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Review

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Nanomedicine-based strategies for treatment of atherosclerosis

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Atherosclerosis is a chronic inflammatory disease of the arterial wall that arises from an imbalanced lipid metabolism and a maladaptive inflammatory response. Despite intensive research on mechanisms underlying atherosclerotic lesion formation and progression during the past decade, translation of this knowledge into the clinic is scarce. Although developments have primarily been made in the area of antitumor therapy, recent advances have shown the potential of nanomedicine-based treatment strategies for atherosclerosis. Here we describe the features of currently available nanomedical formulations that have been optimized for atherosclerosis treatment, and we further describe how they can be instructed to target inflammatory processes in the arterial wall. Despite their limitations, nanomedical applications might hold promise for personalized medicine, and further efforts are needed to improve atherosclerosis-specific targeting.

Atherosclerosis: a lipid-driven chronic inflammatory disorder

Atherosclerosis is a chronic inflammatory disease of the arterial wall resulting from a dysregulated lipid metabolism and a maladaptive inflammatory response. Large clinical studies lend strong support to the association between plasma lipid levels and the risk for cardiovascular events [1,2]. In particular, low-density lipoprotein (LDL; see Glossary) levels correlate with the risk of cardiovascular events in human populations and augment the individual susceptibility to atherosclerosis and its complications. Mechanistically, LDL increases expression of endothelial cell adhesion molecules and primes circulating leukocytes, thus increasing the likeliness for arterial leukocyte infiltration [3]. By contrast, consistent evidence has shown that levels of high-density lipoprotein (HDL) correlate inversely with the occurrence of atherosclerosis and its clinical consequences, and mechanistic studies point towards the strong anti-inflammatory properties of HDL [4]. The accumulation of leukocytes within the arterial wall is a hallmark of all stages of atherosclerosis [5]. During early atherosclerosis, inflammatory monocytes and neutrophils infiltrate the arterial

Glossary

Angioplasty: interventional widening of stenosed blood vessels by inflating a balloon in the narrowed area.
Apolipoproteins (Apos): detergent-like proteins that are capable of binding and solubilizing lipids to transport them through the circulatory system.
Aptamer: a short oligonucleic acid or peptide molecule that binds to a specific target molecule.
Cationic lipids: lipids with an overall positive charge that are located at the hydrophilic head-group.
Efferocytosis: the phagocytosis and clearance of dead cells.
Foam cells: macrophages with an accumulation of intracellular lipids.
Fumagillin: a complex biomolecule that is found in the microbial organism *Aspergillus fumigatus*. It is able to block blood vessel formation.
Glucocorticoids: a class of steroid hormones that bind to the glucocorticoid receptor in order to regulate the immune response to turn down inflammation.
High density lipoproteins (HDLs): particles that are high in apolipoprotein content and are capable of taking up cholesterol from the periphery and transporting it to the liver.
Low density lipoproteins (LDLs): apolipoprotein particles with low protein content but high lipid content. LDLs irreversibly bind cholesterol and are often referred to as 'bad cholesterol'.
Mononuclear phagocyte system: a network consisting of phagocytic cells. These are primarily monocytes and macrophages that are either in circulation or residing in tissues such as the liver, lung, spleen, skin, or brain.
Nanocarrier: molecules or particles on the nanometre scale that have the ability to carry a different molecule.
Nanoparticle: particles on the nanometre scale. They include liposomes, micelles, and polymers.
Polyethylene glycol (PEG): polymer of ethylene oxide. H-(O-CH_2-CH_2)$_n$-OH, where n denominates the number of repetitions, a parameter indicating the polymer size.
Restenosis: the re-occurrence of stenosis following treatment by angioplasty or stent insertion.
Secondary necrosis: an autolytic process of cell disintegration in which the cell contents are released following apoptosis.
Stem cells: undifferentiated cells that can continue dividing, thus giving rise to cells that can either commit to differentiation or remain a stem cell (in the process of self-renewal).
Stenosis: abnormal narrowing of tubular organs or structures such as blood vessels.
Stent: a mesh tube that is inserted into a narrowed blood vessel after angioplasty to prevent restenosis.
Thrombus: the formation of a blood clot, the thrombus, is the final step of the blood coagulation cascade.

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wall by a process that is largely reminiscent of the classical recruitment cascade. Endothelial cell adhesion molecules, such as P-selectin, intercellular adhesion molecule 1 (ICAM1), and vascular cell adhesion molecule 1 (VCAM1), facilitate the firm arrest of inflammatory cells along the arterial wall, while chemokines released from endothelial cells, inflammatory macrophages, or activated platelets crucially facilitate leukocyte adhesion and further guide neutrophils and monocytes into the lesion via mechanisms involving CC-chemokine receptor 1 (CCR1), CCR2, CCR5, and CXC-chemokine receptor 2 (CXCR2) [6,7]. During the later stages of atherosclerosis, accumulation of leukocytes is aggravated by the local proliferation of plaque resident macrophages, and possibly by the hampered egress of inflammatory cells [8,9]. In addition to the accumulation of leukocytes, their state of activation crucially shapes the atherosclerotic lesion. Lesional macrophages ingest modified lipoproteins by engagement of scavenger receptors, thus giving rise to foam cells. Downstream signaling events lead to the activation of transcription factors, including nuclear factor-κB (NF-κB), and the production and release of inflammatory cytokines interleukin-1β (IL-1β), tumor necrosis factor (TNF) and IL-6, as well as CC-chemokine ligand 5 (CCL5), CXC-chemokine ligand 1 (CXCL1) and CCL3. As a result of prolonged endoplasmic reticulum stress, macrophages undergo apoptosis. These apoptotic cells are not effectively cleared owing to an impairment of efferocytosis in advanced atherosclerosis, and thus transit into secondary necrosis. Over time, secondary necrosis feeds the necrotic core and is a driver of plaque destabilization [10]. The plaque is shielded from the blood stream by a matrix-containing fibrous cap that is covered by endothelial cells. Weakening of the fibrous cap by the continuous production and release of matrix-degrading proteases from activated macrophages makes the atherosclerotic lesion more prone to plaque rupture. Consequent exposure of thrombogenic material to the bloodstream causes platelet activation and blood clotting, which is clinically observed as myocardial infarction or stroke.

