Expert Opinion

Liposome technology for cardiovascular disease treatment and diagnosis

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Introduction: Over the past several decades, liposomes have been used in a variety of applications, from delivery vehicles to cell membrane models. In terms of pharmaceutical use, they can offer control over the release of active agents encapsulated into their lipid bilayer or aqueous core, while providing protection from degradation in the body. In addition, liposomes are versatile carriers, because targeting moieties can be conjugated on the surface to enhance delivery efficiency. It is for these reasons that liposomes have been applied as carriers for a multitude of drugs and genetic material, and as contrast agents, aimed to treat and diagnose cardiovascular diseases.

Areas covered: This review details advancements in liposome technology used in the field of cardiovascular medicine. In particular, the application of liposomes to cardiovascular disease treatment and diagnosis, with a focus on delivering drugs, genetic material and improving cardiovascular imaging, will be explored. Advances in targeting liposomes to the vasculature will also be detailed.

Expert opinion: Liposomes may provide the means to deliver drugs and other pharmaceutical agents for cardiovascular applications; however, there is still a vast amount of research and clinical trials that must be performed before a formulation is brought to market. Advancements in targeting abilities within the body, as well as the introduction of theranostic liposomes, capable of both delivering treating and imaging cardiac diseases, may be expected in the future of this burgeoning field.

Keywords: cardiovascular disease, cardiovascular imaging, drug delivery, gene delivery, liposomes

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1. Introduction

Liposomes were first described by Bangham *et al.* (1965) and are defined as artificial vesicles between 20 nm and 10 μm in diameter, made of an aqueous core surrounded by lipid bilayers [1]. Structurally, liposomes are composed of naturally derived phospholipids or synthetic amphiphiles, and are almost always incorporated with sterols, such as cholesterol, to affect membrane permeability. Liposomes are frequently prepared by thin-film hydration, which involves dissolving lipid components in organic solvent, drying down by rotary evaporation and rehydrating in aqueous solution, as well as by freeze-drying, reverse-phase evaporation and ethanol injection [2]. Subsequent processing steps, such as membrane extrusion, sonication and/or freeze-thawing may be employed to control the size distribution.

Liposomes have been used in medicine as delivery vehicles for drugs [3], genetic material [4] and imaging agents [5]. Due to their hydrophilic (aqueous core) and lipophilic (lipid bilayer) domains, liposomes can be used to encapsulate most active agents, irrespective of solubility and physical properties [6]. Encapsulation into liposomes can protect or control the release of an active drug or genetic material and reduce systemic toxicity by

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Article highlights.

- Targeting liposomes specifically to the cardiovascular system can improve the delivery efficiency of therapeutics. Homing mechanisms can include passive targeting, which can be achieved by adjusting liposomal charge and/or size, as well as active targeting, in which functional moieties specific to the vasculature are attached to the liposomes' surface.
- Drug delivery from liposomes has been shown to be an effective means of improving therapeutic outcomes in animal models of cardiovascular disease. Clinical trials are still forthcoming.
- Experimental evidence suggests that the incorporation of genetic material in liposomes may aid to improve gene therapy to treat cardiovascular diseases. Despite some promising studies, much work is required before any systems are viable for use in humans. Liposomes conjugated with hemagglutinating virus of Japan showed promising results in attempts to improve transfection efficiencies in the vasculature.
- Both computer tomography and magnetic resonance imaging of the cardiovascular system can benefit from the application of liposomes in conjunction with contrast agents. Such formulations can be used to improve signal intensity and contrast-to-noise ratios.
- Echogenic immunoliposomes, specifically designed to enhance ultrasound images, have been applied to aid in the diagnosis of cardiovascular diseases. Preliminary studies show promising applications for delineating regions of atherosclerotic plaque.

This box summarizes key points contained in the article.

minimizing dosage requirements. As well, liposomes can help to overcome biological barriers, which is necessary in order to deliver their payload and exert the desired pharmacological effect. Drawbacks include a surface chemistry that can attract proteins and mark liposomes for rapid clearance from the circulatory system. Though much research has focused on stabilizing liposomes by coating with hydrophilic polymers, such as polyethylene glycol (PEG), or other polyelectrolytes that are specifically designed to increase circulation time and reduce clearance *in vivo* [7]. Compared with traditional nanoparticulate delivery vehicles, liposomes also offer the ability to co-currently load both hydrophilic and hydrophobic components within the same system.

Although a great deal of liposome-based research has been devoted to treating cancer [8], work has also been underway to develop liposomal drug and gene delivery devices and imaging agents for cardiovascular disease (CVD) applications. Unlike most tumors, many parts of the vasculature are highly accessible through intravenous injections, although direct administration of various active agents for CVD treatment and diagnosis can be prone to washout due to high shear stresses [9]. By contrast, some forms of CVD, such as atherosclerosis, can cause impaired blood flow and make it difficult to transport therapeutics or contrast materials to sites of interest. In either case, liposomes are particularly well-suited to overcome these challenges since a number of factors, such as size [10], charge [11] as well as the inclusion of targeting moieties [12], can be adjusted to improve delivery efficiency.

In terms of overcoming blood flow blockages, Caride and Zaret (1977) were one of the first to suggest that positively charged liposomes preferentially accumulate in areas of myocardial infarction [11]. Subsequent studies showed that liposomes may exhibit an enhanced permeability and retention effect in certain areas of the vasculature [13], which could be exploited to influence the distribution of therapeutics in the cardiovascular system. Early studies also proposed that liposomes could serve as direct treatments, forming a 'plug' and sealing damaged endothelial membranes to prevent further damage [14]. Furthermore, liposomes are capable of accommodating a wide variety of cardio-specific adhesion molecules and polymers on their surface, which can aid in increasing adhesion to vascular tissues or cells.

This review will highlight the research conducted on the use of liposome technology to treat and diagnose CVD. Targeting liposomes with surface moieties to the vascular system will be explored, as well as their applications in delivering pharmacological agents and genetic material to treat CVD. Finally, the use of liposomes in aiding to image the cardiovascular system will be detailed. An expert opinion section provides perspective and opinion on the current state and future directions in the field.

2. Targeting liposomes to the cardiovascular system

Liposomes are suitable for vascular drug delivery and imaging since they can be precisely tailored to preset conditions by varying the formulation and/or processing steps. In addition, both passive and active targeting can be exploited to improve payload delivery and residence time within the body. Regarding the passive targeting of liposomes, size, charge, and polymeric surface coatings have all been shown to affect blood clearance, cellular uptake and distribution throughout the cardiovascular system [15,16]. Liposome size, for instance, can be altered to passively target a specific population of cells. It has been observed that larger liposomes are more likely to be phagocytosed by macrophages, while smaller liposomes are readily taken up by fibroblasts [17]. Since one of the main drawbacks of liposome use is rapid clearance by the liver and reticuloendothelial system [10], polymer coats, such as PEG, have been employed to affect blood residence time [18]. Indeed, so-called stealth liposomes, named for their ability to improve blood circulation time and avoid clearance, are characterized by the incorporation of PEG into the liposomal formulation by adsorption, conjugation or covalent linkage [19]. It has been postulated that PEG can increase liposomal circulation times by reducing activation of the complement system through steric interactions and enhancing stability. Although it should be noted that results are highly dependent on the molecular weight and grafting density of the polymer.

