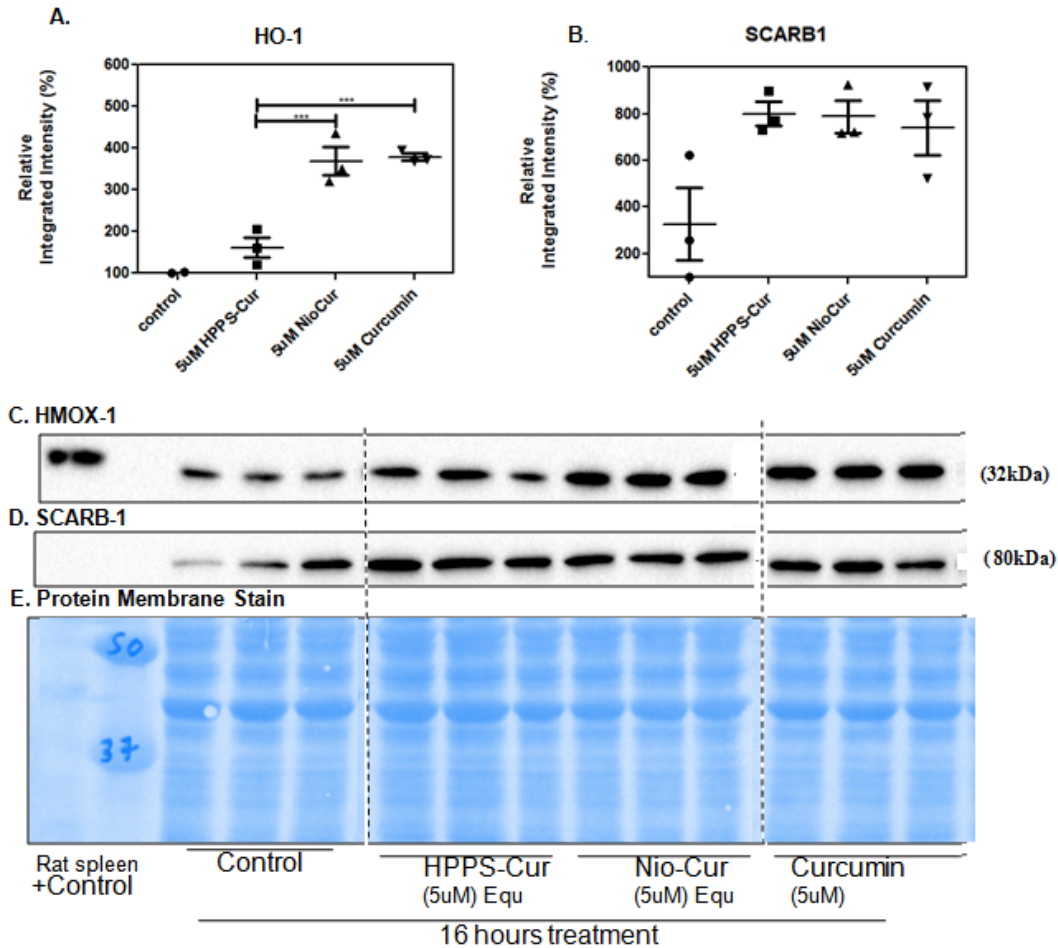
Figure 22 Effect of HPPS-curcumin, Nio-curcumin and curcumin on THP1 cell line.

To detect the effect of HPPS-curcumin, Nio-curcumin and curcumin on cell lines other than HUVEC , THP1 were treated with HPPS-curcumin, Nio-curcumin and curcumin of (5µM)Equ, respectively for 16 hours. (A) HMOX1 expression showed curcumin of 5µM was dose equivalent to Nio-curcumin of 5µM (B) Expression of SCARB1 did not vary much with HPPS-curcumin, curcumin or Nio-curcumin (C) Representative HMOX-1 probed membrane displayed specific prominent HMOX-1 band at 32 kDa. (D) Representative SCARB-1 probed membrane displayed specific prominent SCARB-1 band at 80 kDa. (E). Pierce® Memcode staining demonstrated uniform protein loading on the membrane prior to antibody probe. Representative blot is the triplicate of one independent trial.