Despite the identification of inflammatory mechanisms underlying atheroprogression and plaque destabilization, systemic inhibition has limited potential as a therapy because many of the molecular targets have important roles in host defense. Specific, regionally restricted, nanomedicine-based strategies might be superior for future treatment strategies of atherosclerosis. In this review article, we summarize and discuss nanocarriers and specialized tools for the targeting and treatment of atherosclerosis. We describe how these tools might be instructed to reverse pro-atherogenic mechanisms and, finally, we detail the current status of the clinical use of nanocarriers for treatment of atherosclerosis. Owing to the availability of recent reviews on the use of nanomedicine for imaging of atherosclerosis [11–13], we do not focus on such aspects in this article.

Nanocarriers for the treatment of atherosclerosis

In atherosclerosis the most commonly used nanocarriers for drug delivery are lipid-based. Alternative nanocarriers include carbon- or organometallic-based, virus-like, or inorganic particles, such as gold, silver, and metal oxides, respectively [14]. For a more detailed overview of nanocarriers or polymer-based therapeutics used in the context of atherosclerosis treatment, we refer the reader to recent reviews [13,15]. The dual use of therapeutic and diagnostic tools (theranostics) in one nanoparticle formulation allows for synchronized, site-specific evaluation of disease and the delivery of targeted interventions. In mouse models of atherosclerosis, theranostics have been used to visualize lesional macrophage accumulation in combination with macrophage depletion or inactivation [16,17]. Theranostic nanoparticles are reviewed elsewhere [18,19], and nanoparticles that combine imaging with therapeutic abilities are mentioned throughout this review. Among the lipid-based nanoparticles, there are solid lipid nanoparticles, lipid micelles, nanoemulsions, and nanosuspensions, but the most well studied are liposomes [20]. Liposomes consist of one or more lipid bilayers enclosing an aqueous core. This structure allows the incorporation and delivery of therapeutic and/or diagnostic tools because molecules can be incorporated into the lipid layer or the core, depending on their chemical nature (lipo- or hydrophilic, respectively). In addition, the use of cationic lipids enables the accommodation of polyanions, including nucleic acid structures such as DNA and RNA (Figure 1A). In order to improve blood circulation and cell-specific targeting, the liposome surface can be further modified, mostly by inclusion of polymers, peptides, or antibodies (Figure 1B,C). The primary drawback in the use of liposomes as carriers for drug delivery is the difficulty in encapsulating drugs. Encapsulation of small molecules can generally be achieved by passive loading: for example, by hydration of the dry lipid film with an aqueous solution of the drug. Unfortunately this method is accompanied by encapsulation efficacies below 10%. In this regard, the loading of weak base drugs such as doxorubicin, a prominent cytostatic drug, has been improved by remote loading processes based on a pH gradient across the lipid bilayer [21]. The neutral drug molecule passes the bilayer of preformed vesicles during loading and is immediately trapped inside the liposome after protonation. As result, encapsulation efficiency of >98% is achievable. However, for proteins this method seems to be less suitable. Moreover, the lipid composition significantly affects the capability of the vesicle to release the drug [22], making the design of liposomal nanocarriers a challenge.

By chemical ligation of molecules to the lipid-head group, the surface and therefore the *in vivo* fate of nanocarriers can be manipulated. Surface modification with a hydrophilic polymer, such as polyethylene glycol (PEG), paved the way for a major breakthrough in nanomedicine drug delivery. Nanocarriers with a hydrophilic surface (Figure 1B), so-called 'stealth' nanoparticles, can efficiently bypass the mononuclear phagocyte system. Their hydrophilic surface hinders the recognition by the mononuclear phagocytes of these particles as foreign, and it also prevents blood proteins and opsonins from binding to the particle surface, thereby further avoiding blood clearance [23]. Stealth liposomes circulate for about 8–10 times longer than standard liposomes; however, the circulation time of different nanoparticles depends on various factors, including the size, polymer length, and surface density, as

