National and Kapodistrian University of Athens

Interdisciplinary M.Sc course in Nanomedicine

USE AND EFFICACY OF NANOPARTICLES FOR TREATMENT OF ATHEROMATOSIS IN VASCULAR CONDITIONS.

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1.1 Nanotechnology

Nanotechnology, or Nanoscience, is an interdisciplinary field where natural and life sciences and technology meet for the creation, understanding and use of atomic and molecular structures (0.1-100nm) and eventually the construction of devices or materials in the nanoscopic scale (Liu et al., 2007). Nanotechnology applies the basic principles of engineering, electronics, physics and materials science to create and manipulate structures at the molecular or μm level (Whitesides, 2005). It has already affected and promoted the development of standard industrial products. Many materials that are characterized by their nanostructure, such as nanoparticles (NPs), nanofibers etc., have been developed and are being studied extensively. These materials are usually three-dimensional, and less than 100 nm in size. Most nanomaterials can be categorized into 4 groups: 1) carbon-based materials, 2) materials based on metals, 3) polymeric materials and dendrimers and 4) composite materials (Bobo et al., 2016).

1.2 Applications of Nanotechnology

The unique properties of these nanomaterials, such as electrical, catalytic, magnetic, mechanical, thermal and imaging properties, in respect to their dimensions in the nanoscopic scale, are particularly attractive for commercial, medical, military and environmental applications. Long-term research and development of these materials led to the widespread use of their applications to develop new research fields, such as nanomedicine, nanosurgery, nanobiotechnology, nanotherapy, nanotoxicology and nanomechanics.

Nanobiotechnology is a branch of Nanotechnology with biological and biochemical applications. Nanobiotechnology studies elements existing in nature, in order to create new devices and applications that can imitate nature itself. Nanomechanics is a branch

of nanoscience that studies the basic mechanical properties (elastic, thermal, kinetic) of physical systems on the nanoscale.

The far most important application of nanotechnology in the healthcare industry is Nanomedicine, which is recognized as the application of the principles of nanotechnology for the diagnosis, imaging and treatment of various diseases. Research regarding selective and targeted transport and release of drugs, diagnostic and therapeutic factors are at the forefront of many nanomedicine programs. [3,4] Nanosystems having unique physical and biological properties have been designed and can be used to overcome limitations and obstacles encountered by classical methods of molecular imaging and/or drug and/or gene transfer [1,5].

1.3 Nanoparticles (NPs)

Nanoparticles are particles with a diameter ranging from 1nm to 100nm, although their size can usually cover the entire nanoscale. Sometimes, nanoparticles have a symmetrical shape (eg cube, sphere, polyhedron) and sometimes asymmetric (eg nanorods, branched and complex structures). Since these are surfaces on an atomic scale, their final conformation is determined by various factors, such as the type of material, the crystalline structure of their nuclei and the relative orientation with respect to the crystal symmetry axes. They can be divided into different categories, depending on their physicochemical properties (eg magnetic, "stealth" nanoparticles), their shape (nanospheres, nanotubes, dendrimers, etc.), and the materials used for their synthesis (natural, synthetic, hybrid, gold nanoparticles, etc.) (Liu et al., 2015; Poole and Owens, 2003).

In the dimensional range of nanoparticles (1-100 nm or less) unusual physical and biochemical phenomena take place, making them ideal for regulation and management of matter on an atomic scale and differentiating them significantly from macroscopic "bulk" materials. Their nature is defined by their electronic structure, their magnetic, electrical and optical properties, their surface activity and their comparable size to the size of biological molecules (Poole and Owens, 2003).

1.4 Cardiovascular Diseases (CVDs)

Cardiovascular diseases (CVD) have been the leading cause of death every year since 1900 in the United States and are still the most common cause of death in the United States. More than 50% of these deaths are caused by coronary heart disease, according to the latest data from the American Heart Association and Stroke Statistics based on research conducted in 2008. Effective treatments for cardiovascular disease include preventive lifestyle changes, medication, and surgery (Da et al., 2005; Franco et al., 2004; Studer et al., 2005).

Rapidly evolving innovative applications and the development of nanotechnology have opened new avenues for CVDs management. Several excellent reviews have been written and summarize the existing literature on the application of nanotechnology in the diagnosis and treatment of cardiovascular diseases (Guccione et al., 2004; Wickline et al., 2006; Yang, 2007).

