REVIEW

Bioresponsive nanosensors in medical imaging

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Superparamagnetic iron oxide nanoparticles have been established as sensitive probes for magnetic resonance imaging (MRI). While the majority of specific nanosensors are based on sterically stabilized iron oxide particles, the focus of this review is on the use of very small iron oxide particles (VSOPs) that are electrostatically stabilized by an anionic citrate acid shell. We used VSOPs to develop target-specific as well as protease-activatable nanosensors for molecular MRI.

Keywords: apoptosis imaging; enzyme activity; superparamagnetic; matrix metalloproteinase; iron oxide particles

1. INTRODUCTION

Technical innovations in biomedical research over recent decades have expanded our understanding of the human body and we know more and more about the underlying mechanisms of our physiology and the molecular causes for the development of pathologies.

An important goal of molecular medicine is to diagnose the molecular mechanisms underlying disease for each patient, which is expected to enable us to individually tailor treatment aimed at the molecular root of disease. Such new treatment approaches have the potential to cure patients in the most effective way and may eventually help us save costs by avoiding repetitive, tedious and ineffective treatments over long periods of time. To a large extent, molecular diagnoses will rely on molecular analyses performed by clinical chemistry laboratories applying not only traditional laboratory techniques but also the so-called ‘omics’—techniques such as proteomics, cytomics, genomics, metabolomics, lipidomics or microbiomics. But whenever the concentration of a certain marker in blood or other samples alone is not meaningful without knowledge about its local distribution, or when the site of marker expression is important for interventional treatment, molecular imaging becomes a necessity.

Despite the vast knowledge acquired by basic research, our ability to analyse specific aspects of a biochemical pathway in a certain tissue of an individual patient non-invasively is still highly limited. The field of molecular imaging aims to push the development of imaging tools and especially imaging probes with the goal of characterizing pathologies at a molecular level (Weissleder 2002). Figure 1 highlights three principal types of imaging probes: non-specific probes, targeted probes and activatable ‘smart’ sensor probes. Today, most clinical imaging is done with nonspecific imaging probes.

The first targeted imaging probes were introduced for nuclear imaging techniques decades ago, for example 99mTc for imaging iodine turnover by the thyroid, but they have certain disadvantages such as relative low resolution and radiation exposure. This led to the search for molecular probes for other imaging modalities.

In the last 10 years, near-infrared fluorescence (NIRF) imaging has become very popular for molecular imaging of small animals. Important specific advantages of NIRF are multiple wavelength imaging for simultaneous imaging of several targets, straightforward design of activatable smart probes and straightforward compatibility with numerous classic ex vivo techniques such as fluorescence microscopy and flow cytometry.

In living organisms virtually all biochemical reactions are accomplished and controlled by enzymes. They handle nutritional energy turnover, regulate signal pathways and translate and replicate DNA. Therefore, imaging of enzyme activity has been one of the major goals of molecular imaging. An important group of enzymes that specifically cleave peptides, structure proteins and enzymes themselves are proteases. The first successful, protease-activatable in vivo probe was described by Weissleder et al. (1999) and ignited the development of numerous fluorescent smart sensor probes and instrumentation (Ntziachristos et al. 2005), establishing optical imaging as the modality of choice for in vivo detection of enzyme activity (Weissleder & Pittet 2008). Figure 2 illustrates...
the principal function of a second-generation type NIRF probe.

NIRF imaging is highly suitable for whole-body small animal imaging, surface-weighted imaging techniques such as skin imaging and endoscopy, but the limited depth penetration precludes clinical whole-body imaging.

An imaging modality with non-limited depth penetration is magnetic resonance imaging (MRI). Further advantages are exceptionally good soft-tissue contrast, the high resolution and a lack of harmful radiation. MRI is widely used for diagnostic in vivo imaging as well as for experimental research. Therefore, the hardware is highly accessible. Disadvantages include limited sensitivity in comparison with NIRF and nuclear imaging, often time-demanding imaging procedures, and its sensitivity to object movements.

One option to overcome specific limitations of individual imaging modalities is their combination. Multimodality imaging allows the integration of sensitive molecular information, for example arising from NIRF or positron emission tomography images, with the excellent anatomical images of MRI or computed tomography. Furthermore, multimodality imaging...
probes, for example magnetic nanoparticles with protease-activatable NIRF components similar to figure 2, allow accurate localization of the probe with MRI and simultaneous measurement of protease activity at the probe location by NIRF imaging.