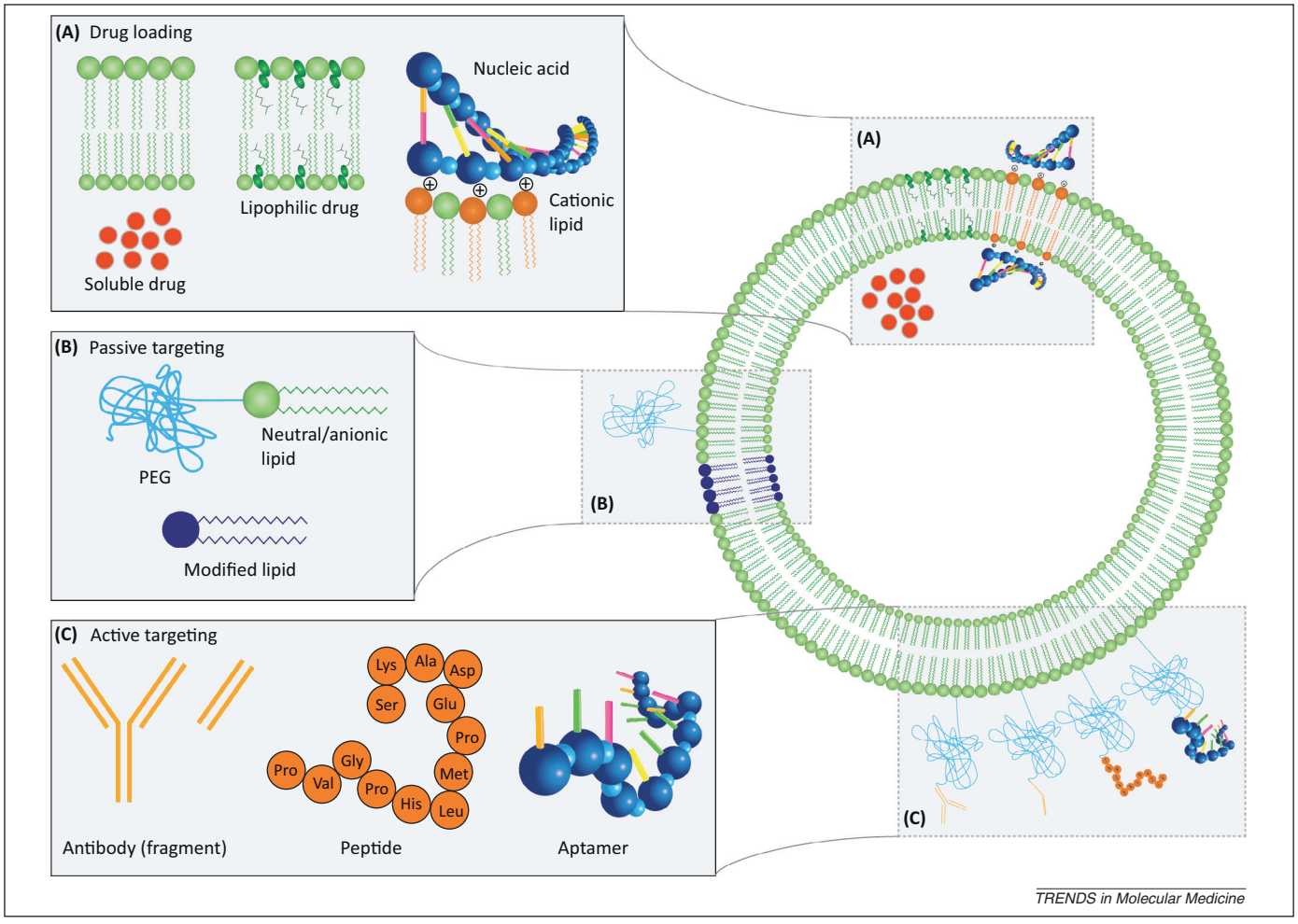

Figure 1. Features of a multifunctional liposome. **(A)** The phospholipid bilayer structure, the basis of liposomes, allows the incorporation of hydrophilic drug molecules into the internal aqueous phase or lipophilic molecules into the membrane. The association of nucleic acid with liposomes is facilitated by the use of cationic lipids (here presented in brown with '+', as opposed to neutral or anionic lipid, which is represented in green). **(B)** Increased blood circulation time of liposomes is needed to allow for targeting. Therefore 'stealth' liposomes are created by surface coating with a hydrophilic polymer such as polyethylene glycol (PEG, light blue). Targeting can be passive, exploiting the abnormalities of diseased tissue (such as leaky vessels or, particularly in the case of atherosclerotic lesions, the changed flow of blood) to trigger drug release. The use of a lipid in which the ester bonds are exchanged with amide bonds (represented in dark blue), makes the lenticular shaped liposomes leak their contents in altered shear stress. **(C)** Liposomes can also be actively targeted through further surface modification with antibodies, antibody fragments, peptides. or aptamers.

well as the overall surface charge [24–26]. Modifications to increase time in circulation have been used in several antitumor therapy strategies for passive drug targeting. In tumor tissues, the vasculature is malformed and leaky, and favors the accumulation of stealth particles with sizes between 20 and 200 nm [27], a phenomenon described as enhanced permeability and retention. At late stages of atherosclerosis, new blood vessels infiltrate atherosclerotic lesions and contribute to their destabilization. These vessels are, however, immature and leaky [28], and can likewise accumulate particles larger than 20 nm: for instance, an albumin-binding contrast agent that allows for the evaluation of the endothelium [29].

Although prolonged circulation time increases the possibilities of therapeutic nanoparticles reaching the site of interest, target-specific strategies are a prerequisite for efficient delivery and are thought to help reduce adverse side effects. To improve on passive targeting, several nanomedical formulations use the abnormal environment of diseased cells, tissues, or organs to induce the release of the loaded drug. For example, low pH in inflammation can be used as a trigger for controlled drug release. So far, such formulations have only been studied in oncology [30], but they could potentially be used for target-specific delivery to sites of inflammation. Atherosclerotic lesions typically form at sites of turbulent flow patterns, such as arterial bifurcations or curvatures [31]. Hence, shear force labile nanoparticles might represent an alternative strategy for exploiting the biophysical characteristics of turbulent areas in arteries. Shear stress-inducible leakage of liposome content can be easily achieved by exchanging the ester bonds in the glycerol backbone into amide bonds in one of the lipids used. This creates lenticular-shaped liposomes with predetermined breaking points on the equator [32]. In another study, micrometer-scale shear-labile particles that consist of poly(lactic-co-glycolic acid) (PLGA) were created [33]. These particles are made of aggregated nanoparticles that are stable under normal blood flow conditions, but break into nanoparticles under shear stress. In comparison to microparticles, these nanoparticles adhere more efficiently to the surface of adjacent blood vessels. When loaded with a thrombolytic drug, the nanoparticles are able to efficiently clear an arterial thrombus. The nanocarrier-assisted delivery of drugs to specific

tissues can also be controlled by magnetism, and sensitivity to heat, light, or ultrasound [22,34]. As an example, magnetofluorescent nanoparticles modified with near-infrared fluorophores combine features for imaging as well as therapeutic options. The phototoxic nanoparticles accumulate in lesional macrophages. Thus they enable imaging and can be light-activated to specifically irradiate macrophages [17]. However, because atherosclerotic lesions develop in unpredictable arterial regions, magnetic targeting or strategies exploiting heat- or light-induced release might only be of circumstantial relevance in advanced preclinical or in clinical settings.

