Chapter-1

Introduction

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and cellular elements in the arteries (Lusis AJ, 2000). Clinically, manifestation of atherosclerosis occur in four major stages, (1) triggering of endothelial activation and inflammation; (2) furtherance of intimal lipoprotein deposition, retention, modification, and foam cell formation; (3) progression of complex plaques by plaque growth, growth of the necrotic core, fibrosis, thrombosis, and remodeling; and (4) precipitation of acute events (Hopkins, 2013). The injured endothelial cells lining under oxidative stress, hemodynamic forces, and modified lipoproteins express and secret adhesion molecules as well as chemoattractant cytokines which results into attachment of monocytes and subsequently, migration of monocytes into the intima of the artery (Moore et al., 2013). Monocytes inside arterial wall differentiated into mononuclear phagocytes that ingest normal and oxidized low-density lipoprotein (LDL) and transformed into foam cells (Libby et al., 2011). The foam cells produce biogenic substances that recruit inflammatory cells, results in more uptake of LDL, initiation of smooth muscle cell proliferation, and the growth of a collagenous fibrous cap over the plaque core (Prueitt et al., 2015). Atherosclerotic plaques cause constriction of the artery lumen, resulting in ischemia. Disruptions of plaques predispose the procoagulant material within the core of the plaque to coagulate proteins in the circulating blood which triggers thrombosis that can block the artery or lodge in distal arteries (Libby et al., 2011). Blockage of an artery leads to prolonged cardiac ischemia resulting into precipitation of acute clinical events like acute myocardial infarction, unstable angina, ventricular fibrillation, or sudden coronary death.

Hypercholesterolemia is an independent dominant risk factor sufficient to drive progression of atherosclerosis even in absence of other risk factors (Glass & Witztum, 2001; Tomkin & Owen, 2012). Hypercholesterolemia is a lipid

metabolic disorder characterized by elevated level of total cholesterol and LDL in plasma. LDL is a heterogeneous class of lipoproteins comprising of triglycerides and cholesterol esters containing hydrophobic core in a hydrophilic shell of phospholipids, free cholesterol, and apolipoproteins (Badimon & Vilahur, 2012). It carries the cholesterol from hepatic region and transports it to the rest of body. Elevated level of LDL in vascular system initiates endothelial dysfunction by reducing NO availability resulting into LDL entry into the arterial intima (Vidal et al., 1998). Moreover, LDL transforms into modified LDL (predominantly oxidized LDL and aggregated LDL) under certain oxidants, proteolytic, lipolytic and hydrolytic enzymes (Oörni et al., 2000). Monocytes entry in the vascular wall is favored by modified LDL inside intima which transforms into macrophages and form foam cell with internalization of cholesterol and modified LDL (Schaftenaar et al., 2016). Foam cells produce several chemotactic agents which lead to proliferation of vascular smooth muscle cells and subsequently migration of these cells toward vascular intima. These vascular cells further favor accumulation of cholesterol and LDL in plaques which finally lead to complication of atherosclerotic events.

High density lipoprotein (HDL) is a small, dense, spherical lipoprotein particle with equimolar ratio of lipid and protein components (Thompson et al., 2004). HDL removes cholesterol from plasma and transports it back to liver for disposal through a process called reverse cholesterol transport (RCT). RCT cause uptake of cholesterol from foam cells in atherosclerotic plaque, which not only prevents atherosclerosis progression but also effectively cause regression of established atherosclerotic lesions (Badimón, 2010; Badimon & Vilahur, 2012). HDL also exhibits anti-inflammatory effects (due to inhibition of synthesis of platelet activation factor and leukocyte adhesion to the arterial wall via attenuation of the expression of cytokine induced cell adhesion molecules), to cause improved endothelial function (due to; stimulation of endothelial NO synthase activity, enhanced endothelium-dependent vasodilation, prevention of endothelial cell apoptosis and stimulation of prostacyclin synthesis), immunomodulatory (modulation of the innate immunity response), antithrombotic (prevention of platelet activation by stimulating nitric oxide and

prostacyclin release and stimulating fibrinolysis), and antioxidative (prevention of LDL oxidation) effects (Chapman, 2006; Choi et al., 2006; Badimon & Vilahur, 2012). Epidemiological evidence studied in men suggested that for every increment of 1 mg/dl of HDL in plasma, there will be 2-3% reduction in coronary risk (Gordon et al., 1989).