Targeting moieties can also be used to improve delivery efficiency by actively targeting a specific location, increasing local concentration and cellular internalization, which is an essential step to achieve the desired therapeutic effects of pharmaceutical agents and genetic material, in particular. Regarding vascular delivery, targeting ligands can help reduce washout by promoting adhesion to vessel walls or atherosclerotic lesions [12,20]. Possible cardiac targeting moieties include lectins, proteins and antibodies. Liposomes conjugated to antibodies, also known as immunoliposomes, are predominately used to target the cardiovascular system and will be discussed in the following section.

2.1 Immunoliposomes

During the mid-seventies, Gregoriadis *et al.* (1975) proposed the concept of homing liposomes to target cells by attaching antibodies to the surface [21]. Since then, immunoliposomes have been employed to treat variety of diseases, including cancer [22] and CVD [23]. Undoubtedly, an important aspect of cardiovascular targeting research lies in acquiring an in-depth understanding of the cellular processes and underlying pathways that occur during each stage of disease evolution or healing. Particularly, information relating to the migration of certain cell types or the expression of receptors can help elucidate which antibodies should be selected to target the liposomal vectors to a desired location. For instance, in the early stages of atherosclerosis, adhesion molecules for leukocytes, such as vascular cell adhesion molecule-1 (VCAM-1), can be targeted since they are expressed on the surface of endothelial cells [24]. Whereas in later stages of the disease, receptors on proliferating smooth muscle cells may be more widely expressed and targeted instead. Depending on the type of drug or imaging agent to be delivered, careful consideration of the target cell population or adhesion molecules, and antibody selection will very likely affect carrier efficiency. **Table 1** lists a selection of publications that feature immunoliposomes designed to target various vascular receptors and regions. As well, **Figure 1A** and **B** schematically depict antibody-conjugated liposomes and a selection of inducible receptors on activated endothelium and platelets, which are common to diseased vascular tissue. In many cases, immunoliposomes targeting the cardiovascular system have been directed to glycoproteins on the activated endothelium or cardiac myosin, both of which will be described further in the following section.

The endothelium is the principal site of cellular infiltration and inflammation during the development of atherosclerotic lesions and injury. Thus, it follows that one of the prime targets for cardiovascular therapeutic delivery and imaging is the activated endothelial lining [25]. During activation, glycoproteins, such as endothelial-leukocyte adhesion molecule (ELAM-1 or E-selectin), VCAM-1 and/or intercellular adhesion molecule-1 (ICAM-1), can be upregulated and expressed on the surface of cells. Depending on the degree and type of disease or injury, these molecules may serve as potential targets for liposomes functionalized with the appropriate monoclonal antibodies. For example, using an *in vitro* model for activated endothelial cells, Lim *et al.* (2011) showed that liposomes, loaded with celecoxib and designed to target VCAM-1, successfully increased liposomal uptake in human umbilical vein endothelial cells compared with unconjugated liposomes [26]. Homem de Bittencourt *et al.* (2007) detailed an *in vivo* study, in which atherosclerotic mice were subjected to a dose of anti-VCAM-1 immunoliposomes [27]. It was determined that the presence of anti-VCAM-1 antibodies improved distribution to the thoracic aorta, while reducing accumulation in the spleen and kidneys. These results demonstrate that antibodies directed toward inducible cell surface glycoproteins could aid in localizing liposomes to areas of vascular disease, thereby improving cellular uptake rates.

Another notable cardiac target is the myosin that is exposed if the membranes of endothelial cells are damaged. Torchilin *et al.* (1979) first described the covalent coupling of antibodies to liposomes in order to specifically target canine cardiac myosin [28]. Subsequent studies demonstrated that antimyosin immunoliposomes improved the survival of H9C2 rat embryonic cardiomyocytes [29] and decreased the mean infarct size in rabbit models of acute myocardial infarction, compared with bare liposomes and nonspecific IgG liposomes [30]. Cytoskeletal-specific antibodies, such as anti-cardiac myosin 2G4, are favorable since they uniquely target exposed myosin. It follows that liposomes are, therefore, less likely to enter healthy myocardial cells, which could minimize exposure of potentially toxic materials to undamaged cells and decrease the overall effective dosage. Owing to the phospholipid components, it has been suggested that myosin-specific liposomes can work to 'plug and seal' damages suffered by cellular membranes, on top of delivering active pharmacological agents, as seen in **Figure 1C** [29]. Given this theory, liposomes may offer a multifaceted treatment alternative to conventional single-component systems and may accordingly improve clinical outcomes. In addition to the activated endothelium and myosin, other cardiac targets, outlined in **Table 1**, have been investigated. For example, Scott *et al.* (2009) chose the cell adhesion molecule P-selectin, which is expressed on platelets and endothelial cells in response to inflammation, to direct liposomes containing vascular endothelial growth factor (VEGF) to sites of myocardial infarction in rat models [23]. Results showed that cardiac functionality was significantly improved compared with unencapsulated VEGF and nontargeted liposomal controls.

Evidently, there are a multitude of target/antibody combinations that can be used to home liposomes to diseased areas of the cardiovascular system. In addition to selecting antibodies based on availability and specificity to a given target, it is important to keep in mind the following: i) targets should be accessible by the vascular system; ii) targets should preferably be selected based on cell surface glycoproteins or receptors that are not present or exposed in healthy cells; iii) antibodies must be able to attach to the liposomal surface without undergoing any deleterious effects on targeting activity; and (iv) antibodies should elicit no immunogenic effect.

Table 1. Selected examples of immunoliposomes used to target specific sites/receptors in the vasculature.

Lipid formulation	Size distribution (nm)	Receptor/ target	Antibody	Ref.
DMPC, glutaryl-N-PE	~ 400	VCAM-1	Anti-VCAM-1-Fab' mAb	[26]
DPPC, DSPE-PEG(2000)-COOH, DSPE-PEG(2000)	132 – 149	ICAM-1	Anti-ICAM-1 mAb	[83]
DOPC, DPPC, N-dod-PE	201 – 205	ELAM-1	Anti-ELAM mAb	[104]
DOPC, DPPC, N-dod-PE	201 – 205	E-Selectin	mAb H18/7	[105]
PC, DSPE-PEG2000, and DSPE-PEG-maleimide	~ 180	P-Selectin	Anti-P-selectin	[23]
PC, PG, PE	500 – 800	Tissue factor	Anti-TF mAb	[75]
PC, PG, maleimido-4 (p-phenylbutyrate)-PE	60 – 100	Fibrinogen	Anti-fibrinogen	[106]
PC, mPEG2000-DSPE, DOTAP	156 – 189	Cardiac myosin	Cardiac myosin 2G4 mAb	[48]

DMPC: 1,2-Dimyristoyl-sn-glycero-3-phosphocholine; DOPC: 1,2-Dioleoyl-sn-glycero-3-phosphocholine; DOTAP: 1,2-Dioleoyl-3-trimethylammonium-propane; DPPC: 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine; DSPE: 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine; ELAM: Endothelial-leukocyte adhesion molecule; ICAM: Intercellular adhesion molecule; mAb: Monoclonal antibody; N-dod-PE: 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-dodecanoyl; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PEG: Polyethylene glycol; PG: Phosphatidylglycerol; TF: Tissue factor; VCAM: Vascular cell adhesion molecule.