CHAPTER 4: Discussion

4.1. Synthesis of Major Findings:

In this thesis, we aimed to synthesize HDL mimetic nanoparticles, HPPS, that encapsulate curcumin as a means of therapeutic substrate delivery to increase the expression of HMOX-1 in HUVEC. Moreover, this study aimed to observe the *in vitro* effect of nanoparticles via cellular uptake and expression of HMOX-1 protein to establish dose-equivalency for the purpose of titrating comparable therapeutic effects. The synthesis of nanoparticles and the expected mechanism of delivery was based materially on the original *in vitro* and *in vivo* studies performed by the laboratory of G.Zheng et al. in two papers: 1. Biomimetic nanocarrier for direct cytosolic drug delivery; [68]. 2. HDL-mimicking peptide-lipid nanoparticles with improved tumor targeting. [105].

The results described in this thesis established the preparation of HPPS and successful loading of curcumin as the purported therapeutic agent. However, HPPS transfer drug payload in a variable manner if the drug is loaded internally compared to surface decorated. The former was reported as necessary in previous literature and the latter was less desirable due to potential limitations in shelf-life (oxidation). Nanoparticles phenotype and drug loading approach (internal vs surface) and cell types are both determinant to establishing dose equivalency. Thus the pharmacokinetics and pharmacodynamics for nanoparticle drug delivery require further study and the mechanisms of variable uptake and intracellular distribution and metabolism are in question. However, the concept of using synthetic nanoparticles, such as those resembling HDL as drug delivery vehicles or components to therapy have not been ruled out.

In general, there is evidence supporting the importance of lipoproteins in controlling lipid metabolism in humans generally. For example, lipoproteins are endogenous nanoparticles for transporting hydrophobic cholesterols and triglycerides to cells throughout the circulatory system [112]. The laboratory of Van Berkel was one of the first groups to study the usage of HDL as a nanocarrier for therapeutics [113]. Similarly, other groups such as Lacko et al. (2008) and Ng et al. (2011) have also worked with HDL as nanoparticles to deliver drugs into the cells. Lacko et al. studied the use of paclitaxel-loaded HDL particles on cancer cells and found an IC_{50} (half maximal inhibitory concentration) to be 5-20 times lower than that of free drug [114] Ng et al.

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have designed nanoparticles in which a fluorescent photo-sensitizer compound was incorporated into HDL to monitor uptake of fluorescent payload over time *in vivo* [111].

The natural, yellow colored solid curcumin (Cur) is a polyphenolic compound isolated from the plant *curcumna longa;* it has been in use as food spice, cosmetics and traditional medicine for many centuries. There is abundant evidence that supports curcumin as an antioxidant, anti-inflammatory, anti-tumoral agent and as well as a fluorescent agent in tracking biological mechanisms at the molecular level [77]. R.Olszanecki et al. (2005) have demonstrated that oral administration of curcumin at low dose (dose of 0.3mg/day/per mouse for four months), incorporated with animal diet, reduced the formation of atherosclerotic lesions in apoE/LDLR double knockout mice [115]. Gupta B et al. (1999) described that curcumin inhibited NFkB in stimulated human umbilical vein endothelial cells [116]. Motterlini R et al. (2000) observed anti-oxidant and anti-inflammatory properties of curcumin in endothelial cells by inducing heme-oxygenase-1 enzyme [117]. Over the years, collective evidence has indicated the significance of HO-1 in protecting cardiovascular system with its anti-inflammatory, anti-apoptotic, anti-oxidant properties. Ishikawa K et al. (2012) studied role of HO-1 in cardiovascular system in one-year old HO-1-knockout mice. Absence of HO-1 gene resulted alteration in HDL composition along with attenuation of an important enzyme (paraoxonase) by 50% and also excess of plasma lipid hydroperoxides [118].