The atherosclerotic disease exerts a substantial economic burden to society. The 2010 global expenditure on cardiovascular disease has been figured at $863 billion USD (equivalent to an average per capita cost of $125 USD) (Barquera et al., 2015). In the European Union, atherosclerotic cardiovascular disease costs annually ~€ 192 billion in healthcare expense (Allender et al., 2008). In 2010, the American Heart Association figured out that the annual expenditure on cardiovascular disease in the U.S. was $503.2 billion USD (1-3% of U.S. GDP). These exorbitant costs effect on the health care system and the national economic growth. Overall, patients with atherosclerosis cost $12,888 in the 12 months after diagnosis (Ohsfeldt et al., 2010). Worldwide, mortality rate of general population is 8.88/1000 from all causes and alone atherosclerotic cardiovascular disease cause 3.21/1000 (Yoshino et al., 2006). It is a leading cause of death (50% death of all cause) in westernized societies. Worldwide, 80% of cardiovascular death occurs in the developing countries and at a younger age (Gaziano et al., 2010). Trend suggests that by the year 2025, cardiovascular diseases will be prevalent (80-90%) in low income and middle income countries (Yusuf et al., 2002).

Statins are the first choice treatment for atherosclerotic cardiovascular disease prescribed by medical practitioners. These are prescribed not only to reduce the elevated total -cholesterol, LDL and triglyceride (TG) levels but also to enhance the HDL level in patients with primary hyper-cholesterolemia and mixed dyslipidemia. Statins are selective, competitive inhibitor of the 3-hydroxymethyl glutaryl co-enzyme A (HMG-CoA) reductase enzyme, which prevent conversion of HMG-CoA to mevalonate (a rate limiting step in cholesterol biosynthesis) and thus limit the cholesterol formation in hepatocytes. Low level of intracellular cholesterol promotes recruitment of LDL receptors on

the hepatocyte surface, resulting in an increased extraction of LDL from the vasculature. Statins reduce the LDL production by limiting cholesterol biosynthesis and resulting in lesser number of LDL particles. In additional to cholesterol lowering mechanism, statins also circumscribe the VLDLs (very low density lipoproteins) level in plasma by inhibiting their synthesis and promoting their catabolism. Atorvastatin calcium (ATR) is second generation statin, which has been block buster drug during patent terms and earned $120 billion to Pfizer Company. ATR possesses an anti-inflammatory property which restricts the recruitment of inflammatory cells in the atherosclerotic plaques. The drug not only inhibits the vascular smooth muscle cell proliferation but also inhibits the platelet function, thereby limiting both the atherosclerosis and the superadded thrombosis (Xu et al., 2002; Labiós et al., 2005). ATR also ameliorates the vascular endothelial dysfunction, by facilitation of nitric oxide generation. It also shows antioxidant properties resulting into enhancement of superoxide dismutase and considerable reduction in oxidized LDL (Sun et al., 2015). All these properties of ATR contribute to protection from atherosclerotic complications. Moreover, ATR also possesses some pleiotropic effects like hypoglycemic (due to activation of peroxisome proliferator-activated receptor-ϒ) and anticancer property (by inhibiting Rhoc function) (Ye et al., 2007; Islam et al., 2013).

In spite of being a blockbuster drug, ATR oral bioavailability is quite low (12% only) due to poor solubility and high presystemic elimination by first pass metabolism as well as intestinal wall efflux pump (Lennernäs, 2003; Shitara & Sugiyama, 2006; Takano et al., 2006). It is a substrate for cytochrome P-450 enzyme and p-glycoprotein efflux pump extensively found in gut wall, and is responsible for limited oral bioavailability of ATR. Generally, atherosclerotic cardiovascular disease and hypercholesterolemia suffering patient require a prolonged ATR treatment regimen. Chronic use of ATR for prolonged period results into skeleton muscle toxicity like mild myalgia to rhabdomyolysis and it compels the patients to switch from statins to other prescribed pharmacotherapy (Magee et al., 2010).