1.4.1 Atheromatous Disease

This term describes the chronic inflammatory process that leads in the gradual formation of atheromatic plaque on the artery wall, consequently disturbing the normal structure and function of the vessel (atherosclerosis). It is a dynamic process that evolves throughout a person's life. It starts from the very first decade of life and is associated with endothelial metabolic dysfunction. In recent years, extensive research and too many publications have been conducted and published, respectively, in order to determine the role of the endothelium in blood vessel homeostasis. Its role in multiple functions of the vessel is crucial, such as the regulation of vascular tone, inflammation, the process of thrombosis and coagulation, nutrient uptake and elimination of products of metabolism etc. Various substances released by the endothelial cells that line the interior surface of vessels, such as prostaglandins, angiotensin II, endothelin and nitric oxide (NO), maintain the balance between vasoconstriction and vasodilation, thrombosis and antiplatelet activity and constitute important regulators of the vascular inflammatory response (Faxon et al., 2004).

In general, the arteries affected by obstructive atherosclerosis have the structure of muscular arteries. Unlike many species of animals that are being used for atherosclerosis experiments, human inner lining consists of smooth muscle cells (SMCs). The middle vascular tunic contains SMCs embedded in a complex extracellular matrix. The outer tunic, the outer layer of the arteries, contains mast cells, nerve endings and microvasculature.

The change in endothelial permeability initially causes the accumulation of lipids (low density lipoproteins, LDL) in the subendothelial space, leukocytes adhere to the activated endothelial layer, and migrate to the inner vascular tunic. LDLs are oxidized by macrophages and the smooth muscle cells of the middle tunic. Macrophages derived from monocytes phagocytose lipoproteins producing foam cells, while T cells, and the factor released by the platelets (Platelet-Derived Growth Factor-PDGF) promote inflammatory cellular response, causing proliferation and migration of the smooth muscle cells of the middle tunic, resulting in the "fatty streaks". As the damage progresses, migration of smooth muscle cells from the middle tunic to the endothelium, proliferation of all SMCs and increased synthesis of macromolecules of the extracellular substance, such as collagen, elastin and proteoglycans, are observed (McDermott and Lloyd-Jones, 2009; Ross, 1995).

In advanced lesions, macrophages as well as SMCs may die by the process of apoptosis. Extracellular lipids derived from dead and apoptotic cells, may accumulate in the central area of the plaque, slowly forming a lipid or necrotic nucleus, which causes an increase in the volume of the lesion as well as its remodeling, creating the fibrous cap. At even more advanced stages, the atherosclerotic plaques also contain cholesterol crystals and microvasculature. The disease, at this stage, can manifest clinically as the increase in atheroma volume can cause hemodynamically significant stenosis or blockage of the lumen of the vessel. During the last phase of its development, the atherosclerotic plaque develops into a complex lesion, where thrombi and calcifications are observed. Thrombosis, the final complication of atherosclerosis, often occurs when there is a rupture of the atherosclerotic plaque. Disruption of the fibrous capsule of the atherosclerotic plaque reveals collagen fibers in blood circulation and causes a blood clot. The final result is either further stenosis or occlusion of the vessel. This occurs due to the inflow of blood in the center of the plaque, resulting in its swelling but also due to the clot itself which occupies a large part of the endovascular lumen (Ross, 1999).

1.5 Nanoparticles in the early diagnosis of atheromatosis

An emerging and very useful application of nanotechnology is molecular imaging. A method of molecular imaging could be defined as any method that attempts non-invasive and real-time visualization of biochemical events that occur at the cellular and molecular level in living cells, tissues and intact organisms. The data produced by molecular imaging studies contribute to the understanding of biological phenomena, the identification of pathological areas and the emergence of the underlying mechanisms of diseases. Thus, molecular imaging contributes significantly to diagnostics, treatment monitoring, drug discovery, and understanding of nanoscale reactions, such as protein interactions and enzyme conversions.

Generally, in the early stages of atheromatosis there are not any symptoms. To date detection methods are not efficient enough to easily and safely identify early-stage lesions and characterize their features (e.g. vulnerability). Molecular imaging approaches have the ability to better recognize patients at risk of acute coronary syndrome (ACS) and provide useful insights of the mechanisms responsible for coronary artery disease (CAD) and ACS, contributing to the design of novel therapeutic interventions.

1.5.1 Fluorescence-Imaging

Many preclinical investigations have applied Nanotechnology, specifically nanoparticles, in combination with fluorescence technologies in order to monitor atheromatosis and inflammation. A notable example is the use of nanoparticles labeled with fluorophores that has significantly contributed to our understanding of remodeling taking place after myocardial infarction as well as inflammation processes (Panizzi et al., 2010). This molecular imaging approach has been limited to a preclinical level due to the short penetration issues and therefore low detection levels of fluorescence that renders it insufficient for clinical research. Additionally, autofluorescence from common vasculatory structures interferes with the detection signal and results in another limitation regarding the *in vivo* use of fluorescence-imaging approach.

