Exploration and application of nanomedicine in atherosclerotic disease
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Chapter 1

General introduction and thesis outline

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The use of nanotechnology for medical purposes — nanomedicine — has grown exponentially over the past few decades. This is exemplified by the US Food and Drug Administration’s approval of several nanotherapies for various conditions, as well as the funding of nanomedical programmes worldwide. Although originally the domain of anticancer therapy, recent advances have illustrated the considerable potential of nanomedicine in the diagnosis and treatment of atherosclerosis. This introduction elaborates on atherosclerotic disease and nanoparticle-targeting concepts and provides an insight into the use of imaging and nanomedicine in atherosclerosis.
Nanomedicine has been defined as “applications of nanotechnology for treatment, diagnosis, monitoring, and control of biological systems”. These applications range from targeted therapy, in vivo imaging and in vitro diagnostics to biomaterials and active implants. Nanoparticles, which are typically in the range of 1–100 nm in size, are similar in scale to biological macromolecules such as proteins and DNA. Importantly, intrinsic physical properties of materials at this level may change, providing fundamentally different properties from the bulk material. Nanoparticles can be composed of organic materials such as lipids, metallic or inorganic materials such as iron oxide and gold, or combinations of these materials. Nanoparticle fabrication can be precisely controlled, which allows their size to be varied and their shape, surface charge, stability and various other characteristics to be modified to ultimately influence particle behaviour in vivo. Moreover, nanoparticles exhibit a high surface to volume ratio, allowing the surface coating to be exploited for derivatization with a variety of molecules. This is of exceptional interest for surface interactions but also facilitates multifunctionality and functionalization for specific applications. Among the different nanomedical applications, nanoparticle-based drug delivery has been most extensively explored. Its advantages include — but are not limited to — improving drug pharmacokinetics and enabling the therapeutic use of drugs that exhibit certain drawbacks, such as low water-solubility, poor bioavailability, fast metabolism, limited specificity or severe adverse effects.

One of the first clinically approved nanomedicinal therapies was a liposomal formulation of doxorubicin, a cytotoxic drug used for cancer chemotherapy. This formulation, branded Doxil, was approved by the US Food and Drug Administration in 1995 for the treatment of Kaposi’s sarcoma and later approved for other cancer types. Subsequently, several other nanomedicinal therapeutics have been approved as anticancer treatments, including liposomal daunorubicin (DaunoXome; Gilead Sciences), applied in patients with advanced HIV-related Kaposi’s sarcoma, and an albumin-bound nanoparticle delivering paclitaxel for the treatment of breast cancer (Abraxane; Abraxis BioScience) after failure of initial treatment for metastatic disease or relapse. Encapsulation of doxorubicin, for example, in liposomes results in favourable pharmacokinetics compared to the free drug by significantly reducing clearance of the drug from the bloodstream, and the main side effect — cardiotoxicity — is substantially reduced.

Besides their therapeutic use, nanoparticles can be incorporated or labelled with small molecules, chelated ions, metals or nanocrystals to enable their detection by diagnostic imaging. For example, nanoparticles can be incorporated or labelled with: the gadolinium ion (Gd$^{3+}$) or iron oxide nanocrystals for magnetic resonance imaging (MRI); electron-dense materials such as gold and bismuth for X-ray and computed tomography (CT) imaging; radiolabels such as $^{18}$F or $^{64}$Cu for positron emission tomography (PET) and $^{111}$In for single-photon emission computed tomography (SPECT) imaging; and fluorophores or quantum dots for optical imaging.

Although its primary application was initially in cancer, nanomedicine has seen tremendous growth and extension beyond oncological applications in the twentieth century. Although still in its infancy, nanomedicine is likely to have a substantial role in the management of cardiovascular disease in general — atherosclerosis in particular.

Key processes in atherosclerosis development

With the advent of preventive medicine, secondary prevention medication and revascularization by interventional procedures, such as balloon angioplasty and drug-eluting stents, considerable advances have been made in the treatment of atherosclerosis. Yet, atherosclerosis remains the major cause of morbidity and mortality in the field of cardiovascular disease, and represents a substantial economic burden. Atherosclerosis can cause clinical complications such as angina pectoris, limb ischaemia, renal impairment and hypertension. However, the most severe complications arise from ruptured atherosclerotic plaques in coronary arteries, which account for ~70% of fatal acute myocardial infarctions and/or sudden coronary deaths, and plaque rupture in carotid arteries, which causes ischaemic stroke.
The build-up of an atherosclerotic plaque starts at lesion-prone areas in large and medium-sized arteries where the endothelium is dysfunctional, induced by cardiovascular risk factors such as chronic smoking, hypertension and chronic hypercholesterolaemia. Dysfunctional endothelium is a crucial pathophysiological factor in atherosclerosis, causing increased permeation of macromolecules such as lipoproteins, increased expression of chemotactic molecules (for example, monocyte chemotactic protein 1) and adhesion molecules (for example, intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1), as well as E-selectin and P-selectin), and enhanced recruitment and accumulation of monocytes.