The introduction of superparamagnetic iron oxide nanoparticles has reduced the sensitivity gap between MRI and nuclear or NIRF imaging to a great extent. Magnetic nanoparticles provide excellent detectability owing to their magnetic properties, which always occurs, with the degree of extravasation into the extracellular space. To keep particles separated, two principal ways of particle stabilization have been developed. The first option is sterically stabilization, where particles are coated with a thick layer of macromolecules that keep the iron oxide cores sterically apart. Dextran and polyethylene glycol (PEG) polymers are extensively used for non-specific and molecular imaging probes. These polymer coats allow covalent coupling of chemical compounds, peptides (Schellenberger et al. 2004a) and proteins without impairing their sterical stabilization. Therefore, sterically stabilized superparamagnetic particles have dominated molecular probe development for MRI. The second option is electrostatic stabilization with positively or negatively charged surface coatings. The advantage of electrostatic over sterical stabilization is the possibility of developing smaller specific superparamagnetic nanoparticles with similar contrast effect. However, direct modification of the stabilizing charged surface frequently results in an alteration of the surface charge causing destabilization and aggregation. Therefore, we applied indirect coupling methods for the electrostatically stabilized very small iron oxide particles (VSOPs) that are described in this review. A broad overview of magnetic iron oxide nanoparticles for medical imaging was given by Corot et al. (2006).

2. NON-SPECIFIC PROBE VERY SMALL IRON OXIDE PARTICLES

In general, non-specific probes generate image contrast based on physiological parameters (figure 1a). Typical non-specific probes for MRI are low-molecular-weight gadolinium-based contrast agents (e.g. Gd-diyethylene trimine penta-acetic acid (DTPA), gadopentetate-dimeglumine or Gd-1,4,7,10-tetra-azacyclododecane-1,4,7,10-tetra-acetic acid (DOTA)), which are injected intravenously and distribute initially after injection in the intravascular space. Therefore, they allow imaging of the blood vessel lumen (MR angiography, T1-weighted imaging) and improve the detection of arterial stenoses or tissues with increased vascularization. Another indicator is the level of extravasation into the extracellular space, which always occurs, with the degree of extravasation depending on the type of imaging probe and the number and leakiness of the blood vessels. Extravasation is increased in pathological tissue with increased leakiness of the vascular endothelium, which is the case in inflamed tissues and many cancers. An exception is the endothelium of the brain. The so-called blood–brain barrier effectively prevents probe extravasation. Only in pathological situations such as stroke or many brain tumours is the blood–brain barrier disrupted, which allows sensitive delineation of these pathologies.

The safety of gadolinium-based contrast agents relies on the formation of stable complexes of the toxic gadolinium ions in chelates and rapid, complete renal elimination of the contrast medium. This is ensured by the small size of low-molecular-weight gadolinium compounds, which also leads to rapid extravasation and therefore early reduction in the contrast between the vessels and the surrounding tissue. Rapid extravasation and rapid excretion after injection limit the time frame for MR angiography, which may limit complete evaluation, for example, of the coronary arteries.

VSOPs were developed to extend the time frame for MR angiography to 1 h and more, which is long enough for an adequate diagnostic evaluation. For that purpose, VSOPs were designed with a favourable ratio of T1- and T2-relaxivity to allow both highly sensitive T2-weighted MRI such as other superparamagnetic iron oxide nanoparticles and additionally T1-weighted MR angiography. Figure 3 shows a model of VSOPs and a high-quality MR angiography of the coronaries of a pig obtained after IV injection of VSOPs (Warmuth et al. 2007). VSOPs have been shown to be safe in preclinical (Wagner et al. 2002) and clinical trials (Taupitz et al. 2004). In contrast to sterically stabilized iron oxide particles, the similar-sized magnetic core of VSOP’s is stabilized electrostatically by a monomeric shell of citrate acid. Electrostatic stabilization allows particle sizes below 15 nm—in the case of VSOPs about 7 nm including the hydrate shell. Therefore, VSOPs can serve as a basis for extremely small superparamagnetic nanosensors, but direct modification of the particle surface can affect the surface charge and consequently the stability of the particle suspensions. Therefore, we employed indirect coupling methods for the design of our VSOP-based nanosensors.

3. TARGETED NANOESENSORS FOR IMAGING APOPTOSIS

Typical targeted imaging probes feature affinity ligands that are specific for the target structure of interest. After injection in the blood stream, the circulating probes accumulate in the target tissue owing to specific binding, causing a signal change once the non-binding probe is sufficiently cleared from the blood (figure 1b). Ligands are frequently antibodies or antibody fragments, other proteins or peptides.