1.5.2 Magnetic Resonance Imaging (MRI)

For the diagnosis of atheromatosis in patients with cardiovascular conditions, characterization of the atheromatous plaque is extremely important. MRI provides characterization of the structure of these plaques in high-resolution as well as discrimination of their main components (Fleg et al., 2012; Kerwin et al., 2013). MR imaging requires administration of contrast agents, including gadolinium (Gd) nanoparticles or nanoparticles based on iron oxide (superparamagnetic iron oxide probes, SPIO and ultrasmall superparamagnetic iron oxide, USPIO). Noteworthy, contrast MRI agents were developed of HDL (high density lipoproteins) like-nanoparticles that show increased affinity for macrophages of atheromatous plaques due to the presence of apolipoproteins, ApoA1 and ApoA2 (Frias et al., 2004). These specific nanoparticles are small in size, consist of endogenous components and therefore are not able to cause immunoreactions and they are not recognizable by the MPS (mononuclear phagocyte system). Another major advantage of these nanoparticles is the ability to carry a significant amount of contrast agents (Frias et al., 2004).

More recently, Tarin et al. prepared iron oxide nanoparticles targeting and detecting by MRI the molecule CD163, which expression levels are elevated at inflammatory sites and has been shown to be useful for the detection of atheromatous plaques (Tarin et al., 2015). In more detail, these nanoparticles were conjugated with an antibody against CD163, resulting in specific nanoparticles that gradually accumulated in atheromatic lesions *in vivo* (Tarin et al., 2015).

1.5.3 Computed tomography (CT)

In order to determine calcification of atheromatous plaques and grade coronary artery stenosis, CT is considered to be the most accurate method. Specificity and contrast of this molecular imaging approach have been recently improved using nanoparticles. Hyafil et al. succeeded noninvasive detection of macrophages in atheromatous plaques using iodinated polymer nanoparticles (Hyafil et al., 2007). Monitoring macrophages has been shown to be crucial for the early detection of plaques, underlining once again

the tremendous contribution of nanoparticles in the early diagnosis of atheromatosis (Hyafil et al., 2007).

1.5.4 Photoacoustic imaging (PAI)

This type of imaging is based on the photoacoustic effect. In photoacoustic imaging, biological tissues are being hit by non-ionizing lasers (pulsed laser) and part of the absorbed energy leads to the generation of ultrasonic waves, which can be detected using ultrasonic transducers and produce images. When compared with high resolution imaging approaches such as OCT, photoacoustic imaging provides larger penetration depth, an advantage that led to its increasing application on atherosclerosis imaging (Fleg et al., 2012; Graf et al., 2011; Wang et al., 2009). Emerging and very promising contrast agents for PAI are Au nanoparticles, which have been reported for macrophages detection, after being conjugated with specific antibody (Wang et al., 2009; Yao and Wang, 2011). Interesting work from Xing et al. resulted in the development of Gadolinium-gold nanords (Gd-GNRs) conjugated to specific antibody (anti-macrophage scavenger receptor, MSR), which could detect and quantify the infiltration of macrophages in atheromatosis (Qin et al., 2013). Overall, for the application of photoacoustic imaging *in vivo*, we certainly need a better understanding of its mechanism and off-target effects.

1.6 Nanoparticles in the treatment of atheromatosis

Therapeutic applications and efficacy of nanoparticles were first developed and tested, respectively, in cancer research (Wang et al., 2012). Advances in nanotechnology extended the application of nanoparticles for the treatment of cardiovascular diseases. In the present study, we focus on nanoparticle-based therapeutic approaches and their efficacy for the treatment of atheromatosis as well as its related complication, thrombosis.

1.6.1 Targeting inflammation

Atheromatosis is a chronic inflammatory disease and is characterized by a dysfunctional interaction between the immune system and lipids. In part, inflammation

is caused and heightened by dysfunctional efferocytosis which in turn leads to the accumulation of apoptotic cells that become necrotic and release proinflammatory substances (Kojima et al., 2014, 2017). This continuous inflammation subsequently can cause lesions with high risk of rupture. Therefore, targeting inflammation in patients with vascular conditions has great potential in the treatment of atheromatosis.

In this line of approach, nanoparticles play a significant role largely because their capability to manage local drug delivery and target specificity, although there are limitations regarding their clinical use, as NP physicochemical properties are not yet completely understood (Allen et al., 2016; Cheng et al., 2012; Pentecost et al., 2016). In the clinical level of atheromatosis, loading anti-inflammatory drugs in nanoparticles has been shown to be a useful tool against various targets of inflammation (Di Mascolo et al., 2013; Jokerst and Gambhir, 2011). For example, nanoparticles loaded with drugs that target immune cells have the potential to control their activities and therefore attenuate or even prevent related disorders, such as increased atheromatosis. It is worth noting a study by Sager et al that aimed to intervene with the recruitment of leukocytes in the atheromatous plaques (Sager et al., 2016). Sager et al achieved combination of 5 siRNAs targeting molecules important for cell adhesion with polymeric nanoparticles. *In vivo* experiments using apoE−/− mice showed significant reduction in vascular inflammation caused by MI, after treatment with the aforementioned nanoparticles, strengthening the possibility of targeting plaque inflammation utilizing targeted NPs (Sager et al., 2016).