These monocytes subsequently differentiate into macrophages, which can then be transformed into foam cells by ingesting apolipoprotein B (APOB)-containing low-density lipoprotein (LDL). Subendothelial retention of lipoproteins and immune cells in the vessel wall comprises the first stage of atherosclerosis. The retention of lipoproteins and immune cells either diminishes as a result of the resolution of inflammation, or it results in plaque progression, cell apoptosis and neovascularization over a period of several years or decades. Advanced atherosclerotic lesions may eventually contain a large volume of lipids and necrotic cells, referred to as the lipid or necrotic core.

In normal vessels the vasa vasorum supplies nutrients to the outer component of the vessel wall, and nutrients are supplied to the intima by their diffusion from the lumen. As an atherosclerotic plaque develops, the intima becomes thicker and, once the distance between the deep layers of the intima and the luminal surface exceeds the oxygen diffusion threshold, local hypoxia occurs. This induces neovascularization as a compensatory defence mechanism to restore nutrient supply to the vessel wall. The microvessels that arise from neovascularization originate from the vasa vasorum in the adventitia and extend into the base of the plaque (Figure 1a). Plaque neovascularization has been correlated with inflammation, thereby promoting plaque progression, and it may even contribute to plaque rupture as it facilitates cellular trafficking and the recruitment of immune cells through the vasa vasorum. Plaque neovessels are fragile structures that are also prone to leakage and rupture, probably because of the lack of mural cells and poorly formed endothelial cell junctions.

The wall of the artery is remodelled during the development of an atherosclerotic lesion. It is either remodelled outwards to allow the continuation of blood flow to distal organs or remodelled inwards causing stenosis and thereby limiting blood flow, which can lead to tissue ischaemia. Atherosclerotic plaques can rupture as a result of the breakdown of the fibrous cap that covers the lipid core via inflammatory processes, which can consequently lead to thrombotic occlusions and clinical events. Lesions that are most susceptible to rupture are characterized by active inflammation, thin fibrous caps with large lipid cores, endothelial denudation with superficial platelet aggregation, fissured plaques or luminal stenosis exceeding 90%. Most occlusions that cause acute coronary syndromes do not result from lesions with severe stenosis; rather, they arise from non-stenotic lesions that are also referred to as vulnerable plaques. The identification and treatment of such rupture-prone plaques in patients before clinical events occur would result in substantial health benefits.
Nanoparticle targeting in atherosclerosis

The processes described above and their accompanying molecular and cellular events create numerous opportunities for targeting the atherosclerotic plaque using nanoparticles. Interestingly, many of these processes also occur in cancer as most of them are inflammation-related, including hypoxia-induced neovascularization, endothelial dysfunction, permeability of the microvasculature and the upregulation of adhesion molecules to facilitate the recruitment of cells. Generally speaking, the key targeting principles in cancer can be divided into nonspecific targeting (through the enhanced permeability and retention effect) and active vascular or tumour cell targeting (using functionalized nanoparticles).

The enhanced permeability and retention effect has been thoroughly investigated since it was first reported in 1986 (ref. 34). Normal vascular physiology results in tight (<2 nm) endothelial junctions, which will restrict nanoparticle distribution, whereas a dysfunctional endothelium leads to large gaps that allow macromolecules and nanoparticles to extravasate from the bloodstream at local sites and remain retained locally owing to impaired lymphatic drainage. However, some investigators have started questioning the validity of this phenomenon, and have proposed that the accumulation of nanoparticles may be the result of a different process.

Although it has never been thoroughly investigated in atherosclerosis, nonspecific targeting can be exploited owing to the previously mentioned permeability of the luminal endothelium, as well as the microvascular permeability and leakiness of the neovessels of the vasa vasorum. In addition, intravenously injected nanoparticles may associate with circulating cells or cells in the spleen that subsequently migrate to areas of inflammation. This observation has been reported in the setting of inflammation related to stroke and myocardial infarction, and may also occur in atherosclerosis.