Based on the studies presented, it is clear that antibodies can provide a degree of active targeting that significantly improves liposomal retention in a given site and improves therapeutic outcomes in animal models of CVD. As a result, the required dosage can be reduced and nonspecific accumulation in healthy areas in the body may be avoided. Ultimately, immunoliposomes can be expected to continue to play a pivotal role in improving the efficiency of drug, gene and imaging agent delivery to treat and diagnose CVD, in conjunction with further advancements in the fields of ligand and cardiac biomarker discovery [31].

3. Liposomal therapeutic delivery to the cardiovascular system

3.1 Pharmacological agents

Conventional pharmaceutical treatments for CVD suffer from a variety of drawbacks related to their method of administration. Systemic delivery of active agents often requires high concentrations owing to nonspecific distributions and short half-lives experienced *in vivo*. These levels can lead to adverse toxic side effects, unsustainable drug levels and developed drug resistance. The application of liposome technology can offer an alternative form of delivery specifically designed to remedy these obstacles by providing the means to control therapeutic delivery over a desired timeline, as well as target specific tissues in the body, and improve cellular internalization rates. An ideal particulate-based drug formulation would specifically target diseased tissue, provide a sufficient dose to elicit the desired therapeutic response and minimize adverse side effects. Liposomes may very well address these conditions, given their ability to protect the active agent from degradation, improve residence time *in vivo* [32], shield the body from toxicity [33], offer control over pharmacokinetics [34-36] and, as discussed, accommodate ligands that can target specific areas of the vasculature [23]. Although there are no liposomal drug formulations currently available on the market to treat CVD, liposomes have made considerable progress to alter the efficiency of cardiovascular drug delivery within the context of *in vivo* animal studies. **Table 2** provides a selection of research that has incorporated pharmacological agents into liposomes with the intention of treating CVD. Of these pharmacological agents, statins, adenosine triphosphate (ATP) and bisphosphonates will be discussed further in the following sections.

3.1.1 *Statins*

3-Hydroxy-3-methylglutaryl (HMG-CoA) reductase inhibitors, also known as statins, are a class of drugs that inhibit the action of HMG-CoA reductase, which is an enzyme that converts HMG-CoA to mevalonic acid for cholesterol synthesis [37]. In addition to decreasing lipid levels, statins have been shown to attenuate endothelial progenitor cell (EPC) senescence and, in some cases, effectively increase EPC proliferation [38]. Statins may also inhibit inflammation and trigger macrophage/monocyte apoptosis [3], which could contribute to the reduction in the rates of restenosis observed after percutaneous coronary interventions [39]. These pleiotropic effects probably explain the benefits of statin therapy in CVD; however, they are difficult to deliver reproducibly owing to their low aqueous solubility. Thus, researchers have examined various methods to improve drug bioavailability and therapeutic efficacy.

In particular, liposomes have also been proposed as a means to increase the efficiency of statin therapy [3,40,41]. For example, Afergan *et al.* (2010) encapsulated simvastatin in liposomes to investigate effects on monocyte/macrophage growth and neointimal formation after balloon injury in rat models [3]. Results showed that both simvastatin liposomes and free simvastatin administered systemically attenuated the monocyte response to injury and decreased neointimal formation compared with saline injections, although liposomes demonstrated significantly prolonged monocyte depletion *in vivo*. However, it should be noted that it cannot be confirmed whether the effect is a result of controlled

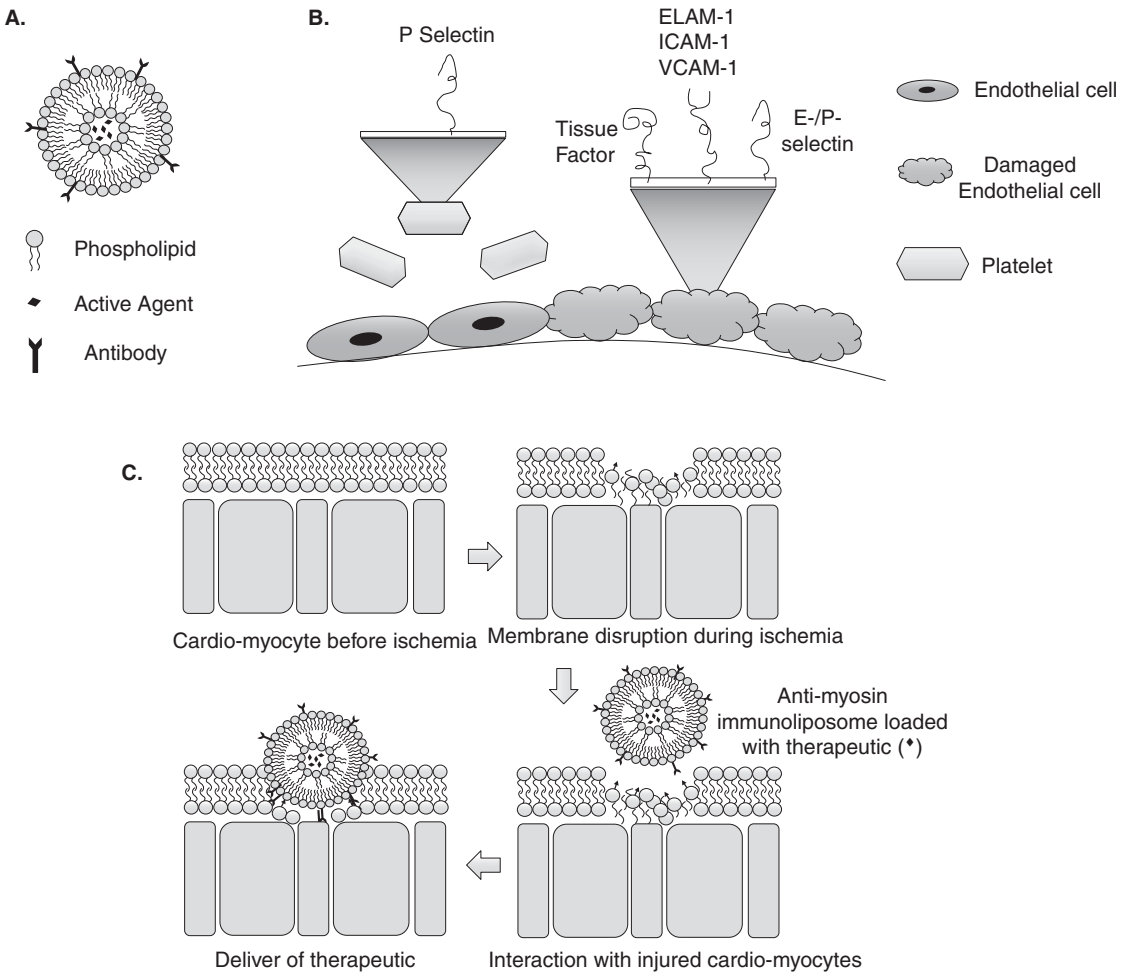

Figure 1. Schematic representation of (A) immunoliposomes and (B) selected targets to damaged endothelium and (C) schematic depicting the proposed 'plug and seal' mechanism of immunoliposomes to ischemic cardio-myocytes.
Part (C) of this figure is adapted with permission from [48].
ELAM: Endothelial-leukocyte adhesion molecule; ICAM: Intercellular adhesion molecule; VCAM: Vascular cell adhesion molecule.

simvastatin release or if there is an intrinsic effect from the liposome constituents since blank liposomes were not used as a control.

