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TOPICAL REVIEW

Exogenous imaging contrast and therapeutic agents for intravascular photoacoustic imaging and image-guided therapy

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Abstract

Intravascular photoacoustic (IVPA) imaging has been developed in recent years as a viable imaging modality for the assessment of atherosclerotic plaques. Exogenous imaging contrast and therapeutic agents further enhance this imaging modality and provide significant benefits. Imaging contrast agents can significantly increase photoacoustic signal, resulting in enhanced plaque detection and characterization. The ability to use these particles to molecularly target markers of disease progression makes it possible to determine patient-specific levels of risk and plan treatments accordingly. With improved diagnosis, clinicians will be able to use therapeutic agents that are synergistic with IVPA imaging to treat atherosclerotic patients. Pre-clinical and clinical studies with relevance to IVPA imaging have shown promise in the area of diagnosis and therapeutics. In this review, we present a variety of imaging contrast agents that are either designed for or are compatible with IVPA imaging, cover uses of therapeutic agents that compliment this imaging modality, and discuss future directions of research in the field.

Introduction

Heart disease is the number one killer in the United States, with an estimated 16.5 million people suffering from coronary heart disease after 2014 (Benjamin *et al* 2018). The disease is inflammation driven (Van der Wal *et al* 1994, Hansson 2005, Skeoch and Bruce 2015, Viola and Soehnlein 2015), and results in the development of fatty plaques (figure 1). These plaques can then rupture (van der Wal and Becker 1999, Virmani *et al* 2000, Kolodgie *et al* 2004) or erode (Farb *et al* 1996, Virmani *et al* 2000, Quillard *et al* 2017), exposing the blood to pro-thrombogenic factors. This causes thrombus formation, vessel occlusion, and ischemia in downstream tissues, the last of which is a common cause of myocardial infarction. One major hurdle in improving treatment of atherosclerosis (AS) is a lack of diagnostic imaging and therapeutic tools in the clinic.

One imaging modality with promise in this area is photoacoustics (Xu and Wang 2006, Wang and Yao 2016). Photoacoustics (PA) uses brief pulses of light to irradiate tissue. This causes localized heating, followed by a brief expansion of the imaging target with the consequent propagation of an ultrasonic wave. The pressure of the photoacoustic wave is linearly proportional to the optical absorption coefficient of the irradiated object, which makes it possible to attain contrast based on the endogenous optical properties of different tissue types. The most important endogenous absorber for imaging and diagnosis of atherosclerosis is lipid. Optical contrast between lipid and water is difficult to attain except for two regions in the near infrared. These two regions are near 1210 or 1720 nm (Jansen *et al* 2014), since at these wavelengths lipid has a higher absorption coefficient than the water-based tissue found in surrounding healthy vessel segments. Innovative catheters have been developed which incorporate a side firing optical fiber and ultrasonic transducer to image arteries from the lumen (Sethuraman