Thus, in the context of atherosclerosis, chemical surface functionalization holds more promise. Nanoparticles can be directed towards their target cells by introducing surface modifications, namely by adding antibodies, antibody fragments, peptides, or aptamers (Figure 1C). In general, the pathways of leukocyte infiltration in atherogenesis,

Table 1. Nanomedical formulations with atherosclerosis-targeting properties[a]

Ligand	Nanoparticle	Purpose as described in the literature	Refs
ICAM1			
Anti-ICAM1 antibody	Liposomes	Stem cell delivery (*in vitro*)	[71]
Anti-ICAM1 antibody	Liposomes	Imaging (*in vitro*)	[88,89]
Anti-ICAM1 antibody	PLGA polymer nanocarriers	Proof of principle (*in vitro*; *in vivo*)	[90]
Anti-ICAM1 antibody	Polystyrene particles	Size and shape dependency (*in vitro*; *in vivo*)	[91]
Peptide (cLABL)	TAT-peptide–DNA complex	Gene delivery (*in vitro*, A549)	[92]
Peptide (cLABL)	PLGA-nanoparticles	Proof of principle (*in vitro*, HUVEC)	[93]
Peptide NNQKIVNLKEKVAQLEA [binding sequence of fibrinogen (γ_3)]	Polystyrene particles	Proof of principle (*in vitro*, HUVEC; *in vivo*, C57BL/6)	[94]
VCAM1			
Cyclic peptide VHSPNKK	Crosslinked iron oxide nanoparticle	MRI imaging (*in vitro*, MCEC; *in vivo*, *Apoe*$^{-/-}$)	[95]
Peptide VHPKQHR	Monocrystalline magnetic nanoparticle	MRI imaging (*in vitro*, human carotid artery specimens; *in vivo*, C57BL/6 ear inflammation, *Apoe*$^{-/-}$)	[96]
Cyclic peptide NNSKSHT	Gd-DOTA-contrast agent	MRI imaging (*in vivo*, *Apoe*$^{-/-}$)	[97]
Anti-VCAM1 antibody	Liposomes	Drug delivery (*in vivo*, *Ldlr*$^{-/-}$)	[98]
Anti-VCAM1 antibody	Liposomes	siRNA delivery (*in vitro*, HUVEC/HAEC)	[99]
PECAM and E-, P-selectin			
Anti-PECAM antibody	Polymer nanocarriers	Enzyme delivery (*in vitro*; *in vivo*, C57BL/6)	[100]
Anti-E-selectin antibody	Liposomes	siRNA delivery (*in vitro*, HUVEC/HAEC)	[99]
Anti-P-selectin antibody	Cu-DOTA contrast agent	*in vivo*, *Ldlr*$^{-/-}$	[101]
Monocytes/macrophages			
Phosphatidylserine	Liposomes with Gd-DTPA-SA	MRI imaging (*in vitro*, RAW 264.7; *in vivo*, *Apoe*$^{-/-}$)	[102]
Mucic acid polymer (targeting scavenger receptor A)	Micelles	*in vitro*, THP-1/HEK-SRA; *in vivo*, Sprague Dawley rats	[56]
Poly-guanidine oligonucleotide (targeting scavenger receptor AI)	Nanoparticles	Proof of principle (*in vitro*, RAW264.7/THP-1; *ex vivo*, *Apoe*$^{-/-}$ plaques)	[103]
Others			
Peptide CREKA (targeting clotted plasma proteins)	Micelles	Drug delivery (*in vivo*, *Apoe*$^{-/-}$)	[39]
Peptidomimetic vitronectin antagonist (US Patent 6,322,770) (targeting angiogenesis via $\alpha_v\beta_3$ integrin)	Paramagnetic nanoparticles	Imaging, drug delivery (*in vivo*, rabbits)	[69,76]
3,5-dipentadecyloxybenzamidine hydrochloride (TRX-20) [targeting the subendothelial matrix via chondroitin sulfate proteoglycans (CSPGs)]	Liposomes	Drug delivery (*in vivo*, rabbits)	[75]
PLGA-PEG-polymer (targeting collagen-IV)	Nanoparticles	Drug delivery (*in vivo*, C57BL/6J) (peritonitis, hind-limb ischemia)	[65]
Recombinant IL-10 (target molecule/receptor unknown)	Liposomes	Imaging (*in vivo*, *Apoe*$^{-/-}$)	[104]
C-terminal globular domain of adiponectin (target molecule/ receptor unknown)	Liposomes and proticles (protamine-oligonucleotide nanoparticles)	Imaging (*in vivo*, *Apoe*$^{-/-}$)	[105]

[a]Abbreviations: Apoe, apolipoprotein E; cLABL, cyclo-(1,12)-PenITDGEATDSGC; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; Gd, gadolinium; Gd-DTPA-SA, Gd-diethylenetriaminepentaacetic acid distearylamide; HAEC, human aortic endothelial cell; HEK-SRA, human embryonic kidney cells stably transfected with human scavenger receptor A; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; IL-10, interleukin-10; Ldlr, low density lipoprotein receptor; MCEC, murine cardiac endothelial cell; MRI, magnetic resonance imaging; PECAM, platelet endothelial cell adhesion molecule; PLGA, poly(lactic-co-glycolic) acid; RAW264.7, abelson murine leukemia virus transformed macrophages; siRNA, small interfering RNA; TAT, threonine-alanine-threonine; THP-1, monocytic cell line derived from an acute monocytic leukemia patient; VCAM, vascular cell adhesion molecule.

including adhesion molecules such as ICAM1, VCAM1, and selectins that are expressed on the activated endothelium of the luminal wall [35], can be used to target the atherosclerotic lesion (Table 1). However, because these adhesion molecules are expressed on the surface of any inflamed endothelium, more specific molecular targets need to be identified. Recently identified flow-dependent translocation of the junctional adhesion molecule A (JAM-A) at atherosclerotic predilection sites might be a feasible and more specific target [36]. In addition, cells that are constantly recruited during atherosclerosis, such as monocytes and neutrophils, have been targeted in the circulation [37,38] and might serve as 'Trojan horses' for shuttling drugs into the lesion. In addition, the non-cellular components within plaques, such as extracellular matrix components, allow for the specific targeting of plaques with functionalized nanoparticles (Table 1). Finally, structures such as clotted plasma proteins that are exposed on the plaque at late stages of atheroprogression can also be targeted [39].

Apart from chemical surface functionalization of nanoparticles, approaches exploiting lipid components involved in disease progression have also been reported as delivery vectors in the field of atherosclerosis. For instance, nanocarriers derived from natural or synthetic HDL, or HDL- and apolipoprotein AI (ApoA-I)-mimetic peptides can spontaneously home to atherosclerotic lesions and could be used for imaging and the delivery of drugs, nucleic acids, and therapeutically active proteins or peptides [40–42]. Finally, functionalized stents [43] implanted into stenosed arteries can be used to specifically deliver anti-inflammatory mediators [44].