Another liposome-statin delivery system, formulated to target injured myocardium, was detailed by Aso *et al.* (2007) [40]. Liposomes were loaded with pravastatin and conjugated with a cardiomyocyte targeting lectin, *N*-acetylglucosamine. Since pravastatin is hydrophilic, cells sparingly take it up, thus liposomes were proposed as a means to increase cellular internalization. **Figure 2** shows electron micrographs of the *N*-acetylglucosamine liposome uptake by cardiomyocytes. It was determined that the targeting moiety significantly increased delivery efficiency, which may aid in to improve delivery of statins, as well as other cardioprotective drugs, to the myocardium. These results indicate that liposomes may be used to promote statin delivery to diseased myocardium; however, it remains to be seen whether these treatments can be translated to a clinical setting.

3.1.2 Adenosine triphosphate

During myocardial ischemia, levels of ATP can quickly become depleted, owing to a restricted blood supply and associated loss of oxygen [42]. Low levels of ATP can affect contraction and functionality and, thus, researchers have looked into methods of delivering exogenous ATP to sites of injury in an attempt to treat and prevent damage to cardiac myocytes [43]. Encapsulation into liposomes offers a convenient means to improve delivery efficiencies compared with free ATP, which exhibits a short half-life *in vivo* since it can be degraded by plasma endonucleotidases and is poorly permeable through hydrophobic membranes [42].

An early study by Xu *et al.* (1990) determined that positively charged ATP-loaded liposomes accumulated at ischemic areas in an experimental canine model of myocardial infarction [44]. Since then, Vladimir Torchilin and his colleagues have published several papers detailing promising results of both PEGylated liposomes and immunoliposomes and their effects

Table 2. Selected publications detailing the use of pharmacological agents encapsulated into liposomes to treat cardiovascular disease (CVD).

Pharmacological agent	Lipid formulation	Liposome size (nm)	Experimental model	Experiment outcomes	Ref.
Simvastatin	DSPC, DSPG	~ 164	Carotid-injured rat model	Inhibited neointimal growth	[3]
ATP	PC, PEG-DSPE, DOTAP	167 – 189	Rabbit model of myocardial infarction	Reduced damage to myocardium	[46]
Alendronate	DSPC, DSPG	176 – 183	Carotid-injured rat model	Suppressed intimal growth	[107]
Nitric oxide	DPPC, DOPC	NA	Rabbit model of atherogenesis	Neointimal hyperplasia was attenuated	[108]
Prostaglandin E1	NA	NA	Rat model of myocardial infarction	Reduced neointimal hyperplasia and lipid accumulation	[27]
Tissue-plasminogen activator	PC, DPPG, -(maleimidophenyl)butyrate-PE	NA	Rabbit aorta thrombus model	Improved recanalization of the abdominal aorta	[97]
VEGF	PC, DSPE-PEG2000, and DSPE-PEG-maleimide	~ 180	Rat model of myocardial infarction	Improved cardiac function	[23]
Streptokinase	PC	~ 330	Rabbit model of carotid artery thrombosis	Reduced reperfusion times and residual clot mass, improved arterial blood flow	[109]
Prednisolone	HSPC, 3,5-dipentadecyloxybenz-amidine hydrochloride	~ 100	Rabbit model of atheroma	Reduced neointimal growth	[110]

ATP: Adenosine triphosphate; DPPG: 1,2 Dipalmitoyl-sn-glycero-3-phosphoglycerol; DOTAP: 1,2-Dioleoyl-3-trimethylammonium-propane; DSPE: 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine; DSPG: Distearoyl phosphatidyl glycerol; NA: Not available; PC: Phosphatidylcholine; PEG: Polyethylene glycol; POPC: 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphotidylcholine; VEGF: Vascular endothelial growth factor.

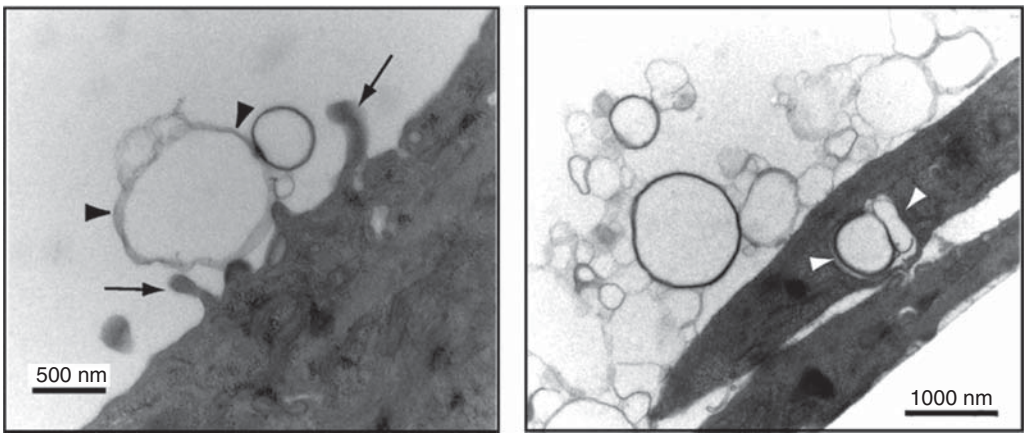

Figure 2. Photomicrographs of the internalization process of liposomes conjugated with *N*-acetylglucosamine by cardiomyocytes. Black arrowheads indicate the microvilli of the cells; white arrowheads indicate liposomes internalized by the cells.
Reprinted with permission from Elsevier [40].

on myocardial ischemia [45-48]. For instance, Verma *et al.* (2005) demonstrated that liposomal-ATP improved contractile function in a Langendorff isolated rat heart model of ischemia compared with free ATP, as well provided superior protection against enzymatic attack [47]. An *in vivo* rabbit study also concluded that liposomal-ATP diminished the proportion of irreversibly damaged tissue after myocardial infarction [46]. In a follow-up study, Verma *et al.* (2006) determined that the inclusion of a cardiac myosin-specific antibody to ATP-loaded liposomes enhanced myocardial protection, compared with nonspecific liposomes. A dual mechanism of treatment was proposed in which the immunoliposomes may 'plug and seal' the membranes of damaged myocytes while delivering exogenous ATP (**Figure 1C**) [48]. Though research is still ongoing, it is clear that the pharmacological delivery of ATP by liposomes may offer cardioprotection during ischemia and reperfusion [43].

3.1.3 *Bisphosphonates*

Bisphosphonates (BPs) are a class of drugs typically used to treat osteoporosis and other bone diseases, since they decrease the resorptive capacity of osteoclasts and promote apoptosis [49]. BPs have also been shown to affect the activity of immune cells [50] and vascular smooth muscle cells [51], which may explain some of their beneficial effects in treating certain cardiovascular afflictions, such as restenosis [52]. However, due to a bulky bisphosphonate group and anionic charge, BPs are not easily internalized by cells. To increase delivery efficiency, BPs were encapsulated in liposomes, which could mediate transfer through the cellular membrane.

Immune cell infiltration to sites of vascular injury may play a significant role in restenosis and, as a result, strategies aimed to reduce macrophage/monocyte activation and proliferation have been proposed [53]. The effects of free BP as well as liposomal-BP on inflammatory cells and macrophages have been well documented [50,54]. For instance, Ylitalo *et al.* (1998) studied the effects of liposomal clodronate, etidronate and pamidronate on macrophages, macrophage-like RAW 264 cells and the formation of LDL-derived foam cells [55]. Results showed that clodronate- and etidronate-encapsulated liposomes reduced the release of degradation agents from RAW 264 cells, which also inhibited the degradation of acetylated LDL. Since free bisphosphonates did not produce the same effects on the degradation of acetylated LDL, it could be concluded that liposomes increased their effectiveness *in vitro*.