By attaching antibodies, proteins, peptides or other ligands to its surface, a nanoparticle can be targeted to single or multiple receptors that are expressed on the surface of (or inside) a tumour or an atherosclerotic plaque. For example, vascular targeting can be accomplished using nanoparticles that have been functionalized with specific ligands to adhesion molecules such as VCAM1, selectins or integrins such as αVβ3 integrin, as these adhesion molecules are expressed on the activated endothelium of the luminal wall or on the endothelium of newly formed microvessels. Once the nanoparticle is attached to the specific receptor being targeted, it can either bind to endothelial cells or become internalized, depending on the receptor that is targeted and the properties of the nanoparticle used, such as its size.

In addition, the range of cellular and non-cellular components within plaques, such as the extracellular matrix and lipids, allow for specific targeting of plaques with functionalized nanoparticles. Various research groups have established that using functionalized nanoparticles does not enhance the percentage of the injected dose of the nanoparticle that reaches the specific lesion, but it does allow for better distribution of the nanoparticle among pathological lesions and increases its internalization by targeted cells. Therefore, it can be concluded that the physicochemical properties of nanoparticles will largely determine their biodistribution, although the presence of targeting ligands can substantially enhance intracellular uptake, which can increase therapeutic activity. Although this is a form of active targeting, nanoparticle accumulation (at the diseased site) is primarily dependent on vascular permeability as the nanoparticle must first extravasate from the circulation.

To enable efficient targeting of specific processes, several factors should be taken into account — most of them related to pharmacokinetics and/or biodistribution. Upon their intravenous administration, nanoparticles can be distributed over organs and tissues, followed by rapid renal clearance when the hydrodynamic diameter is less than 5.5 nm, as shown in rodent models. Most nanoparticle agents have a hydrodynamic diameter that is considerably larger, causing them to be cleared from the bloodstream predominantly by the mononuclear phagocyte system of the liver and spleen. The initial removal of the nanoparticles occurs through their binding to opsonin proteins in the circulation and their subsequent uptake by the mononuclear phagocyte.
system, which can be useful for targeting the liver but represents a substantial obstacle for efficient targeting of atherosclerotic plaques.\textsuperscript{47} To ensure efficient targeting of plaques, rapid removal of nanoparticles by the mononuclear phagocyte system should be prevented to prolong their circulation time in the blood, enabling nanoparticles to reach and accumulate in tissues and exert their effects. Specific size, surface charge and stability can all contribute to enhanced circulatory half-life.\textsuperscript{6} One of the most commonly used methods to evade rapid clearance via the mononuclear phagocyte system is the addition of hydrophilic polymers such as polyethylene glycol (PEG) to the surface of the nanoparticle.\textsuperscript{48,49} In addition to PEG, various alternative polymers — some of which are biodegradable — are under investigation for the same purpose.\textsuperscript{50} Detailed discussions of how nanoparticle design affects pharmacokinetics have previously been published (reviewed in references 51,52).

Specific nanoparticle accumulation can also be accomplished via inherent targeting of natural nanoparticles. Lipoproteins, including high-density lipoprotein (HDL) and LDL, interact with plaques through a natural conduit. Exploiting or mimicking this inherent plaque affinity of lipoproteins has been shown to be a powerful approach for targeting plaques effectively.

Within the key plaque-targeting mechanisms outlined above and summarized in Figure 1, the distribution of nanoparticles will vary considerably at the cellular level. Nanoparticles with a long circulating half-life will extravasate or accumulate in the plaque by nonspecific targeting and will be subsequently sequestered by macrophages within the plaque (Figure 1a). Nanoparticles that target the activated endothelium, however, are mostly prone to stay confined to the vasculature\textsuperscript{53} (Figure 1b) but they have also been observed to extravasate and target cells within the plaque,\textsuperscript{54} similarly to nanoparticles that specifically target components of the plaque (Figure 1c).

In addition to the targeting methods described above, the formation of a blood clot in an artery allows a thrombus to be targeted using nanoparticles. Thrombi are easily accessible and express a rich variety of cellular and molecular components. When relating these targeting principles to cancer, there is a considerable difference; cancer is commonly a focal disease, whereas atherosclerosis is a systemic vascular disease that develops at various sites throughout the body simultaneously. This further motivates the development of specific agents that efficiently target atherosclerotic lesions following their intravenous administration.
Figure 1. Targeting principles in atherosclerotic plaques. The vessel walls of larger arteries are supplied with nutrients by the lumen and the vasa vasorum — a network of small microvessels. In the lesioned vessel wall the vasa vasorum undergoes angiogenic expansion, with neovessels reaching into the base of the plaque, which is accompanied by the upregulation of cell-surface receptors and increased permeability of the endothelium. The upregulation of receptors and the increased permeability also affect the endothelium on the luminal side of the plaque. The main targeting principles can be classified into nonspecific targeting of the plaque (part a), specific targeting of the vasculature (part b) and specific targeting of components (part c) of the plaque (for example, the extracellular matrix or macrophages) with either synthetic nanoparticles or via interaction through a natural conduit. The targeting of the plaque occurs via both the vasa vasorum and the main lumen at lesioned sites, and is exemplified on the figure with corresponding arrows. Depending on the targeting principle applied, the cellular distribution of nanoparticles in the plaque will vary considerably.
**Atherosclerosis — imaging and nanomedicine**