After synthesis of HPPS nanoparticles, we analyzed the physical properties i.e. measuring size-morphology and the entrapment efficacy of drug payload within the nanoparticles. TEM image revealed empty HPPS to be discoidal and cargo loaded HPPS to be more spherical in shape. Similar data has been reported by the laboratory of Gang Zheng et al. (2009) [68] when they synthesized biomimetic nanocarriers for direct cytosolic drug delivery. It is important to note that results of our DLS (dynamic light scattering) analysis were not in complete agreement with the TEM result. The possible explanation for such variation could be DLS and TEM are that these are very different techniques with their own advantages and limitations - DLS measures the hydrodynamic size of nanoparticles whereas TEM measures the size of nanoparticles in dried form and gives the actual material size. A common issue with DLS measures are air bubbles in media giving false positive, relatively small sizes and agglomeration/aggregation properties of nanoparticle, which in turn can give false positive, relatively large sizes in a sample. Surface

charge and functional groups of a nanoparticles determine their abilities to circulate and play essential role to maintain monodispersity. During the TEM process, the sample processing could shrink or distort the shape and size of nanoparticles. Thus, the combination of both methods should be employed to ensure certainty of the hydrodynamic and material size of well dispersed nanoparticles.

Here, we used UV-spectrophotometery to determine the successful loading of curcumin in HPPS. However, this approach does not indicate whether the curcumin is encapsulated/incorporated into the hydrophobic core of HPPS or simply decorated on HPPS surface. In this thesis, with a slight variation in the nanoparticles-cargo payload step, we sought to internalize the cargo (curcumin) within the core of the nanoparticles. As curcumin is a light-sensitive polyphenol that tends to oxidize rapidly, this modification was deemed necessary in improving the shelf-life of the active compound by enveloping it in stable nanoparticles. It is possible that, high-resolution confocal microscopy images would have confirmed whether curcumin is encapsulated within the core or simply embedded on the outer layer of HPPS. This could also be used to track the drug distribution once take up by cells.

After characterizing the physical properties of drug loading, we analyzed the biocompatibility of these nanoparticles on our cell line human umbilical vein endothelial cells (HUVEC) via cell cytotoxicity assay and JC1-FACS analysis. Results of our study showed no cytotoxic effect on endothelial cell lines. Cell toxicity or cytotoxic effects of an empty nanoparticle is also a vital factor to consider. After delivering the cargo or drug within the cytoplasm, the empty nanoparticle may remain on the cell surface or enter the cell by being engulfed by vesicles and then stored, transported or degraded in lysosomes in the cell cytoplasm. Therefore, empty HPPS as a vehicle as well as cargo-loaded HPPS must be cell friendly. Several published studies such as Gupta B et al. (1999) and Motterlini R et al. (2000) reported that curcumin is a safe therapeutic agent for endothelial cells. Endothelial cells were exposed to different concentrations of curcumin (0–30 μM); it was observed that curcumin (1–15 μM) for 18 h resulted in a concentration-dependent increase in heme oxygenase activity with a maximal enzymatic activity (12-fold increase) at 15μM curcumin. Motterlini R et al. (2000) used ethanol as vehicle for curcumin whereas we have used DMSO. Kurien and Scofield (2009) mentioned in their paper that neither DMSO nor ethanol is ideal for dissolving curcumin in case of *in vivo* experiments.

Although there is no information regarding *in vitro* experiments [119], it would be interesting to explore the effect of DMSO and ethanol as a vehicle for curcumin through *in vivo* and *in vitro* experiments. After investigating biocompatibility via FACS, we further confirmed the relative uptake of variable doses of curcumin and the nanocarrier HPPS in HUVEC. Curcumin exposure to endothelial cells induces heme-oxygenase-1 enzyme in both dosage and time dependent fashion. Similar results were expected by HPPS nanoparticle-loaded curcumin (HPPS-curcumin).