Danenberg *et al.* (2002) proposed the application of clodronate-encapsulated liposomes to reduce restenosis *in vivo* [54]. Liposomes were formed by reverse-phase evaporation and injected intravenously to rabbits, which had undergone a standard balloon injury procedure. Morphometric analysis of carotid arteries showed that smooth muscle cell proliferation, extracellular matrix formation and luminal stenosis were significantly decreased in animals treated with liposomal-clodronate compared with free clodronate, blank liposomes and buffer controls. It was found that liposomal-clodronate did not directly inhibit endothelial or smooth muscle cells, but rather attenuated the increase in circulating monocytes observed shortly after injury, affecting macrophage numbers and activity in the liver, spleen and injured arteries. Similarly, alendronate encapsulated into liposomes was shown to decrease circulating monocytes and attenuate neointimal formation after balloon injury and stent deployment in rabbits [56]. Liposomes were again selected as a delivery vehicle since they are thought to increase BP delivery efficiency to macrophages, resulting in apoptosis once the liposomes are metabolized. Epstein *et al.* (2008) demonstrated the favorable effect of liposomal alendronate on restenosis in a rabbit carotid injury model [57], as seen in **Figure 3**.

As of February 2010, the Biorest Liposomal Alendronate with Stenting sTudy (BLAST) Phase II clinical trial was underway to

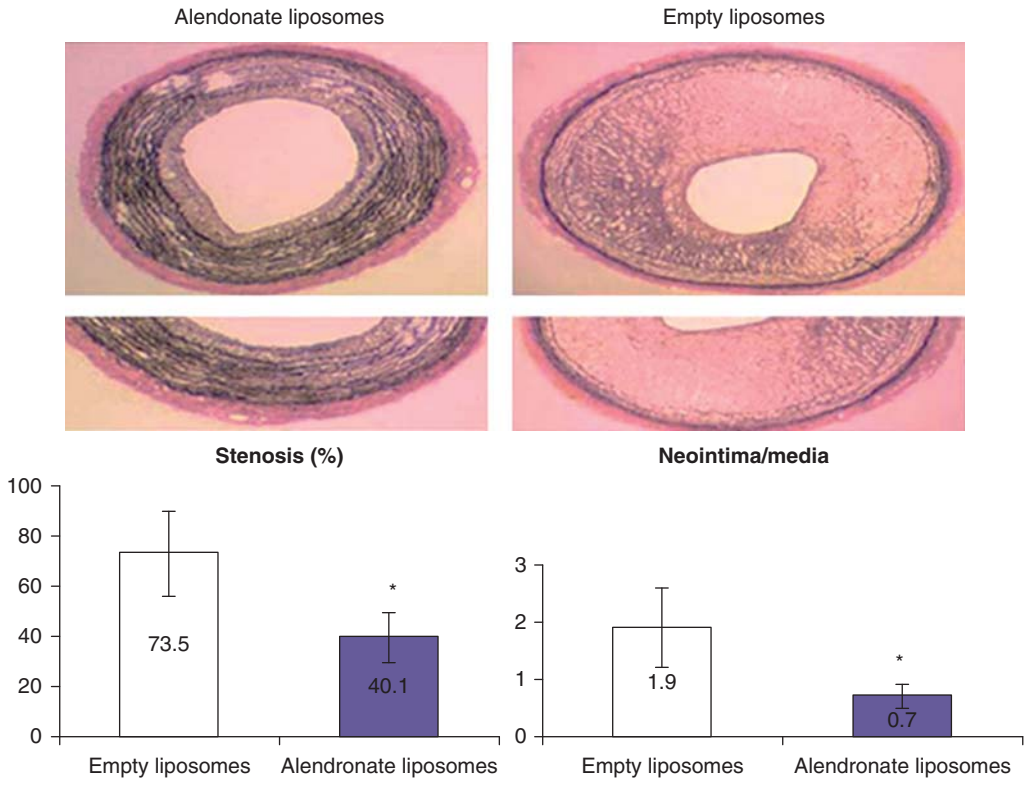

Figure 3. Photomicrographs of Verhoeff tissue elastin staining of untreated and alendronate-treated sections in hypercholesterolemic rabbit models of carotid artery injury. Bar graphs depict the inhibition of stenosis and neointimal formation in liposomal alendronate-treated rabbits.
Reprinted with permission from AAPS [57].

Table 3. Publications detailing a selection of genes used for liposomal delivery to models of vascular disease.

Gene encoded	Experimental model	Experimental outcomes	Ref.
iNOS	Porcine model of ischemia	Delivery by intramyocardial injection did not significantly affect cardiac functionality; showed moderate improvement in neovascularization	[64]
Human tissue inhibitor of metalloproteinase-1	Rat model of vascular injury	Successful transfection and associated decrease in neointimal hyperplasia	[111]
Heat shock protein 90 cDNA	Rabbit model of chronic ischemia	Improved hindlimb perfusion by influencing nitric oxide	[112]
VEGF	Porcine occlusion model	In coronary and peripheral arteries, treatments resulted in angiogenesis/arteriogenesis	[113]

iNOS: Inducible nitric oxide synthase; VEGF: Vascular endothelial growth factor.

determine the effectiveness of liposomal alendronate to reduce restenosis in patients having undergone a PCI with a bare metal stent [58]. The estimated study completion date is not set until January 2015, but preliminary results indicate that there were no statically significant differences in vascular response between patients receiving the treatment and controls [59]. However, some of the subgroup analysis did indicate a differential patient response in diabetics and those with high monocyte counts. No short-term safety concerns were reported. Based on the experimental evidence detailed in this section, liposomes may indeed be promising vehicles for the delivery of BPs to treat CVD, though it is clear that a viable formulation is still several years away from being brought to market.

3.2 Genetic material

Gene therapy can offer local and sustained production of molecules by incorporating exogenous DNA or oligonucleotides into the genome of target cells. Although direct gene transfer with naked DNA has been investigated, it is clear that genetic carriers can offer superior rates of transfection efficiency.

Genetic material can benefit from encapsulation into liposomes by improving transfer across biological membranes, increasing residence time and reducing degradation *in vivo*. Cationic liposomes, in particular, have commonly been used to promote gene transfer, since they can condense plasmid DNA to form stable complexes, called lipoplexes [60].

Gene therapy has been widely used to treat CVD [61]. The vast majority of gene carriers for CVD research and clinical trials are viral vectors [62]; however, a number of groups have also utilized liposomes, owing to their non-immunogenic and relatively low-toxicity profiles in the body [61]. For example, Khurana *et al.* (2004) investigated the application of liposome-mediated VEGF gene transfer to collar-induced intimal thickening of the carotid artery in rabbits [63]. Results showed that VEGF expression exhibited cardioprotective effects, successfully attenuating thickening, as well as diminishing local macrophage infiltration. In another study, Abegunewardene *et al.* (2010) studied the expression of the inducible nitric oxide synthase transgene, which was delivered by liposomes to a porcine model of chronic myocardial ischemia [64]. Only moderate recovery in ischemic areas was reported compared with control procedures, which suggests the need for further study and optimization. **Table 3** presents selected *in vivo* studies of liposomal gene therapy for CVD. A handful of clinical trials have also detailed liposome-mediated transfer of genes for the treatment of restenosis and ischemia [65,66], which produced some promising results but have yet to be translated to any viable product.