1.6.2 Preventing plaque angiogenesis

Atheromatous plaques in advanced stages display extensive neovascularization. In patients suffering from ACS or symptomatic carotid stenosis, lesions demonstrate increased plaque angiogenesis, when compared to patients with stable disease (Dunmore et al., 2007; Gössl et al., 2010). Therefore, targeting and preventing plaque vascularization serves as a promising approach for the treatment of atherosclerosis (Guo et al., 2017). A potential anti-angiogenic drug is Fumagillin, which is hypothesized to act by stabilizing or causing regression of atheromatous plaques. Delivery of Fumagillin on specific sites of angiogenesis in the atheromatous plaque has been achieved using nanoparticles targeting integrins (Winter et al., 2006).

1.6.3 Targeting macrophages

Macrophages and monocyte-derived foam cells play a significant role in atherosclerotic lesion sites, accumulating lipids and triggering inflammatory responses (Moore et al., 2013). In the process of investigating effective therapies for atheromatosis, targeting foam cells and macrophages serves as a very promising avenue to achieve inhibition of lesion development.

The majority of macrophages are derived from the differentiation of circulating monocytes (Moore et al., 2013). Circulating monocytes are divided into inflammatory monocytes, that differentiate to classical macrophages (M1 macrophages) and increase inflammation and non-inflammatory monocytes that differentiate into alternative macrophages (M2 macrophages) and decrease inflammation (F et al., 2011). For distribution to the walls of blood vessels and invasion into intimal layers, only inflammatory and not non-inflammatory monocytes rely on the CCR2 (CC-chemokine receptor 2) (F et al., 2011). Based on these data Leuschner et al. prepared nanoparticles loaded with siRNA against CCR2. After their administration to apoE−/− mice results regarding inflammatory monocytes showed a significant decrease in mRNA and protein levels of CCR2, as well as in lesion size (F et al., 2011). Although additional studies presented encouraging results in this research field, more intensive investigation regarding the underlying mechanisms is required.

1.6.4 Targeting lipid metabolism

Lipid accumulation in the arterial wall and stimulation of inflammatory responses are hallmarks of atherosclerosis (Ross, 1999). Delivery systems using nanoparticles have been designed in order to target cholesterol metabolism in the liver utilizing the technology of RNA interference (RNAi). A therapeutic approach with great potential for the reduction of LDL cholesterol is silencing of PCSK9. Naked siRNA targeting PCSK9 has been shown to be very unstable in the bloodstream and unable to cross the membrane of cells. Contrariwise, administration of lipid-based NPs loaded with siRNA against PCSK9, resulted in successful suppression of the synthesis of PCSK9 in the

liver. It is worth noting, that compared with current treatments including PCSK9 antibodies which require repeated injections, siPCSK9-loaded nanoparticles caused durable and rapid outcomes with only one dose on preclinical as well as clinical levels (Fitzgerald et al., 2014, 2017; Frank-Kamenetsky et al., 2008; Ray et al., 2017, 2018).

1.6.5 Targeting thrombosis

In advanced stages plaque degenerates and ruptures and subsequently thrombosis occurs resulting in myocardial ischemia and eventually infarction (Libby et al., 2011). A promising therapeutic approach includes targeted delivery of anticoagulants and thrombolytic agents, such as tissue-type plasminogen activator (tPA) and urokinase, using nanoparticles targeting the thrombus (Bi et al., 2009; Kawata et al., 2012; Ma et al., 2009; Marsh et al., 2011; Zhou et al., 2014). A notable example are nanoparticles which are coupled with PPACK, an irreversible thrombin inhibitor, that were administrated in thrombosis mouse models and successfully prevented arterial thrombosis (Myerson et al., 2011).

1.7 Conclusions

Atheromatosis is a progressive disease, not easy to detect at its early stages using the currently available imaging approaches. Till today, treatment of atheromatosis is systemic and in most cases with low efficacy and multiple side effects. Local delivery of diagnostic or therapeutic agents to atheromatous plaques and cells actively participating in the process of atherosclerosis, using specific nanoparticles represents a promising approach for diagnosis as well as treatment of atheromatosis. Nanoparticles-mediated delivery increases the bioavailability and stability of drugs and the sensitivity of detection, while decreasing side effects. However, it is important to conduct further *in vivo* experiments to evaluate the efficacy of NPs and eventually proceed to clinical trials.

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