Various strategies for therapeutic and diagnostic targeting in atherosclerosis have emerged over the past few years, but the major limitation in these approaches seems to be the lack of specific delivery to atherosclerotic sites. In consequence, future developments need to integrate environment-specific information more closely.

Targeting of proatherogenic mechanisms

Interference with lipid-driven proatherogenic mechanisms reduces atherogenesis

With the clear correlation of plasma LDL concentrations and the incidence of cardiovascular events, and with the abundance of apolipoprotein B (ApoB) in LDL particles, targeting ApoB may hold an important role in reducing LDL-dependent vascular inflammation. Because ApoB is not accessible with conventional therapies, liposome-encapsulated small interfering RNA (siRNA) can be used to silence ApoB and consequently reduce LDL [45]. A single injection of 2.5 mg/kg of a stable nucleic acid lipid particle (SNALP) containing siRNA directed towards ApoB into cynomolgus monkeys reduced the ApoB mRNA levels by over 90% in the liver, and the effect lasted for more than 11 days. Subsequently, ApoB, cholesterol and LDL plasma levels were reduced, whereas HDL levels remained stable [45]. Proprotein convertase substilisin/kexin type 9 (PCSK9) is an endogenous regulator of LDL receptors in the liver. Gain-of-function mutations of the human PCSK9 protein lead to higher circulating LDL cholesterol and increase the incidence of cardiovascular diseases, whereas loss-of-function mutations have opposite effects. Therapeutic silencing or genetic knockdown of PCSK9 induces the expression of LDL receptor levels in the liver and is thus atheroprotective [46]. The delivery of PCSK9-targeting siRNA in liposomes reduces plasma LDL cholesterol concentrations up to 60% of normal, without having a negative effect on HDL cholesterol or triglyceride levels. This effect lasts for 3 weeks after a single intra venous administration in nonhuman primates [47], and the formulation succeeded in a phase I clinical trial discussed later in this review [48].

An alternative target in the lipid-driven inflammation is cholesterol efflux, a process that critically controls leukocyte production and lesional macrophage activation [49,50]. HDL and its mimetics are powerful cholesterol acceptors and hence promote cholesterol efflux from macrophages and stem cells. In this context, the PEGylation of HDL particles improves their plasma half-life and therefore enhances their anti-atherogenic properties *in vivo* [51]. A mutation in the major structural protein of HDL ApoA-I, designated 'ApoA-I Milano', shows atheroprotective effects in an Italian family. Hence, the infusion of ETC-216, a mixture of recombinant ApoA-I Milano with palmitoyl-2-oleoyl phosphatidylcholine-mimicking HDL, induces higher reverse cholesterol transport and has more potent anti-inflammatory properties, and thus can stimulate plaque regression more prominently than the infusion of non-mutated HDL [52]. Furthermore, liposomes consisting of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) have a 10 times higher affinity for plasma HDL compared to liposomes made of egg- or soy-derived phosphocholine. In this complex, the capability of HDL to solubilize cholesterol is improved, and a weekly infusion of liposomes for 5 weeks in an atherosclerotic rabbit model led to a reduction of aortic cholesterol content and plaque volume [53]. Serum amyloid A 2.1, an acute-phase protein that associates with HDL, is able to suppress the storage of cholesterol through its esterification, and it stimulates cholesterol ester hydrolyzation with subsequent cholesterol efflux *in vitro* and *in vivo* [54]. *Apoe*$^{-/-}$ mice treated with two serum amyloid A 2.1-derived peptides capsuled in a liposomal formulation show the prevention of aortic lipid accumulation [55]. In a different study, a mucic acid polymer can target and block the family of scavenger receptors (Figure 2A), and thereby prevent the uptake of oxidized LDL [56]. This macromolecule is used to target macrophages with a micellar formulation of a liver X receptor agonist inducing the efflux of oxidized LDL. When delivered after carotid injury in rats, this formulation reduces cholesterol and macrophage content in atherosclerotic lesions. In a subsequent study, this system was formulated into serum-stable nanoparticles that prevent the thermodynamic disruption of the polymers into monomers [57].

Inhibition of inflammation to prevent atheroprogression

As leukocyte accumulation to a great extent defines the atherosclerotic lesion, the blockade of chemokine-mediated leukocyte locomotion is a mechanism that is used to interfere with arterial leukocyte accumulation. The recruitment of classical monocytes (Ly-6C^{high} in mice and CD14^{+}CD16^{-} in humans) is mediated by the chemokine–chemokine-receptor pair monocyte chemotactic protein 1–chemokine