Based on recent evidence, liposomes are still vastly inferior to viral vectors in terms of transfection efficiency, and make up a minority of the gene carriers used in cardiovascular clinical trials [67]. Thus, alternative complexes, formed between liposomes and viral components, have been investigated with the aim of improving the transfection capabilities. A hybrid vector commonly applied to treat cardiovascular symptoms is hemagglutinating virus of Japan (HVJ) liposome.

3.2.1 *Hemagglutinating virus of Japan liposomes*

The inactivated Sendai virus (hemagglutinating virus of Japan; HVJ) was complexed to liposomes preloaded with genetic material in an attempt to improve transfection efficiency and safety profile relative to bare liposomes and complete viral vectors [68]. The viral envelope of HVJ contains two glycoproteins, hemagglutinating neuraminidase and fusion proteins, which promote cellular attachment and subsequent gene delivery directly into the cytoplasm. The genetic material is complexed with DNA-binding proteins to enhance expression and incorporated into liposomes, which are subsequently fused with UV-inactivated HVJ [68]. This method can avoid endocytosis and the associated lysosomal degradation, which is widely believed to significantly impede transfection rates.

Early results demonstrated that HVJ liposomes could improve DNA transfer in cultured cardiac myocytes compared with unconjugated liposomes [69]. In later studies, another version of HVJ-artificial viral envelope (AVE) liposome hybrid vectors were loaded with the tissue factor pathway inhibitor (TFPI) gene, in rabbits after balloon angioplasty [70]. Since tissue factor (TF) is a glycoprotein involved in coagulation, it was postulated that the gene encoding for TFPI might lessen TF expression in turn attenuating restenosis. Indeed, HVJ-AVE liposomes were shown to successfully mediate TFPI gene transfection since TFPI mRNA and protein were detected locally. Moreover, rabbits that received HVJ-AVE liposomes loaded with TFPI cDNA exhibited significantly reduced rates of stenosis, as shown in **Figure 4**. A follow-up study reported that a combination of TFPI genes delivered by HVJ-AVE liposomes and recombinant TFPI produced an even greater inhibitory effect on restenosis as compared with either method alone [71]. This positive effect of co-delivering genes and pharmacological agents could be further explored in future studies.

These studies seem to indicate that HVJ liposomes may be well suited to mediating gene delivery in the vasculature. However, one drawback associated with HVJ liposome preparations is that they are not specifically targeted to a given cardiac cell population, in contrast to the application of myocardial or smooth muscle cell-specific antibodies. Forthcoming studies may perhaps consider functionalizing HVJ liposomes with targeting antibodies to determine whether transfection specificity in the vasculature could be further enhanced.

As a final point, despite the widespread use of viral vectors and viral derivatives in the literature, they can pose serious toxicity and immunological issues [72]. Evidently, prior to treating cardiac diseases with genes loaded into liposomes or hybrid liposomes, it is vital to rigorously test *in vivo* safety and immune reactions over the long term. In addition, there are major obstacles related to the production aspects of genetic material and significant variability issues in treatment outcomes that must be addressed before patients with CVD can benefit from gene therapy [62]. Overall, preliminary studies seem to indicate that liposomal gene therapy will continue to pervade the area of CVD treatments, with a focus on improving transfection efficiencies, toxicological profiles and reproducibility.

4. Liposomes in cardiovascular imaging

While drugs and gene delivery can be used to treat CVD once symptoms have manifested, a more conservative approach, involving early diagnosis and intervention prior to reversible damage, may be favorable [73]. Thus, the importance of improving imaging technologies to detect early signs of atherosclerosis and heart disease cannot be understated. Currently, there exists a multitude of cardio-imaging techniques, including cardiac catheterization, computer tomography (CT), magnetic resonance (MR), echocardiography and chest radiography. Of these, liposomes have been used as contrast agents to improve image resolution in CT [5], MR [74] and echocardiography [75]. Recent research in the

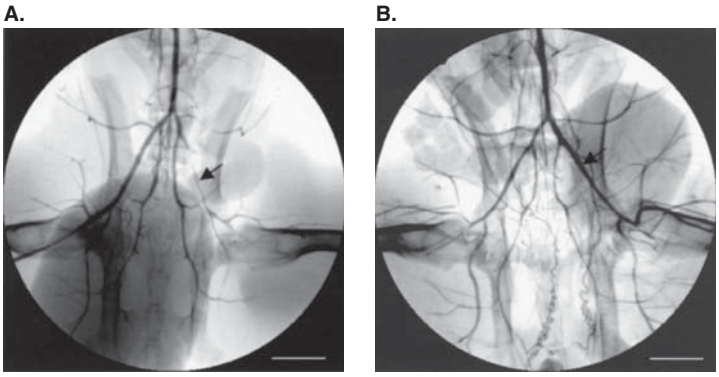

Figure 4. Angiographs depicting the effects of liposomal tissue factor pathway inhibitor genes on neointimal formation and stenosis in rabbit models of angioplasty. (A) Angiogram of an animal that received the control plasmid and (B) angiogram of an animal that received the tissue factor pathway inhibitor gene. Arrows indicate gene-transferred iliac arteries, bar represents 1.05 cm.

Reprinted with permission from Oxford University Press [70].

area of liposomes and cardiac imaging will be detailed in the following sections.

4.1 Liposomal contrast agents

Atherosclerosis is characterized by a complex cascade of events that involve a number of molecular factors, chemokines and various cells types [24]. Since coronary events, such as myocardial infarction and stroke, can occur from relatively small lesions, early and accurate diagnosis is necessary in order to begin appropriate treatments and prevent acute complications [76]. Indeed, the key to reversing atherosclerosis may lie in locating sites of plaque formation before blood flow has even been affected [77]. Although X-ray coronary arteriography is currently the standard for diagnosing atherosclerosis, the procedure is invasive, can often fail to spot vulnerable atheromas due to negative remodeling of the vessel and may also introduce a risk of bleeding at the site of catheter insertion [24]. Noninvasive imaging technologies, such as conventional ultrasound, CT and MR, may offer lower-risk alternatives to facilitate the characterization of atherosclerotic plaque, though technical advancements are still needed before they may be widely employed.

One of the advancements in the field of MR angiography is the use of contrast agents or tracers, which can improve image clarity, reduce artifacts and decrease scan time [78]. Optimal contrast agents should be able to accumulate preferentially at sites of disease for a sufficient amount of time, so as to allow for enhanced image acquisition, while improving the signal-to-noise ratio [79]. Since MR signals are generated from protons, most contrast agents are used to improve the signal by accelerating proton relaxation [24,73]. For example, gadolinium chelates are currently administered intravenously to image vessels in conjunction with MR; however, owing to high throughput *in vivo*, it can be challenging to obtain good-quality images. In addition, agents can rapidly diffuse through vascular tissue, thus diminishing the ability to visualize the border between lesions and healthy tissue [80]. To overcome this challenge, it has been proposed to employ nanoparticulate delivery vehicles that could transport imaging agents and control their distribution. In the same way that nanoparticles can improve delivery efficiency of drugs and genes, they can also reduce the toxicity of tracers, as well as bear surface targeting ligands to improve residence time and target cell/tissue delivery specificity. Liposomes are of particular interest to this application, owing to their characteristic ability to improve residence time and stabilize contrast agents in the blood pool [81].