At this stage, we began a two pronged examination aimed at measuring dose-equivalency between curcumin and HPPS-curcumin for HO-1 induction and then determining the potential therapeutic efficacy. Moreover, we planned to use the determined dose-equivalency to investigate cell rescue experiments and demonstrate HPPS-curcumin to protect the cells *in vitro* from oxidative stress (treating the cells with H_2O_2 and TNFα) conditions. By treating endothelial cells for 16 hours with 5µM curcumin and (5µM)Equ HPPS-curcumin, we tried but failed to measure the dose-equivalency. Our study showed induction of HO-1 was easily achieved with free curcumin but not with HPPS-curcumin. This suggests that despite uptake, bioavailabilty was not established. It could be the nanoparticles were not internalized or escaped the lysosome degradation and failed to release the drug payload. With this finding, we decided not to proceed any further with cell rescue experiments and/or to measure expression of pro-inflammatory cytokines VCAM-1 (CD106) and ICAM-1 (CD54) on endothelial cell surface as planned. Rather, we performed troubleshooting experiments in terms of solving the dose-equivalency issue. Besides HO-1 enzyme, SCARB-1 receptor was also our subject of interest. Being the only HDL receptor available in hepatic cells, endothelial cells, macrophages, SCARB-1 may allow access of HPPS and or HPPS-curcumin right into atherosclerotic lesion. However, our *in-vitro* western blot results indicate no effect of curcumin, HPPS and/or HPPS-curcumin on SCARB-1 receptors. As such, it was not likely that the nanoparticles were down-regulating the SCARB-1, however our ability to detect the receptor on the surface interacting with the nanoparticles is limited. It is important to keep in mind that the scavenger receptors family consists of eight different subclasses of structurally unrelated receptors, which can bind to modified forms of LDL [120]. Class A, B, E, F and G are suggested to be connected with atherosclerosis. Under scavenger receptor Class B, there are two subgroups: SCARB-1 and CD36 [121]. Therefore, we need to acknowledge the fact that SCARB-1 may not be the appropriate/sufficient receptor to be

targeted for HPPS or HPPS-curcumin. In future studies, it is necessary to explore CD36 as well as remaining subclasses alongside class B scavenger receptors.

By synthesizing HPPS with a hydrophobic inner core, we aimed to retain the hydrophobic drug curcumin within the nanoparticle. There are two reasons behind that concept: 1. to improve the bioavailability of curcumin which further improves the therapeutic efficacy of curcumin and 2. HPPS is presumed to follow same pathway as native HDL to deliver curcumin inside the atherosclerotic plaque. Even after generating affirmative TEM, UV-vis and drug entrapment efficacy results, the release of curcumin from HPPS in terms of elevating HO-1 expression was not satisfactory. One possible explanation could be, once loaded into the HPPS, curcumin encountered trouble getting out of the HPPS, such as through lipid rafting as previously reported to occur with lipophilic drugs. Immunoblot data of surface coated HPPS-curcumin were better than HPPS-curcumin, suggesting that lipid rafting may be a physiochemical limitation that is prevented by incorporating the drug deep inside the nanoparticle.

Niosomes are composed of non-ionic surfactants and cholesterol; they are very much similar to that of a liposome. They are stable in nature as well as improve stability of the drug as entrapped and delivered to the target side [107]. Niosome carrying curcumin can easily penetrate the cell without any expected specific targeting or any specific receptors. By comparison, HDL-mimetic nanoparticles such as HPPS-nanoparticles are expected to carry the payload via the SCARB-1 receptor. Our data show that HUVEC could be targeted by niosomes, surface decorated HPPS-curcumin but not HPPS-curcumin with variable efficacy. We established also that macrophages could process niosomes and HPPS-curcumin and were more readily targeted by nanoparticles. Targeting of a molecular pathways is therefore dependent on the nanoparticle formulation and cell dependent features such as variable receptors and nanoparticle internalization and metabolism, which are not limited in solvent mediated drug delivery.

4.2. A Prospective View of Nanotechnology in Atherosclerosis Management

Atherosclerosis is an inflammatory disease of the arteries, which is also the underlying pathology for other cardiovascular diseases such as heart attack, stroke, peripheral vascular diseases. Other than healthy life-style practices, usually a therapeutic goal is to lower or control the cholesterol level, blood pressure level, sugar level and or to prevent inflammation. Statin medications are the most commonly used, popular treatment for atherosclerotic patients. Statins are

hydroxymethylglutaryl CoA reductase inhibitors, which have proven to be highly effective in lowering blood LDL level in patients with cardiovascular diseases [122]. Although, statins (e.g. Atorvastatin) are the best-selling prescription drugs, their adverse effects also severely affect patient's quality of life. The most commonly reported adverse effects of statins are muscle pain, fatigue and weakness as well as rhabdomyolysis [123].