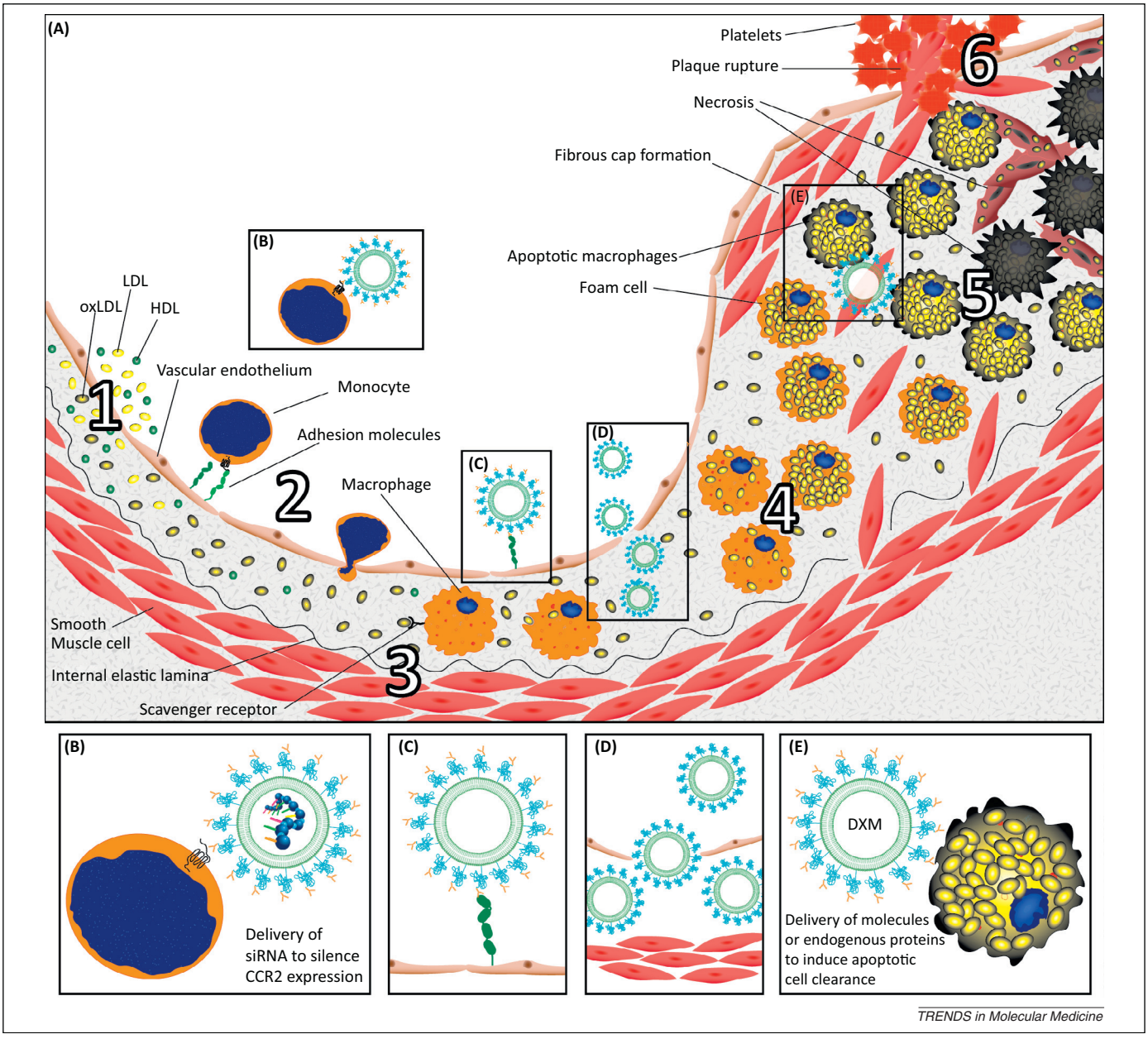

Figure 2. Nanomedicine-based therapeutic targets in atherosclerosis. **(A)** Summary of mechanisms underlying atherogenesis, atheroprogression, and plaque destabilization. The leakiness of endothelial cell junctions in areas of low shear stress permits low-density lipoprotein (LDL) to enter the intima, where it is oxidized (oxLDL) (1). LDL mediates the upregulation of adhesion molecules, such as P-selectin, intercellular adhesion molecule 1 (ICAM1), and vascular cell adhesion molecule 1 (VCAM1), to recruit leukocytes, such as monocytes and neutrophils (2). Recruited macrophages engulf oxLDL via scavenger receptors and give rise to foam cells (3). Atheroprogression is characterized by further accumulation of leukocytes by local proliferation, ongoing recruitment, and hampered egress (4). The fate of atherosclerotic plaques is determined by the failed clearance of apoptotic cells, which leads to secondary necrosis and plaque destabilization (5). The plaque is shielded from the bloodstream by a matrix-containing fibrous cap that is covered by endothelial cells. At late stages, the fibrous cap is weakened by matrix-degrading proteases from macrophages, leading to plaque rupture and the exposure of thrombogenic material to the bloodstream, causing platelet activation and blood clotting, which is clinically observed as myocardial infarction or stroke (6). **(B)** To intervene in leukocyte recruitment, circulating monocytes can be targeted to deliver nanoparticles to the lesion as 'Trojan horses' or to knock down surface receptors, such as CC-chemokine receptor 2 (CCR2), which is crucial for the adhesion to endothelial cells, by siRNA. **(C)** Natural ligands (as well as mimetics or antibodies) of adhesion molecules can be used as targeting entities to direct nanoparticles to atherosclerotic tissue. **(D)** Particles such as high-density lipoprotein (HDL) and LDL naturally home to atherosclerotic lesions, and synthetic equivalents or mimetics can be used as nanocarriers for drug delivery or as cholesterol acceptors to stimulate cholesterol efflux. The increased permeability of endothelial cells or neovessels not only allows lipoproteins to enter the lesion but also permits the entry of (untargeted, long-circulating) nanocarriers within a certain size range. **(E)** The fate of the stability of atherosclerotic lesion is determined by defects in the clearance of apoptotic cells. Inducing this clearance by anti-inflammatory and pro-resolving drugs [such as dexamethasone (DXM)] encapsulated into liposomes could therefore stabilize the lesion.

ligand 2 (MCP-1–CCR2). In this context, recent studies successfully use systemic siRNA encapsulated in a liposome in order to mediate the silencing of CCR2 expression and block the accumulation of inflammatory monocytes in atherosclerotic lesions, as well as in myocardial infarcts [37] (Figure 2B). Functionally this results in reduced atherosclerotic lesion sizes and improved infarct healing in mice [38]. With the importance of cell adhesion molecules throughout all stages of atherosclerosis, these may stand out as promising therapeutic targets for inhibition of leukocyte influx (Figure 2C). However, because these cell adhesion molecules are also important during myeloid cell recruitment in acute inflammatory responses, tailored delivery strategies are needed. At this point, no attempt

has been made to integrate the delivery of adhesion molecule-directed therapy with particles specifically targeting the inflamed arterial wall. Statins are known for their lipid-lowering ability, but they also harbor potent anti-inflammatory properties that cannot be fully effective owing to low systemic bioavailability. In a recent published study, statins were made bioavailable and at the same time targetable to atherosclerotic lesions by encapsulation into synthetic HDL particles derived from recombinant human ApoA-I. In a long-term treatment, this synthetic HDL nanoparticle showed that it is able to reduce plaque inflammation and decrease inflammatory protease activity in atherosclerotic plaques when delivered in four high-dose injections per week [58] (Figure 2D). Another recent study shows, that statins delivered in poly (lactic-co-glycolic acid) nanoparticles decreases monocyte recruitment by inhibiting MCP-1 chemotaxis and increases plaque stability by inhibiting matrix metallopeptidase 9 secretion [106].