An interesting biological target for molecular imaging is programmed cell death, also known as apoptosis. Apoptosis is an extensively regulated programme for the safe removal of cells and is essential for normal tissue homeostasis and tissue differentiation (Kerr et al. 1972). Only if the apoptosis rate equals the rate of cell division (mitosis) does the number of cells in an organ remain constant (homeostasis). Changes in apoptosis play a very important role in the pathology of many diseases such as atherosclerosis, myocardial...
infarction, stroke, neurodegenerative diseases and cancer. Moreover, the induction of apoptosis is the goal of most cancer therapies. Currently, the effectiveness of anti-cancer treatments is evaluated by measurements of size reductions of the tumours. Usually, it takes weeks or months until these changes become significant, leading to long delays before ineffective treatment regimens can be recognized and changed. The induction of apoptosis and necrosis usually occurs within hours after treatment begins (Schellenberger et al. 2004b; Yagle et al. 2005). Therefore imaging of apoptosis allows ‘real-time’ monitoring of treatment outcome and identification of the most effective anti-cancer regimen for an individual patient. The broad range of possible applications is the reason why apoptosis is such an attractive target for molecular imaging.

A promising target for molecular imaging is the externalization of phosphatidylserine by apoptotic cells. During early apoptosis, negatively charged phospholipids (especially phosphatidylserine and phosphatidylethanolamine), which are normally distributed at the inner cell membrane, translocate to the outer cell membrane. Annexin A5, a human protein (36 kDa), binds with receptor-like affinity to these exposed negative phospholipids and is widely used as an apoptosis marker in flow cytometry and fluorescence microscopy. Moreover, annexin A5 was successfully used as a radiotracer for monitoring chemotherapy of different cancer types and predicting response to treatment (Belhocine et al. 2002, 2003). Similar good results were achieved when patients with follicular lymphomas were treated with radiation only (Haas et al. 2004). Meanwhile, radiolabelled annexin A5 was used for monitoring of chemotherapy and radio-chemotherapy in several studies with different tumour types (Kartachova et al. 2004; Hoebers et al. 2008; Kurihara et al. 2008). In addition to oncology, apoptosis imaging with technetium annexin A5 allowed vulnerable atherosclerotic plaques to be distinguished from stable plaques in carotid arteries of patients undergoing carotid endarterectomy. In patients with acute stroke, it has been used as a marker to identify damaged tissue in ischaemic brain regions, which is important for selection of the appropriate therapy (Lorberboym et al. 2006). One theoretical limitation is that annexin A5 binding cannot distinguish apoptosis from necrosis; since the cell membrane of necrotic cells becomes leaky annexin can bind to the negative phospholipids at the inner leaflet of the membrane. But in most cases, including cancer therapy monitoring, it is advantageous to use annexin A5 as a damage marker for detection of both distinct induction of apoptosis and/or necrosis.

We used sterically stabilized cross-linked iron oxide particles (CLIOs) as a basis for developing annexin A5–CLIO-Cy5.5 particles for the MRI of apoptosis (Schellenberger et al. 2002, 2004c). These particles have a hydrodynamic size of about 50 nm (figure 4a) and were used for MRI of myocardial apoptosis secondary to ischaemia–reperfusion injury in mice. Ischaemia was induced by transient (30 min) ligation of the left anterior descending coronary artery, a model that results in apoptosis of up to 20 per cent of cardiac muscle cells (Bartling et al. 1998; Dumont et al. 2000; Muni & Kitsis 2003).

T2*-weighted MRI was performed 24 h after IV injection of AnxCLIO-Cy5.5 or unlabelled CLIO-Cy5.5 as control. Figure 4b shows the signal decrease on MR images induced by the accumulation of AnxCLIO-Cy5.5. The areas of decreased signal intensity matched apoptotic areas of the myocardium in histology. No significant signal change or accumulation were observed for the control probe (figure 4c) (Sosnovik et al. 2005).

Using the VSOPs as a starting point, we have recently developed annexin A5–VSOP by electrostatically coupling annexin A5 to VSOP via protamine. Protamines are highly positively charged peptides (about 22 out of 32 amino acids are arginines) that are purified from fish sperm and used clinically to counter
anticoagulation with heparin. Figure 4d shows a model of annexin A5–VSOP in size comparison with AnxCLIO-Cy5.5 (figure 4a) and an IgG antibody (figure 4e). For the synthesis, at first protamine is site-specifically coupled to a single cysteine in the region opposite the binding site of annexin A5. In the second step, annexin–protamine is mixed with the VSOPs, which leads to electrostatic coupling of the positively charged protamine at the negatively charged citrate surface of VSOPs. With this method, we could couple about five annexin A5 molecules per VSOP, resulting in annexin A5–VSOP with a hydrodynamic size of only 14 nm. The small size of the particles should improve extravasation and therefore increase the bioavailability in target tissues, which should ultimately result in more potential applications for MRI of apoptosis. Currently, we are trying to increase the coverage of the VSOP with annexin A5 to minimize the surface not covered by the human protein. A complete annexin shell should help to further reduce non-specific binding and the risk of allergic reactions, prerequisites for successful in vivo imaging.