The recent progress of nanotechnology has created dynamic opportunities to create novel classes of therapeutics and/or enhance the efficacy of existing therapies. Even with nanotechnology, the game-plan of treating atherosclerosis remains focused on reduction of plaque burden, stabilization of vulnerable plaque, prevention of plaque rupture or platelet activation and thrombosis [124]. Broz et al. developed target specific polymer vesicles loaded with pravastat that targeted inflammatory macrophages to deliver the drug-loaded vesicles with better tolerability (15-fold reduction in cytotoxicity in muscle cells *in-vitro*). Yet, *in vivo* efficacy and safety studies still need to be determined [125]. In 2008, Cormode et al. reported to synthesize nanocrystal core HDL and nanocrystal-PEG particles; both *in vitro* and *in vivo* (injected in apoE knockout mice) results demonstrate multimodal characteristics of the nanocrystal core HDL lipoproteins [126]. HDL is the endogenous nanoparticle in our body, which via reverse cholesterol pathway removes cholesterol from peripheral tissues. Therefore, HDL is a preferable target of approach, moreover, nanotechnology is already involved in synthesizing biomimetic HDL to treat atherosclerosis. Cho et al. 2010 reported, liposomal formulation with DMPC infused into cholesterol-fed rabbits, which enhanced the extraction of cholesterol from peripheral tissues [127]. In 2011, Gang Zheng et. al reported to synthesize HDL-mimicking peptide phospholipid scaffold (HPPS) nanocarrier, that was able to bypass the lysoendosomal route via targeting SCARB-1 mediated selective transport pathway (nonendocytic pathway) for reaching the cytosol of cells to treat cancer [111]. In terms of synthesizing HDL-mimicking peptide phospholipid scaffold (HPPS) nanocarrier we followed the protocol as described, however, to more effectively encapsulate the payload to improve shelf-life, we dried curcumin as a thinfilm prior to addition of remaining components. This was to improve increase the certainty of internalization of the therapeutic agent to increase it's stability by reducing the chance degradation by oxidation. This internalization was in our understanding the outcome achieved previously (ie not to have surface decoration). However, it has become clear that the previous

literature may have underestimated the amount of surface coating as our results demonstrate this to be critical to achieving dose equivalency and may be essential to drug entry by lipid rafting.

In the past few years, many research groups investigated target-specific mechanism of actions of nanoparticles for atherosclerosis. Some of these recent papers have been addressed here, regarding types of nanoparticles being synthesized for combating atherosclerosis at the cellular level. Pan et al. (2018) synthesized peptide-siRNA nanoparticles targeting JNK2 expression; data from *in vivo* experiments indicated attenuation in JNK2 expression, macrophage population and inflammatory signaling (preventing endothelial barrier degradation in an atherosclerotic plaque) [128]. Y.Song et al. (2018) prepared platelet membrane-coated nanoparticles which binds to adhesion molecule in atherosclerosis, their nanoparticles showed potential in reducing side-effect and increasing therapeutic efficacy of the drug (Rapamycin). Similar to previous investigators, Y.Song et al. nanoparticles targeted macrophages to reduce inflammatory signaling, their population. These nanoparticles show a mode of action by activating macrophage autophagy in the atherosclerotic plaque [129]. Seijken et al. (2018) synthesized reconstituted HDL (rHDL) nanoparticles containing small molecular inhibitor that showed anti-inflammatory activity by targeting TRAF6. As per *in vitro* and *in vivo* data, the nanoparticles also reduced activity of monocytes/macrophages recruitment [130]. Y. Guo et.al (2018) synthesized phospholipid reconstituted apoA-1 peptide (22A)- derived synthetic HDL (sHDL), which has been claimed as multi-functional nanocarrier targeting atherosclerotic plaques. This nanoparticle was capable of increasing ABC transporter and improved cholesterol efflux. *In vivo* results indicated sHDL reduced side-effects, while delivering the cargo in the liver [131]. Pont I et al. (2018) synthesized liposome nanoparticles, which were able to carry within its hollow core a drug (Fumagillin) with poor solubility and instability in the system. As Fumagillin is highly hydrophobic, the author claimed that drug has been entrapped within the nanoparticles, but they have not shown any data regarding drug entrapment efficacy in nanoparticles [132]. So far, none of the above papers have mentioned neither the term 'dose-equivalency' nor its importance in nanopharmacology or shown any data regarding dose-equivalency between the drug and the nanoparticles version of drug. Most of the work has focused on macrophage targeting and little cell type comparison has been made. Although, the above papers have done *in vivo* experiments on mice models, not all conducted *in vitro* experiments. They did not show any data related to pharmacokinetics or biodistribution of the nanoparticles in animal models either.