Traditionally, clinical investigations that were used to detect atherosclerotic plaques focused on the degree of stenosis in the blood vessel lumen. However, with the advent of the concept of vulnerable plaques, the early 1990s marked a change in the understanding of the underlying pathophysiology of atherosclerosis in relation to plaque instability. This resulted in a shift towards visualizing plaque morphology and biology using novel imaging techniques, either stand-alone or — more recently — combined. Developments in the field of molecular imaging allow the characterization of biological processes in cardiovascular disease at the cellular and molecular level. This approach heavily relies on the development and application of contrast-loaded probes — often nanoparticles — that are specifically designed to target cell types and epitopes of interest.

**Nanoparticle-associated imaging applications.** Molecular imaging allows the study of subclinical plaque inflammation and other cellular processes in atherosclerosis, as well as neovascularization and enzymatic activity. Nanoparticles can also be useful for probing mechanisms of uptake in atherosclerotic cells, thereby obtaining new biological insights, whereas imaging techniques themselves can advance the development of nanoparticles (Figure 2, Box 1). The complementary features of certain imaging modalities have motivated the development and application of multimodality contrast agents — nanoparticles in particular — as they allow the facile incorporation of several types of contrast-generating material within the same probe. Validation of targeting is an important motive for the development of multimodal nanoparticle contrast agents, as it allows the detection of the nanoparticle by imaging on clinical scanners and by *ex vivo* techniques — including confocal microscopy and flow cytometry for nanoparticles possessing a fluorochrome. This not only enables confirmation of nanoparticle accumulation in targeted tissues but also the investigation of their cellular fate.

Besides diagnosing subclinical atherosclerosis, nanoparticles carrying contrast-generating material can be used to track drug delivery or enable the quantification of expression of cellular markers after treatment. Many of the studies using nanoparticle therapeutics make use of these possibilities. This type of approach enables a sensitive readout of an early response to therapeutic activity before the onset of changes in atherosclerotic plaque size. This can be especially valuable for early phase clinical trials, providing insight into functional changes and dose response.

The development of novel nanomedical strategies for atherosclerosis can be facilitated by medical imaging at various levels and within different frameworks. Novel nanomaterials need to be thoroughly characterized with respect to their biodistribution within the body following their intravenous administration, preferably *in vivo* and in a longitudinal fashion. Labelling of nanoparticles with a radionucleotide provides a unique tool for quantitatively visualizing the biodistribution of the nanoparticle over time via nuclear imaging, most notably via PET. Different constituents — such as the carrier material itself or the payload — of the nanoparticle can be labelled, which allows for a focused determination of the ultimate fate of the formulation. In the setting of atherosclerosis, the systemic nature of the condition further supports the approach mentioned above, and also facilitates the use of imaging as an end point for nanotherapeutics. In this context, both anatomical and functional imaging strategies, including dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) and PET, are becoming increasingly valuable. Additionally, nanoparticle-facilitated molecular imaging is an important category within nanomedicine. The capability to include high payloads of contrast-generating materials makes nanoparticles attractive as probes for molecular imaging by MRI or CT imaging in atherosclerosis, whereas their affinity for atherosclerotic lesions and the favourable biodistribution of certain nanomaterials makes them well suited to serve as PET tracers.

**Box 1.** How imaging can facilitate nanomedical applications for atherosclerosis
**Nanoparticle-enhanced imaging.** Nanoparticle probes targeting atherosclerotic lesions have been developed for MRI, CT, PET and SPECT imaging, as well as ultrasound and optical techniques. MRI and CT are excellent techniques for macroscopically depicting atherosclerotic plaques in a non-invasive manner, and are continually being improved for the purpose of accurately assessing plaque size and composition." MRI is recognized as a technique that excels in visualizing different components of the plaque, including the fibrous cap and lipid core, whereas CT allows the visualization of calcifications. Recent developments in contrast-enhanced coronary CT angiography allow the detection of positive remodelling, increased plaque volumes and lipoprotein deposition in non-calcified plaques. For molecular imaging purposes both techniques were initially considered to lack sufficient sensitivity for the detection of exogenously administered molecular imaging agents, but with the rise of new and potent nanoparticulate agents MRI and CT are increasingly being explored for this purpose.58