Glucocorticoids have the ability to effectively suppress inflammation, but their major drawback derives from their systemic side effects, including insulin resistance, osteoporosis, glaucoma, skin atrophy, and disturbed wound healing, which particularly limit long-term administration. Therefore, several attempts have been made to deliver glucocorticoids in liposomal formulations to reduce the side effects by decreasing the systemic drug concentration. In one study, uncoated anionic liposomes loaded with dexamethasone (DXM) were able to decrease the cholesterol levels in the lesion of atherogenic mice [59]. Liposome-encapsulated DXM had a significantly more potent anti-atherosclerotic effect than the application of free DXM, even when the latter was applied at tenfold higher doses. In another study, the glucocorticoid prednisolone along with a contrast agent was encapsulated in a long-circulating liposome to monitor the delivery of liposomes into atherosclerotic plaques [60]. Within 7 days a reduction in arterial inflammation could be documented by magnetic resonance imaging and positron emission tomography–computed tomography imaging. An interesting approach in the delivery of glucocorticoids is the local delivery via a bioadhesive gel, adapted from marine mussels, to the vascular wall. The gel shows *in vitro* stability against shear forces higher than the ones in blood flow and is still present after 4 months. When incorporated into degradable microparticles that are delivered to the lesion in the bioadhesive gel, DXM shows the characteristics of slow drug elution. Lesions treated using such a gel exhibit reduced inflammation, as measured by decreased VCAM1 expression on the endothelium, attenuated matrix metallopeptidase-9 activity, and a thicker fibrous cap [61].

Mechanistically, glucocorticoids might not just reduce arterial leukocyte influx but also promote clearance of dead cells, and hence contribute to plaque stabilization. In this context, glucocorticoids stimulate the expression of milk fat globule-EGF factor 8 protein (MFG-E8), a bridging protein, which links apoptotic cells to phagocytes and hence promotes their uptake [62]. DXM, when incorporated into liposomes and targeted specifically to the area of apoptotic macrophages (Figure 2E), could promote efferocytosis and thus prevent the transition into secondary necrosis and the clinical outcome of acute atherosclerosis. However, anti-inflammatory treatment strategies might come at the expense of immunosuppressive side effects [63], so attempts have been made to stimulate endogenous mechanisms orchestrating the resolution of inflammation [64]. The advantage of endogenous pro-resolving molecules is their ability to promote a variety of anti-atherogenic effects, such as clearance of dead cells or leukocyte egress. Besides broad-acting glucocorticoids, there are several more specific approaches for stimulating the endogenous resolution processes. Many of these converge at formyl peptide receptor 2, a receptor that has both pro-inflammatory and pro-resolving ligands. Annexin A1 and lipoxin A4 are among its resolving ligands. Annexin A1-mimetic peptide-loaded nanoparticles coated with a peptide that targets collagen IV successfully inhibit tissue damage while promoting tissue repair in a peritonitis and hind-limb ischemia–reperfusion injury model [65,66]. Nanoparticles loaded with annexin A1 or a lipoxin A4 analogue reduce neutrophil influx and shorten the resolution intervals in a mouse peritonitis model [67]. The potential of lipoxin A4 in terms of the inhibition of neutrophil influx and the production of cytokines (TNFα) and chemokines (KC/CXCL1) has been further proved in a chronic arthritis model [68], and if lipoxin A4 was applied as a targeted nanomedical formulation, similar effects might be observed in models of atherosclerosis.

Strategies for stabilization of atherosclerotic lesions

The clinical outcome of atherosclerosis is predominantly determined by the stability rather than the size of the lesion. Novel strategies to induce plaque stability include the inhibition of inflammatory cytokine signaling, the blockade of matrix-degrading proteases, and the stimulation of dead cell clearance [10]. Although nanoparticle-assisted delivery of such compounds is rare, alternative nanomedical strategies have been used to induce plaque stability. Advanced human atherosclerotic lesions are characterized by a dense network of intralesional microvessels which have a strong negative impact on plaque stability [10]. Those microvessels are characterized by a high expression of $\alpha_v\beta_3$-integrin and can be addressed by targeted nanoparticles with peptidomimetic agonists. Such nanoparticles loaded with fumagillin, an endothelium-selective anti-angiogenic compound, can stabilize or reverse atheroprogression with a single treatment in atherosclerotic rabbits. A second injection of those nanoparticles leads to a reduction of neovascularization by 60 to 80% after only 1 week [69].

Another alternative approach for stabilizing atherosclerotic lesions is the delivery of stem and progenitor cells, which can give rise to cell types that are associated with plaque stabilization, such as collagen-producing fibroblasts or smooth muscle cells, or endothelial damage-repairing endothelial cells [44,70]. In contrast to mature differentiated cells, stem and progenitor cells typically carry CD34 on their surface. In a CD34-instructing approach, liposomes were coated with an anti-ICAM1 antibody to target the atherosclerotic lesion and an anti-CD34 antibody to bind $CD34^+$ stem cells. First the liposomes are delivered to aortic segments so that they can bind to

ICAM1, then $CD34^+$ stem cells are added to bind to the liposomes. In this case, the liposomes carry small air pockets, so the adherence, penetration, and migration of $CD34^+$ cells to the intima can successfully be improved by applying a continuous wave of ultrasound to the aortic segments. Nevertheless, the effect of such treatment on overall lesion stability requires further evaluation [71].