On the other hand some studies went much further, Beldman TJ et al. (2017) not only described the synthesis of hyaluronan nanoparticles that specifically target plaque-related macrophages capable of improving plaque stability in atherosclerosis but they also presented interspecies difference between two animal models (mice and rabbit) regarding pharmacokinetics and biodistribution data, along with parameters such as blood-clearance kinetics, clearance kinetics, distribution of radiolabeling of nanoparticles in different organs and also time-dependent distribution of nanoparticles. Nevertheless, they have not mentioned anything about dose-equivalency [133]. Duivenvoorden et al. (2014) synthesized a statin-loaded reconstituted HDL nanoparticles, [S]-rHDL that could prevent atherosclerotic plaque inflammation. Rather than aiming rHDL itself to be therapeutic, they aimed to observe the drug delivery ability of the nanoparticles. They conducted *in vitro* (on macrophages, hepatocyte, ECs and SMCs) to study efficacy of [S]-rHDL in dose-dependent and time-dependent manner and *in vivo* (apolipoprotein E-knockout mouse model) experiments, to show [S]-rHDL directly affects plaque macrophage and accumulates in atherosclerotic lesions. However, they did not investigate the mechanism by which plaque macrophage takes up [S]-rHDL as they are assuming the nanoparticle is interacting similar to that of native HDL via SCARB-1 and or ABCA1, ABCG [134]. Nakashiro et al. (2016) prepared poly(lactic-co-glycolic-acid) nanoparticles containing pioglitazone and controlled plaque destabilization and rupture via targeting monocytes/macrophages differentiation in ApoE knockout mice models. These PLGA-nanoparticles delivered the drug pioglitazone to prevent macrophage activation and plaque rupture which was not achievable with control-NP and oral drug treatment. However, Nakashiro et al did not present any data regarding dose-equivalency or cell toxicity assays [135]. Kheirolomoom et al. (2015) have developed coated, cationic lipoparticles (CCL) encapsulating anti-miRNA agent and targeted VCAM-1. In their work they showed that CCL-anti-miRNA had 5-fold more antiatherogenic effects than naked anti-miRNA via subcutaneous injection. By using a specific VCAM-1-internalizing peptide incorporated with the nanoparticles, Kheirolomoom et al. showed they could successfully deliver the nucleic acid to the specific target cells, therefore, minimizing potential off-target tissue effects [136]. Sanchez-Gaytan et al. (2015) prepared HDL-mimetic nanoparticles with a PLGA core. Other than exploring the characteristics of these hybrid PLGA-HDL nanoparticles, they were macrophage-specific with cholesterol efflux capacity. The *in vivo* experiments (in ApoE knockout mouse) showed hybrid PLGA-HDL nanoparticles to accumulate