**Figure 2.** Imaging and nanomedicine in atherosclerosis. This figure provides examples of the ways in which imaging is associated with nanomedicine. A. The figure depicts the systemic arterial vasculature, highlighting the carotid arteries, coronary arteries and peripheral arteries, which are prone to developing vulnerable lesions that can be visualized by imaging. B. State-of-the-art imaging techniques that are used to visualize plaque morphology and plaque processes are shown. Ba. The image shows a T1-weighted contrast-enhanced magnetic resonance imaging (MRI) scan of a carotid artery, showing thickening of the arterial wall. MRI can provide information on anatomical features and can be used to differentiate between the fibrous cap and lipid core. Bb. The image depicts the same carotid vessel illustrated in part Ba, which has been assessed using dynamic contrast-enhanced MRI; this imaging technique provides information on vascular permeability and neovascularization. Bc. An intravascular ultrasound image is shown in the figure; intravascular ultrasound imaging can be used to obtain information on plaque area within coronary arteries in response to treatment. Bd. A fused image, obtained using [18F]-fluorodeoxyglucose imaging in combination with positron emission tomography (PET) and computed tomography imaging, is shown. The white arrow in the image points to the right carotid artery, showing a high amount of signal, which is indicative of metabolic activity within an atherosclerotic plaque. C. The figure shows a nanoparticle attaching to an endothelial cell lining the atherosclerotic plaque. The contrast payload within the nanoparticle can render it detectable by an imaging modality of choice. SPECT, single-photon emission computed tomography. Image Bc is modified, with permission, from ref. 71 © (2006) American College of Cardiology.
Outline of this thesis

In this thesis we describe the development of nanotherapeutics with the final aim of reducing atherosclerotic — and therefore — cardiovascular disease. Various aspects of nanomedicine are studied, from the development of novel nanoparticles, to plaque targeting concepts, the development and implementation of new imaging techniques and finally, clinical translation.

The first part of this thesis focuses on a natural nanoparticle, high-density lipoprotein (HDL). In Chapter 2, we describe the development, characterization and application of an HDL nanoparticle with statins incorporated to specifically inhibit atherosclerotic plaque inflammation. In Chapter 3, we further investigate mechanisms by which this statin-loaded HDL nanoparticle inhibits inflammation and explore a possible clinical scenario. In Chapter 4, we incorporate poly(lactic-co-glycolic acid), a biodegradable polymer, into the core of HDL to prompt controlled release properties. We assess the pharmacokinetics, biodistribution and metabolism of radiolabeled HDL in a variety of animal models with PET/CT and PET/MRI in Chapter 5.

The second part of this thesis is centered on long-circulating nanoparticles. Chapter 6 describes the methods to non-invasively image atherosclerosis with 18-fluorine-fludeoxyglucose PET/CT and DCE-MRI, methods to produce liposomal nanoparticles according to good manufacturing practice (GMP) and ways to induce atherosclerosis in a rabbit model. A new technique, three-dimensional DCE-MRI, to assess microvascular permeability in atherosclerotic lesions over a large vascular territory is described in Chapter 7. In Chapter 8, a microfluidic chip that aims to simulate microvascular permeability in an atherosclerotic plaque neovessel is reported and we assess if this model can evaluate nanoparticle translocation. The plaque targeting mechanism of long-circulating nanoparticles is investigated by multimodal imaging and reported in Chapter 9. Liposomal nanoparticles are radiolabeled with the isotope 89-zirconium in Chapter 10, where the biodistribution and accumulation of long-circulating nanoparticles in the atherosclerotic vessel wall is quantified noninvasively by PET/CT and PET/MRI.

The third part of this thesis is focused on the use of liposomal nanoparticles to reduce inflammation in atherosclerosis. In Chapter 11, the anti-inflammatory drugs, glucocorticoids, are encapsulated in liposomal nanoparticles and their therapeutic efficacy is assessed by noninvasive multimodal imaging in a rabbit model of atherosclerosis. In Chapter 12, the scale up to GMP production of glucocorticoid encapsulated liposomal nanoparticles is commenced, and pharmacokinetics, toxicity and therapeutic efficacy is assessed in rats and rabbits. A Phase I/II clinical trial is reported in Chapter 13, in which delivery and therapeutic efficacy of liposomal nanoparticles is assessed in patients with atherosclerosis.
References