4. PROTEASE-SPECIFIC IRON OXIDE PARTICLES FOR IMAGING PROTEASE ACTIVITY

Matrix metalloproteinases (MMPs) represent an especially interesting target for imaging enzyme activity. Several MMPs with up to about 30 members (VanMeter et al. 2001) have been identified as important regulators of pathological remodelling of the extracellular matrix in cancer and inflammation and represent increasingly interesting targets for enzyme inhibition treatment (Hu et al. 2007). Tumour extension and proliferation into existing tissue, the invasion and migration of tumour cells and the neo-angiogenesis of growing tumours all depend on remodelling of the extracellular matrix, mainly by MMPs. Another important pathology in which MMPs play a role is the destabilization of atherosclerotic plaques, as MMPs contribute to the degradation of the stabilizing extracellular matrix. The rupture of such destabilized plaques triggers acute events such as stroke or myocardial infarction. Therefore, imaging of specific MMP activity with the potential for monitoring MMP inhibitor therapy is extraordinarily attractive in many fields. This concept was applied by the Weissleder Laboratory in 2001 with successful imaging of MMP-2 inhibition in cancer in vivo by NIRF imaging with an activatable probe as shown in figure 2 (Bremer et al. 2001).

On the basis of VSOPs, we designed novel protease-specific iron oxide particles (PSOPs) by coating the nanoparticles with peptide–PEG copolymers, turning the electrostatically stabilized VSOPs into PSOPs, which are sterically stabilized by the PEG shell (Schellenberger et al. 2008). PEGs, which have been proven to be biocompatible (listed in the pharmacopoeia), can provide stealth properties to particles, which is a prerequisite for sufficiently long blood circulation times of nanoparticles (Shi et al. 2006).

The synthesis of PSOPs is done in two steps. First, amine-reactive N-hydroxysuccinimide (NHS)–mPEG is covalently linked with the peptides. In the second step, the peptide–PEG copolymers are mixed with the VSOPs, which leads to electrostatic adsorption of the arginine-rich cationic coupling domain of copolymers at the anionic citrate surface of VSOPs. Figure 5a shows a model of the resulting PSOPs. At the centre is the magnetic iron oxide core of about 5 nm (grey)
coated by a monomeric citrate shell (red). The peptide–PEG copolymers are electrostatically coupled to the citrate coat. Between the coupling domain (blue) and the PEG (light blue), the peptides contain the cleavage site (yellow), in this case specific for MMP-2/9. PSOPs have a hydrodynamic size of about 24 nm, including the PEG shell.

Figure 5b,c illustrates the function of PSOPs. When PSOPs contact a protease (brown) specific to the cleavage site, the protease cuts the peptide, thereby releasing the PEG molecules (figure 5b); PSOPs lose the sterically stabilizing PEG shell and expose a mixed-charge surface with positive coupling domains and the negative citrate shell. This and the magnetic iron oxide cores lead to aggregation (figure 5c). Aggregation of superparamagnetic particles increases the T2 and especially T2*-relaxation effects of the particles ("magnetic relaxation switch") (Josephson et al. 2001; Perez et al. 2002); the resulting contrast effect is detectable by MRI.

The first protease-sensitive magnetic relaxation switches presented by Josephson et al. (2001) and Perez et al. (2002) were clusters of CLIO monomers before activation, which were cleaved into approximately 45 nm-sized monomers by caspase-3, leading to a decrease in relaxivity and contrast effect. Therefore, these CLIO nanosensors were designed and useful only for in vitro applications. In contrast, PEGylated PSOP monomers (24 nm) can be used in vivo because they aggregate and cluster upon activation, resulting in increased relaxivity in potential target tissues.