For example, Ayyagari *et al.* (2006) encapsulated gadodiamide in liposomes coated with polyethylene glycol, in an attempt to improve MR angiography [80]. As shown in **Figures 5A** and **5B**, results indicate that liposomes improved residence time in blood vessels with enhanced image contrast. In regard to further improving residence time and homing accuracy, it has been proposed that inflammatory cells, such as macrophages and monocytes, are ideal targets for imaging agents since they are capable of taking up nano-sized particulates and play an active role in the development of atherosclerotic plaque [73]. Maiseyeu *et al.* (2009) showed that liposomal-gadolinium enriched with exteriorized phosphatidylserine residues, known to promote macrophage recognition and apoptosis, enhanced the MR imaging signal of plaques in $ApoE^{-/-}$ mice compared with un-targeted liposomal-gadolinium (**Figure 5C**) [82]. Thus, formulating liposomes loaded with tracers that are specifically engineered to be attracted to macrophages at atherosclerotic lesions could facilitate imaging and enable better resolution for MR scanning. CT imaging can also benefit from targeted liposomal imaging agents. For example, Conyers *et al.* (2009) formulated ICAM-1-specific immunoliposomes loaded with iodinated contrast media [83]. Preliminary results *in vitro* showed that immunoliposomes selectively bound to inflamed endothelium, which may prove to enhance CT image resolution in future studies.

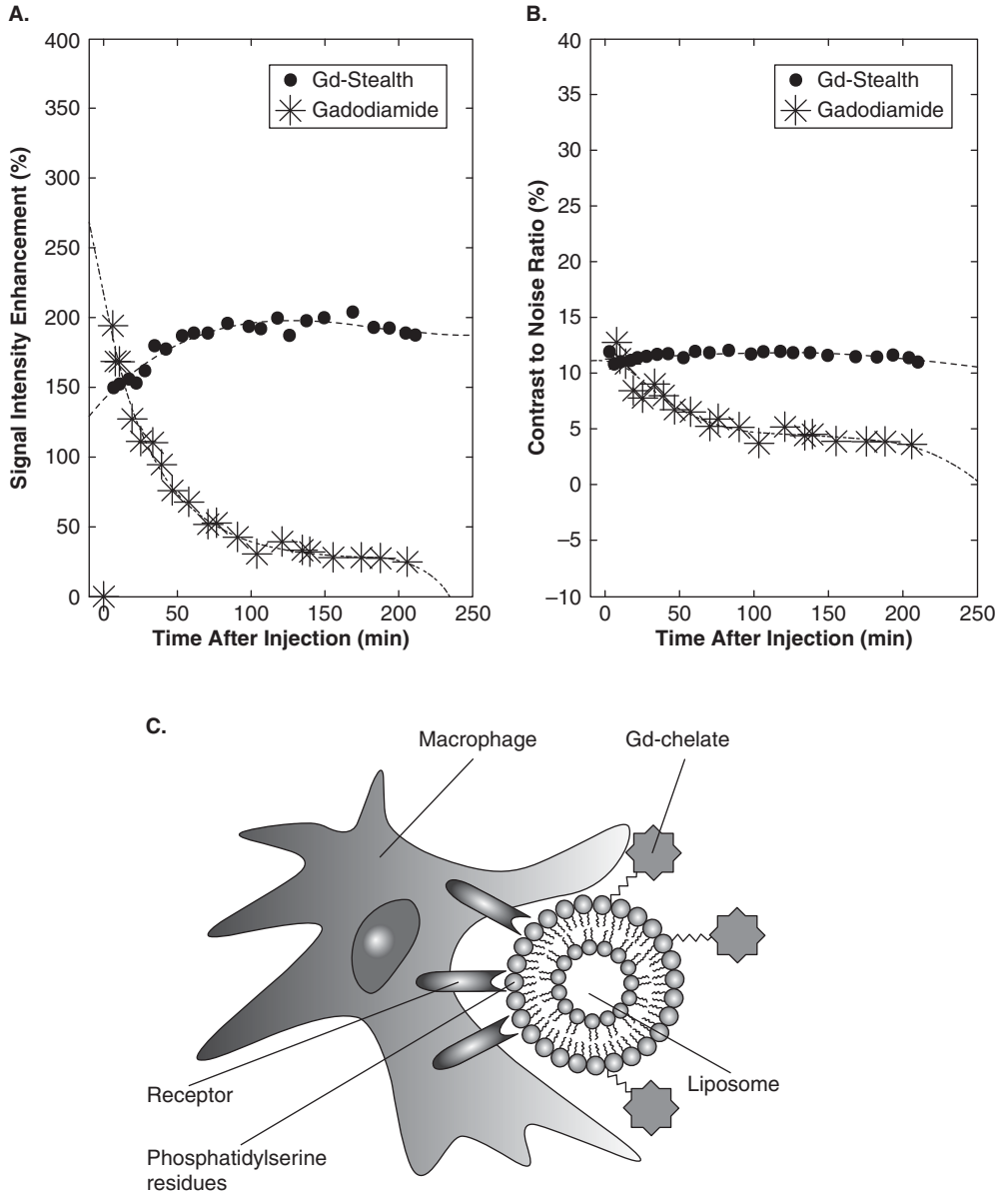

Figure 5. Graphs depict the signal intensity. A. Contrast-to-noise. **B.** Enhancements obtained from gadodiamide encapsulated in liposomes (Gd-stealth) as compared with gadodiamide controls in the magnetic resonance imaging of blood vessels. **C.** Proposed interaction between gadolinium liposomes studded with phosphatidylserine residues and macrophages.

A. and B. Reproduced with permission from John Wiley & Sons [80]. **C.** Reproduced with permission from ASBMB [82].

Advanced studies involving animal models will surely continue to probe the use of liposomal contrast agents in CT and MR cardiovascular imaging applications. Liposomes hold great promise and potential in improving image properties by increasing half-lives and imparting homing abilities to contrast agents that may otherwise be nonspecifically taken up by the reticuloendothelial system. In addition, liposomes may make it possible to co-encapsulate materials that enable dual imaging by CT and MR [84], and/or synergistic targeting of two or more receptors [85], which could help to further elucidate CVD progression, improve diagnosis and subsequent treatment.

4.2 Echogenic liposomes

Ultrasound has widely been used for imaging and diagnostic purposes, since it can be directed to specific regions of interest, enables real-time image acquisition, is widely available and economical [73,77]. Ultrasonic contract agents were

introduced with the aim of improving image quality, sensitivity and tissue characterization [86]. In addition, by improving the signal-to-noise ratio, contrast agents can decrease the amount of ultrasound energy directed to tissues, thus minimizing any peripheral damage. Unlike MR or CT contrast agents, ultrasonic contrast agents can simply be liposomes that contain gas within the core, which significantly improves the imaging contrast by altering backscatter intensity and ultrasound wave reradiation [77,87]. Alternative forms of acoustically sensitive liposomal contrast agents, devoid of gas, were also developed to overcome the inherent instability of the two-phased systems [88]. For these formulations, lipid compositions were varied to produce carriers that could still exhibit echogenicity and enhance image contrast.