Per oral drug delivery is the highly chosen route of drug administration due to convenience, cost-effectiveness and patient compliance (Yamanaka & Leong, 2008). Promising advantages of polymeric nanoparticles (PNs) have attracted various research groups working in oral therapeutic delivery system (Rao & Geckeler, 2011). PNs have been proved as a competent novel delivery system toward improved stability, sustained release profile, oral bioavailability and efficacy along with reduced toxicity of incorporated therapeutic agent (Soppimath et al., 2001; Daglar et al., 2014; Juneja & Roy, 2014). PNs can easily be fabricated by elementary, robust and non-invasive various techniques with miniscule uniform particle size and better storage stability. Surface property of PNs can be functionalized in order to achieve protection from opsonization and target the drug loaded PNs to particular organs or tissues (Patil et al., 2009; Hubbell & Chilkoti, 2012; Kowalczuk et al., 2014). PNs protect the incorporated sensitive drug from harsh environment (hydrolytic and enzymatic degradation) of gastro-intestinal tract (GIT). Absorption of PNs from gastrointestinal region not only follow paracellular and transcellular pathway via epithelial cells but also the lymphatic pathway via M-cells of Payer's patches in GIT to bypass the first-pass metabolism (Swarnakar et al., 2011; Aprahamian et al., 1987; Jani et al., 1989; Lamprecht et al., 2006). These advantageous properties results in to the enhancement in bioavailability of incorporated therapeutic agents. Moreover, enhanced bioavailability require dose adjustment i.e. either reduction in dose size or dose frequency. Reduced dose size or dosing frequency and sustained release action of PNs lead to amelioration of toxicity profile of incorporated drug.

Traditional approach of optimization of a pharmaceutical product or process using changing one variable at a time (OVAT) has been proved to be not only uneconomical in terms of time, money, and effort, but also unfavorable to fix errors (Singh et al., 2005). Conventional OVAT method of formulation and process optimization ascertains the effect of individual factors on the responses which exclude the estimation of interaction effects among the factors. In order to circumscribe the shortcomings of OVAT, a holistic design of experiment (DOE) approach has been preferred which express the response as a function of various factors involved including interaction terms not only using mathematical

expression but also with graphical illustration. By applying DOE one can ensure the quality built into the product and is not merely established by testing the end product (Yu, 2008). DOE identifies the various critical variables and collect maximum information of critical variables using lesser number of runs in order to rapidly avail the high quality pharmaceutical products (Verma et al., 2009). Formulation development and its optimization involves thorough understanding of the effect of the formulation independent variables viz. polymer content, concentration surfactant, volume of organic solvent, agitation speed, etc. on the formulation response variables such as particle size, entrapment efficiency, etc. Response surface methodology (RSM) is a collection of mathematical and statistical techniques based on fitness of a polynomial expression to the experimental data, which must describe the behavior of a data set with the aim of making statistical anticipation. Central Composite Design (CCD) is an RSM tool having factorial or fractional factorial design with centre point, augmented with axial points (star points) that estimate the extent of curvature. CCD has been extensively used to identify the critical variables of nanoparticle formulation and to optimize the independent variables parameters in order to achieve desirable response variables of formulation (Chaubey et al., 2014; de Carvalho et al., 2013; Chawla et al., 2014; Hao et al., 2012)

On the basis of availability, frequent usage as nanocarriers and other beneficial advantages; Eudragit RSPO, poly lactic-co-glycolic acid (PLGA) and poly (ε-caprolactone) (PCL) were selected as nanocarriers to encapsulate ATR using D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) and polyvinyl alcohol (PVA) as stabilizer to stabilize nanocarrier colloidal system (Woodruff & Hutmacher, 2010; Lopedota et al., 2009; Elshafeey et al., 2010; Ubrich et al., 2005; Adibkia et al., 2011; Danhier et al., 2012; Kumari et al., 2010; Siepmann et al., 2001). Surface adsorbed TPGS and PVA imparts hydrophilic character over the nanocarriers surface resulting into prolonged retention of nanocarriers in blood circulation (Lee et al., 1999; Vuddanda et al., 2014). In this study, ATR loaded various polymeric nano-particles have been hypothesized to improve bioavailability, efficacy and toxicity profile of ATR. ATR loaded polymeric nanocarriers have been prepared by nano-precipitation and emulsification

solvent evaporation method. The nanocarriers were optimized by using CCD as RSM tool using polymer content (mg), stabilizer concentration (%), volume of organic solvent (ml) and agitation speed (rpm) as independent variables to get minimal particle size (PS) and higher entrapment efficiency (EE) as response dependent variables. Further, these nanocarriers were evaluated for extensive *in vitro* characterization like solid state characterization (Fourier transform infrared spectroscopy, differential scanning calorimetry and X-ray diffraction), morphology (Atomic force microscopy and transmission electron microscopy), *in vitro* drug release study in phosphate buffer (pH 7.4) and storage stability study. Further optimized nanocarrier systems were extensively evaluated for *in vivo* study in Charles Foster rat for measuring pharmacokinetic and pharmacodynamic (plasma lipid profile and glucose level) profile and also for the toxicity study (various toxicity indicating biochemical parameters).