Proof-of-principle experiments of PSOP activation are shown in figure 6 (Schellenberger et al. 2008). Measurement of hydrodynamic size (Z-sizer, Malvern) before and after the addition of MMP-9 reveals rapid aggregation of PSOPs about 3 min after the addition of MMP-9 from a single PSOP of 24 nm to clusters of activated PSOPs of 3 μm or more (over 3 μm the clusters precipitate and measurements become inaccurate). Pre-treatment with an MMP-9 inhibitor prevents the activation of the particles, confirming that activation is due to specific enzyme activity of MMP-9. A similar MRI experiment is shown in figure 6b. Five reaction vials contained equal concentrations of PSOP and no or increasing concentrations of the MMP-9 inhibitor (MR image from left to right). Without an inhibitor, the signal intensity in the T2*-weighted images drops over time owing to aggregation of particles activated by MMP-9. Activation is prevented by addition of increasing concentrations of MMP-9 inhibitor. The MR image is a snapshot at 32 min. After some time, the T2* signal intensities increase again. This is partially attributable to precipitation of aggregates. Additionally, the fraction of water protons influenced by the magnetic field of the particles decreases from a certain time onwards as the aggregates continue to increase in size while their number decreases (Koh et al. 2008). Although precipitation due to aggregation is problematic for these in vitro experiments, it is an intended and important mode of function for the in vivo application.

Both experiments prove that PSOPs are activated specifically by the protease MMP-9, demonstrating the potential for imaging real activity of proteases by MRI in vivo. These results remain to be confirmed by in vivo experiments. PSOPs are designed to allow straightforward modification of parameters such as PEG length, PEG number per peptide, coupling domain length and the ratio of copolymer to VSOP, which together determine the in vivo properties of the particles. At the moment, we are optimizing the extent of the PEG shell and the stability of the electrostatic coupling between the citrate shell and the copolymers by modifying the coupling domains to allow optimal blood circulation times and activation kinetics in vivo.

Besides the detection of other proteases, future directions could be the incorporation of two distinct protease cleavage sites in a logical arrangement to either increase or decrease the specificity of the PSOPs. Another direction could be the combination of the specific accumulation of PSOPs in tumour targets with magnetic thermo-ablation (Thiesen & Jordan 2008) or the adaptation of other designs for particle accumulation, for example a clotting amplification system (Simberg et al. 2007).
5. REQUIREMENT OF NANOSENSORS FOR CLINICAL APPLICATION

Our intention in developing molecular imaging probes has always been to theoretically allow translation into clinical application, for all three types of imaging probes—non-specific, target specific and activatable.

One principal requirement is the safety of the probes. In particular, it is necessary to avoid toxic probe components and their potential accumulation. Otherwise, one needs to ensure that toxic compounds (e.g. gadolinium-containing contrast agents) are cleared from the body completely. Consequently, we used only clinically approved or biocompatible compounds. The underlying platform, VSOPs, has emerged as a good imaging probe for MR angiography and has been proved to be safe in preclinical (Wagner et al. 2002) and initial clinical tests (Taupitz et al. 2004). It has been shown that iron oxide nanoparticles with suitable composition are degraded predominantly in Kupffer cells of the liver and other macrophages, with the iron incorporated into the body’s natural iron stores (Corot et al. 2006). VSOPs are currently undergoing further optimization to yield a more robust synthesis and reduction of hazardous chemical compounds.

Annexin A5 is a recombinant human protein that is undergoing clinical testing for nuclear imaging of apoptosis (Hofstra et al. 2000; Kemerink et al. 2001; Belhocine et al. 2003; Lorberboym et al. 2006; Rottey et al. 2006; Hoebers et al. 2008; Kurihara et al. 2008). In general, radiolabelled annexin A5 was safe. In one case a patient developed an allergic reaction (rash and nausea) after the first injection of a 99mTc-HYNIC-annexin V formulation (Rottey et al. 2006).

Protamine has been in clinical use as a heparin antidote for years (Jaques 1973; Arén 1990; Patel et al. 2007). As stated before, we are optimizing the surface of annexin A5–VSOP to cover the citrate/protamine surface completely with annexin A5 to improve the pharmacokinetics and minimize the risk of allergic reactions. The PEG cover of the PSOPs (figure 5a) should fulfil a similar antiallergenic effect by masking the particles with the extensive water shell of the PEG. Numerous PEGs are listed in the pharmacopoeia and are part of many drugs and cosmetics because of their high water-binding capacity and lack of chemical and biological reactivity. Administration should be very safe because PEGs are eliminated by the kidneys, especially when they are of low molecular weight.

Another important point for future clinical translation is the balance of development costs and the spectrum of potential applications in the clinics. Especially for the first nanosensors, the costs will be high. Therefore, we chose cell apoptosis and MMP-2/9 activity as targets for our nanosensors, since both are important players in the pathophysiology of widespread, severe diseases such as atherosclerosis and cancer.

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