In terms of cardiovascular applications, echogenic liposomes have been employed to improve the characterization of atherosclerotic plaque [89]. Echogenic liposomes that attach specifically to the plaque via conjugated antibodies, also called echogenic immunoliposomes (ELIPs), can be used to identify specific surface morphologies and delineate diseased regions *in vivo*. In addition, ELIPs can be tailored to specific sizes (< 1 μm), which are required to adequately perfuse vascular tissues while avoiding accumulation in pulmonary capillaries [89].

Demos *et al.* (1999) demonstrated that anti-fibrinogen ELIPs can be directed to thrombi and fibrin-rich areas of atheromas, while anti-ICAM-1 ELIPs attached to early plaque formations and retained acoustic sensitivity in miniswine [89]. Hamilton *et al.* (2004) showed that anti-VCAM-1 ELIPs could enhance echocardiography images of atheroma in the left carotid artery of miniswine [75]. In another study, Hagisawa *et al.* (2010) described the application of liposomal microbubbles coupled with Arg-Gly-Asp (RGD) peptides in an attempt to bind activated platelets. Results showed that these contrast agents, coupled to liposome delivery vehicles, could markedly enhance ultrasound imaging in rabbit models of acute thrombotic occlusion [90].

Molecular imaging of the cardiovascular system with liposomes will certainly help to enhance current imaging technologies. Indeed, the versatility of liposomes as carriers of imaging agents and gases, as well as their innately ultrasonic properties, renders them well suited for use in a variety cardiac imaging applications and may indeed push the limits of CVD diagnosis in the future.

5. Conclusion

Currently, although there are no liposome-based drug, gene or imaging formulations clinically available to diagnose or treat CVD, this review presents a wide range of ongoing research in the field. It highlights innovative approaches from actively targeting liposomes with antibodies to their use as inherently echogenic contrast agents. The majority of the research indicates that liposomes may indeed allow for therapeutic and diagnostic efficiencies not previously possible with conventional formulations, owing to improved blood circulation times and increased rates of cellular internalization, though it is clear that a great deal of work and advancements are still required.

6. Expert opinion

The application of liposome-based technologies to diagnose and treat CVD has not been as widely investigated compared with applications within oncology; nevertheless, it is evident that this field of research presents great possibilities. As detailed in this review, liposomes have facilitated the delivery of drugs and genetic material to a variety of CVD models, in addition to improving cardio-imaging for CT, MR and ultrasound technologies. In terms of drug delivery to the vascular system, it is clear that liposomes can enhance therapeutic outcomes in animal models of disease for a number of formulations. In particular, poorly soluble drugs, such as statins, and large bulky agents, such as bisphosphonates, can benefit from encapsulation to improve cellular permeability and therapeutic effectiveness. As well, actively targeting liposomes to sites of injury or disease can further the desired outcomes, within the context of CVD treatment and diagnosis.

However, despite this mostly positive progress, further steps must be made in order to improve effectiveness and translate these findings to clinical relevance. For instance, preliminary results have indicated that applying multiple targeting ligands to liposomes could further enhance homing and cellular uptake, compared with single-ligand systems [4,85]. As demonstrated by Kluza *et al.* (2010), liposomes complexed with two antibodies specific to receptors present on activated endothelial cells, synergistically improved particulate uptake compared with either moiety alone [85]. In another study, it was determined that liposomes specific for CD34 antigens and ICAM-1 significantly improved the adherence and penetration of $CD34^+$ adult bone marrow stem cells in aortic tissues [91]. Ma *et al.* (2011) also showed that liposomal gene delivery vectors could benefit from multi-ligand targeting, by combining cellular and nuclear homing molecules to improve transfection [4].

In terms of further improving the delivery success of liposomes, it has been suggested that exogenous triggers, such as temperature and ultrasound, may be able to provide better control over the release of active agents [92], enhance tissue perfusion [93] and cellular permeability [94]. Specifically, ultrasound has widely been used to modify the delivery profile of active agents from liposomes, by inducing thermal or mechanical effects [94,95]. As well, ultrasound has been shown to improve the expression of transgenes delivered from echogenic liposomes, perhaps through cavitation effects [96]. Ultrasound frequency can also affect the cellular uptake of liposomes by temporarily disrupting membranes or tight junctions [95]. In terms of delivery to the cardiovascular systems, these capabilities can lend to enhancing pharmacological effects in future formulations, though it

will be vital to closely test and monitor ultrasound dosage to avoid peripheral tissue damage.

While liposome-encapsulated drug and gene delivery can be used in CVD treatment, and liposomal imaging agents can help elucidate and diagnose similar afflictions, the most important developments in the next few years can be expected to come in the form of theranostic agents, which combine both 'therapy' and 'diagnostic' components into a single system [9]. Indeed, theranostic agents could facilitate the drive toward personalized medicine and thereby improve the outcome of treatments. Liposomes are ideally suited for this form of research, since they comprise separate compartments that can house more than one substrate or active agent. The inherent echogenic properties of liposomes could also make it possible to, for example, simultaneously image thrombi by echocardiography and deliver thrombolytic agents. In a recent study, Laing *et al.* (2011) demonstrated the multifaceted capacities of echogenic liposomes by imaging the delivery of liposomal tissue plasminogen activator to thombi in rabbit aorta [97]. In the future, the ability to co-currently image diseased cardiovascular tissue and deliver cardiovascular drugs by the application of liposome vehicles may have a positive effect on the clinical outcomes for millions of patients suffering from CVD.

In addition to improving vascular targeting and control over therapeutic release timelines, it is imperative that the toxicological and inflammatory effects of liposomes be rigorously investigated. Indeed, one of the current limitations of liposome use *in vivo* is related to immunological reactions, which are characterized by activation of the complement system and inflammatory cells on administration [98]. To address this issue, researchers have studied the effects of the liposome size, surface charge and phospholipid components, all of which can alter the degree of immunological activation [98]. It has been proposed that surface coatings can also improve liposomal stabilization and reduce aggregation [99], which may consequently affect biocompatibility [100]. However, toxicity issues still remain one of the biggest barriers to the success of the liposomal delivery of therapeutic or diagnostic agents. Therefore, further research must be conducted with the aim of minimizing adverse reactions and undesirable side effects before significant advancements can be made toward developing clinically relevant formulations for CVD applications.

Considering the recent success and FDA approval of several liposome-based formulations to treat various forms of cancer, it is likely that liposomes will continue to be probed as a medium for the delivery of active agents to the cardiovascular system [101]. Future work in the area of CVD pharmaceutical delivery can be expected to focus on improving control over pharmacokinetics and biodistribution, in conjunction with a drive to decrease immunological reactions. Specifically, modifications to the liposomal surface by highly specific targeting moieties or alternative polyelectrolytes could decrease toxicity, promote cellular internalization and, in turn, improve therapeutic efficacy. In regard to gene delivery, it is likely that liposomes will continue to be studied as an alternative to viral vectors, which often present larger toxicity and regulation issues. Much of the work in this area can be expected to be devoted to improving transfection efficiencies in the vasculature region. Further advancements in the field of liposomes and CVD may include multicomponent systems that combine liposomal technology with medical devices, such as stents [102], and attempts regenerate the microvascular environment by improving local cell viability [103] or interactions with stem cells [91]. Overall, based on the work detailed in this review, it is clear that liposomes present interesting therapeutic and diagnostic capabilities that will be explored and developed in the future with the ultimate aim of improving CVD treatment and diagnosis.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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