in atherosclerotic plaque, in plaque macrophages [137]. Zhao et al. published a paper in 2013, reporting on the synthesis and functionality of HDL mimetic nanoparticles with multivalent peptide structures. The authors showed that instead of using synthetic peptides with monomeric, amphiphilic α-helical structure in HDL-mimetic nanoparticles synthesis, when multimeric α-helical structure were used- the nanoparticles demonstrated exceptional properties in biophysical characterization, stability, pharmacokinetics, cholesterol efflux [138]. Unlike all the above papers, which mostly focused on developing nanoparticles with anti-inflammatory mechanism, precisely targeting single molecule and or specific receptor they mostly relied on the macrophages natural ability to scavenger the nanoparticles, whereas my thesis aimed in measuring drug (curcumin) entrapment efficacy within the nanoparticles (HPPS) along with in vitro experiments data to explore the dose-equivalency of HPPS and or HPPS-curcumin in compare to free curcumin. Despite the successful synthesis of these nanoparticles, dose-equivalency remained elusive and cell dependent. Though other studies have been performed with HDL mimetic nanoparticle, the pharmacology is too often trivialized. In future studies, we could try to use multimeric peptides in the synthesis of HPPS and investigate change in characteristics of our nanoparticles. We should also explore by super-resolution microscopy where the nanoparticles distributed over time in cells. We could also establish knock-down or inhibitor studies of SCARB1 or CD36 to determine their contributions to making drug payloads available. Similarly, it would be important to monitor lysosomal activity and function as a requirement to release the drug payload from a nanoparticle.

4.3. Study Limitations

There were limitations that may have influenced the outcome of the present study. To begin with, a limitation of using human umbilical vein endothelial cells (HUVEC): its ability to recover from cryostate and retaining their continuous passaging affected our ability to rapidly conduct experiments. Bouis et al. (2001) reported in one of their review papers that HUVEC has a short life-span of 10 serial passage and afterwards cells enter the phase senescence where cells stop proliferating, become multinucleated and finally die. Therefore, it is not convenient to perform long-term *in vitro* experiments with HUVEC for the sole purpose of establishing nanopharmacology if other cell lines are available [128]. Instead of HUVEC, future experiments should confirm key mechanisms and evaluate human coronary or aortic endothelial cells as these

are more prone to atherosclerosis and their complications. Our decision to use HUVEC was solely as a model cell which was more cost effective. Furthermore, the photosensitive and rapid oxidizing nature of curcumin could be considered as a limitation to the overall therapeutic strategy, if internalization is not an affective option. However, if the sole objective is to target macrophages and not endothelial cells, it raises a unique opportunity to do so. Handling curcumin and treating cells in a dark-room as well as covering every apparatus and/or equipments with aluminum foil must be practiced. The shelf-life of our nanoparticle HPPS may also pose a limitation. With addition of apoA1 mimetic peptide HPPS is in good condition at 4°C for a week. Without the synthetic peptide, HPPS is good for only a month. It will need to be determined how to achieve commercial translatability if this remains a feature of the formulation.

4.4. Summary and Conclusion

In pharmacology, knowing the therapeutic doses and/or dose-equivalency is an important aspect. However, we were not able to clearly establish the dose-equivalency between curcumin and nanoparticles-curcumin easily in our target cell type. This limited the ability to establish further insight into how we could use HPPS as a means of delivering curcumin as a therapeutic agent. Consequently, we abandoned that line of investigation and did not perform the experiments as planned for: 1) in vitro cell rescue experiments and 2) measuring pro-inflammatory cytokines which could be more informative about the mechanism of action of HPPS-curcumin at the molecular level in endothelial cells. So where does this leave us? To summarize (Table1: summary table), we have prepared HPPS and loaded curcumin as a payload successfully and characterized the physical nature of the nanoparticle. We have shown that HPPS-curcumin is non-toxic to endothelial cells. Relative uptake of variable doses with nanocarrier to endothelial cells was evident (although whether this was surface binding or internalized remains to be determined) and induction of HMOX-1 (an inducible, stress-responsive enzyme) was achieved when HUVEC were treated with nanoparticles of another type. Although our preliminary data showed no appreciable change in SCARB-1 expression, there could be other classes of scavenger receptors responsible in part for internalizing nanoparticles or result in different intracellular processing, such as through the lysosome or endolysosomal escape. Future studies with super-resolution microscopy could address these particular challenges formally, using lysosome tracers with labeled nanoparticles. According to Watari Y et al. (2008) removal of bach-1 gene leads to