Nanomedicine-assisted prevention of restenosis

Percutaneous transluminal angioplasty with stent implantation is used to dilate arteries that have been narrowed by atherosclerotic plaques and to revascularize coronary arteries that have been occluded by atherothrombosis in myocardial infarction. Commonly applied drug-eluting stents release anti-proliferative or anti-inflammatory agents in order to reduce the incidence of in-stent stenosis. However, in-stent stenosis and late stent thrombosis still occur, and ongoing research aims to address the problem with different nanoparticle formulations. It is proposed that systemically administered nanoparticles accumulate in the stented area primarily via the local damage and the increased permeability thereby created. For example, albumin-stabilized nanoparticle-loaded paclitaxel, an anti-proliferative agent, reduces in-stent stenosis [72]. Paclitaxel delivered in this manner has a lower toxicity compared to systemic delivery, and can hence be administered in higher doses [72]. Different nanoparticles were also delivered locally to the balloon-injured area during angioplasty. For example, the local delivery of an amino acid-based nanoparticle containing siRNA targeting NOX2, a component of the NADPH oxidase, reduces restenosis [73]. This study proves that therapeutic reduction of oxidative stress in the vessel wall is feasible. However, so far updates on this concept have been limited. A novelty of liposomal delivery is the possibility of delivering therapeutic gaseous molecules. Nitric oxide (NO) has vasodilatory, anti-inflammatory, anti-thrombotic, anti-proliferative, and anti-atherogenic effects. However, natural scavengers, including hemoglobin, have a high affinity for NO, making its therapeutic use difficult. Encapsulating NO into liposomes can prevent NO binding to hemoglobin, thus rendering it therapeutically available. After carotid injury in rabbits, the local delivery of NO together with argon loaded into liposomes attenuated intimal hyperplasia and reduced arterial wall thickening by 41% [74]. To reduce possible side effects arising from systemic treatment with non-targeted nanoparticles, formulations that are specifically directed towards molecular moieties exposed in the subendothelial matrix after angioplasty have been developed. For example, a novel cationic lipid used for the synthesis of liposomes containing prednisolone allows for accumulation in the subendothelial matrix [75], and targeting $\alpha_v\beta_3$ is achieved with a peptidomimetic antagonist-coated nanoparticle enclosing rapamycin [76]. All formulations are able to inhibit in-stent restenosis after stent implantation or balloon injury.

The long road towards clinical studies

Basic and preclinical studies on the use of nanomedical strategies for the treatment of atherosclerosis are growing, but the translation into clinical studies is still in its infancy. To be therapeutically useful, nanoparticles have to fulfill many criteria. The formulation should remain stable in the circulation, and then when it reaches the diseased area it should release the drug in therapeutically effective concentrations (here it should be highly unstable) without efflux into healthy tissue. Additionally, in many cases the drug has to reach its intracellular target, for instance the cytoplasm, cell nuclei, or other cell compartments, which is often the problem for targeted liposomes ingested via endocytosis, because they then need to escape the endosome. Another major limitation is that atherosclerosis is a chronic disease, so repetitive treatment over a long time is inevitable. In this regard, there is upcoming evidence that nanoparticles, in particular PEG polymers, can have immunogenic properties. A second injection of PEG-coated liposomes in the same animal, when administered after a certain time interval, can be rapidly cleared [77], but owing to the lack of standardized methods for detection, there is great controversy regarding the existence of PEG antibodies and the mechanisms by which these nanoparticles can stimulate immune responses [78]. Hence, standardized tests for assessing immunotoxicology are as important as the assessment of therapeutic efficiency, and clinical studies have to take the accelerated blood clearance phenomenon into consideration [79]. Additionally, a recent study describes complement activation after administration of PEGylated nanoparticles, explaining the nanoparticle related hypersensitivity reactions in humans [80]. Nanoparticles might also aggravate inflammatory processes that are relevant to atherosclerosis. In this context it has been shown that nanoparticles can induce oxidative stress, which is an important promoter of atherosclerosis progression and lesion destabilization [81]. Despite these concerns, several attempts have been made to launch nanomedicine-based clinical trials. The vast majority of these studies focus on the treatment of restenosis after stent insertion. This restriction is likely to be the result of the short time period between stent insertion and treatment. Nanoparticle-mediated delivery of plasmids encoding the angiogenic vascular endothelial growth factor after angioplasty significantly increases myocardial perfusion in patients [82]. Delivery of ApoA-I Milano allows for a significant regression of coronary atherosclerosis [83]. The results of the first human safety trial of systemic nanoparticle paclitaxel (nab-paclitaxel) for in-stent restenosis (SNAPIST-I) were published in 2007. These results show no significant adverse advents attributable to the nab-paclitaxel at 10 or 30 mg/m^2, although moderate neutropenia, sensory neuropathy, and mild to moderate reversible alopecia occurs at higher doses [84]. A liposomal formulation containing alendronate is currently in a clinical phase II trial for the prevention of restenosis. Alendronate is anti-inflammatory and anti-proliferative, and when applied in a liposomal formulation it seems to be exclusively phagocytized by monocytes. So far there is only a difference in restenosis between the treatment and placebo group in patients with high baseline monocyte counts [85]. In addition, the aforementioned silencing of PCSK9 [47] led to a reduction of plasma LDL cholesterol levels by 40% in a recently published phase I clinical trial [48].

Box 1. Outstanding questions

- How can the chronic inflammatory processes of atherosclerosis be targeted without impairing host defense in acute inflammatory situations?
- How can processes that occur within the atherosclerotic lesion be targeted specifically?
- How can side effects associated with long-term, repetitive application be circumvented?

Finally, mipomersen, an antisense oligonucleotide inhibitor of ApoB, has recently been approved by the US Food and Drug Administration for the treatment of familial hypercholesterolemia. Clinical studies have shown its potential in reducing lipid levels in patients at risk for coronary heart disease not controlled by existing therapies [86,87].

Concluding remarks and future perspectives

Many of the exciting preclinical findings with nanoparticles in animal models of atherosclerosis have not progressed beyond the developmental phase. A major reason might be the incongruence between human and murine atherosclerosis, with atherosclerosis in mice being vastly accelerated. Nevertheless, when compared to small molecule drugs, nanoparticles have an improved bioavailability and can hold the ability to be specifically designed to target molecular structures. However, with these advantages come disadvantages such as limited diffusibility, as well as possible toxic, immunostimulatory, or immunosuppressive properties. Nanoparticles might also be retained in the body for prolonged periods, and hence extensive toxicological, long-term studies are required before translation into the clinic becomes realistic [78]. In addition, several important questions remain to be answered, such as the possibility of specifically targeting chronic inflammatory responses in atherosclerosis without impairing host defense in acute inflammation (Box 1). Nonetheless, nanomedicine in atherosclerosis holds promise for personalized medicine, and further efforts are needed to improve tissue-specific targeting and to limit the toxicity for the designated patients.

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