suppression of atherosclerosis in bach-1 and apolipoprotien E double knockout mice. Their study showed augmentation of HO-1 in endothelial cells and macrophages in comparison to VSMC. The effects of bach-1 ablation were found by using an inhibitor of HO-1 activity i.e. Sn protoporphyrin. Interference of bach-1 in apo-E knockout mice showed prevention of atherosclerosis via augmentation of HO-1 expression [139]. Therefore, in near future besides HO-1, we should also expand our protein of interest and consider nanoparticles that can target bach-1. In terms of dose-equivalency, endothelial cells (HUVEC) did not respond to HPPS-curcumin (with 5μM)Equ as we expected, although higher concentrations of surface coated HPPS-curcumin did show dose-equivalency at (25μM)Equ and one possible reason could be curcumin can only enter the cell in this less stable formulation of nanoparticles. In the case of niosome-curcumin (15μM)Equ showed dose-equivalency with reference drug curcumin. The possible reason might be niosome is receptor-independent to cells variable use of receptors on their cell surface. This might be one possibility for us to observe HO-1 induction in THP-1 niosome-curcumin dose-equivalency with our reference drug i.e. curcumin at 5μM concentration.

This thesis indicates that HPPS-curcumin could be a promising novel strategy in reducing atherosclerotic lesions via reverse cholesterol transport (RCT) pathway if we explore targeting a different cell type, such as macrophages, rather than endothelial cells. Although we have not explored the preparation and mechanism of action of niocurcumin in detail in this thesis as it was used for trouble shooting determinations, our results from these troubleshooting experiments did show treating endothelial cells with niocurcumin may be a potential nanoparticle to be considered as a novel therapy in treating our near-future cardiovascular diseases. This may require consideration as to whether pH is acidic in atherosclerotic plaques or other targeting moieties be added to niosomes.

Future studies must be directed toward elucidating the anti-inflammatory and anti-oxidant effect of any nanoparticle-curcumin and for these nanoparticles the dose equivalency and mechanisms of uptake and/or internalization need to be clarified in general. In terms of observing time dependent induction of HO-1 in cells, we should verify that a time course was not shifted (either earlier or later than 16hours) as the variations by HPPS or niosomes may be influenced by binding kinetics or uptake variably. In future, we may investigate effect of apoA alone and/or

HPPS without apoA on endothelial cells and/or macrophages as effect controls to isolate their specific contributions to cell uptake or competitive binding to eliminate uptake of the whole nanoparticle. We believe our results could be used as a platform or preliminary evidence for further investigations of pharmacodynamic and pharmacokinetic effects of nanoparticles *in vitro* before we start designing any *in vivo* experiments.

Vehicle	DMSO	HPPS	Niosome	Decorated HPPS
Drug	Curcumin	Curcumin	Curcumin	Curcumin
Cellular Uptake Detected	Yes	Yes	Yes	N/D
Cellular Toxicity Likely	No	No	No	N/D
Dose Response (for HO-1 expression)	Yes	No	Yes	N/D
SCARB1 expression	Consistent	Consistent	Consistent	N/D
Dose Equivalency	[5µM]	HUVEC=No THP1=[15µM]Equ	HUVEC=[15µM]Equ THP1=[5µM]Equ	HUVEC=[25µM]Equ

Table 1: Summary of major findings

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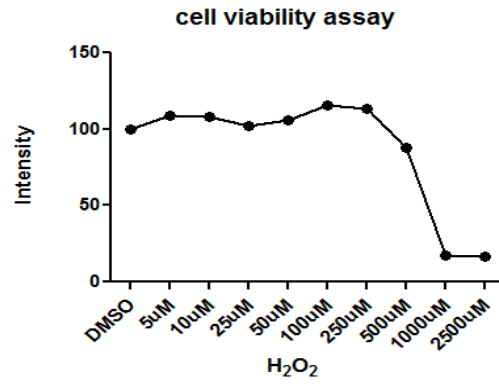
Figure 1 Effect of H_2O_2 on cells viability.

HUVEC are seeded in 96 well plate and treated with H_2O_2 at various concentrations for 16hours. Percentage of viable cells were determined. The intact cell viability level was taken as 100%. Independent assay performed in triplicates