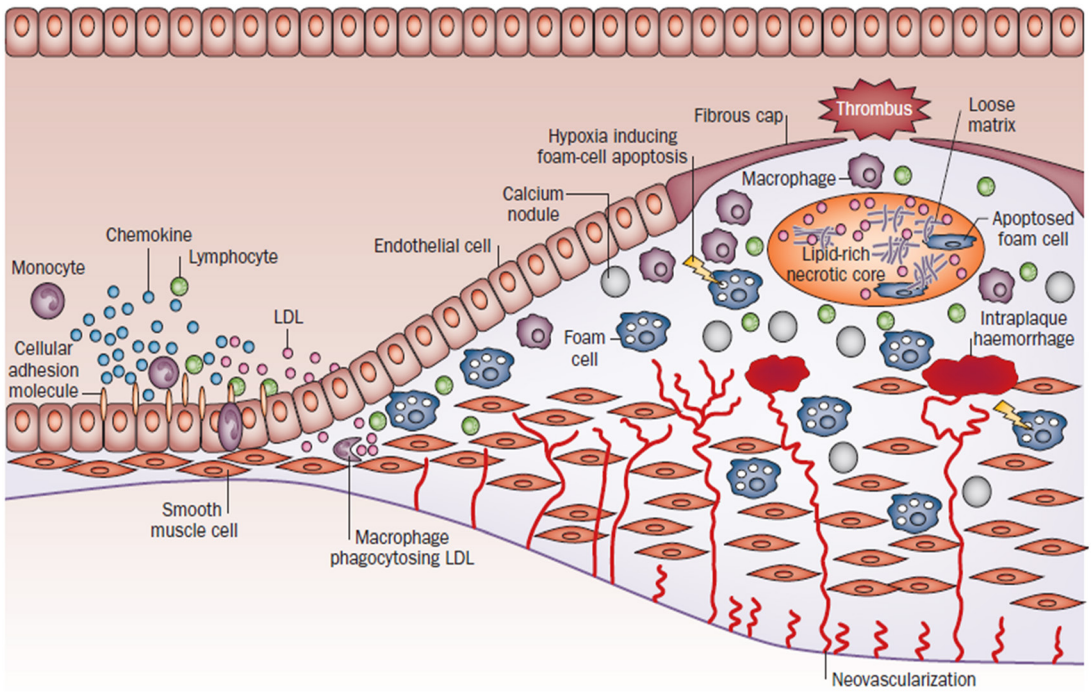

Figure 1. Development of an atherosclerotic plaque. Endothelial activation leads to chemokine production and expression of molecular markers on the surface of endothelial cells, which cause migration and adhesion of immune cells, including monocytes, to the endothelium. Increased endothelial permeability enables cells and LDL to cross into the vessel wall. Monocytes differentiate into macrophages, which take up modified LDL to form foam cells. Proinflammatory cytokines promote cell recruitment, smooth muscle cell proliferation and neovascularization. Both foam-cell formation and smooth-muscle proliferation cause a localized thickening of the vessel wall, which becomes a plaque. The fragile neovessels can bleed, causing intraplaque haemorrhage that can accelerate growth. Hypoxia and oxidative stress lead to foam-cell apoptosis and the formation of a lipid-rich necrotic core. Calcium is deposited within the plaque and a fibrous cap forms over the top of the plaque, shielding the thrombogenic content of the plaque from the circulation. As the plaque enlarges, it not only causes narrowing of the lumen, but can also lead to outward vessel-wall remodeling, a feature of plaques at high risk of rupture. The fibrous cap thins and the plaque eventually ruptures, which can lead to acute thrombosis and clinical events. High levels of inflammatory cells are found in the plaque at the time of rupture. Skeoch *et al* (2015) Copyright © 2015, Springer Nature. With permission of Springer.

et al 2007, Karpiouk *et al* 2010). This imaging technique is referred to as intravascular photoacoustic (IVPA) imaging (Wang *et al* 2010a, Jansen *et al* 2011, Wu *et al* 2016).

In the last decade, this technique has seen steady development. Multiple catheter designs have been developed, enabling small footprints and enhanced overlap of the ultrasound transducer and laser pulse footprint (Cao *et al* 2016), for example. Spectroscopic imaging has been used to differentiate lipid in plaques from adipose tissue (Wu *et al* 2015). Additionally, the combination of IVPA imaging with other imaging modalities (Abran *et al* 2014, Dai *et al* 2017, Mathews *et al* 2018) promises more accurate identification and characterization of atherosclerosis. Other more recent developments include real-time IVPA imaging (VanderLaan *et al* 2017) and *in vivo* real time imaging of non-native plaque in swine (Wu *et al* 2017).

Despite the advances in IVPA imaging instrumentation, imaging of native lipid is difficult due to the high attenuation of blood in the lumen at lipid relevant wavelengths. In addition, this modality is not inherently sensitive to all environmental markers present in the atherosclerotic plaque. Thus, another encouraging area of development is the use of imaging contrast agents. These are small agents, on the scale of a few to hundreds of nanometers, which can be used to enhance plaque identification and characterization. They can be broadly categorized as either dyes, inorganic particles (metallic and carbon-based), and organic particles (liposomes or micelles). It has been reported that many of these agents show preferential accumulation in areas of disrupted endothelium due to increased endothelial permeability (Kim *et al* 2014), although the mechanisms of transport have not been thoroughly studied in atherosclerosis (Lobatto *et al* 2011). These contrast agents have been used by a variety of researchers for atherosclerosis imaging. Molecular targeting of these agents using antibodies for specific molecular markers of atherosclerotic disease is common. Moreover, the use of nanoparticles as a therapeutic platform is also being increasingly explored.

A variety of imaging modalities are inherently compatible with IVPA due to similar hardware and instrumentation. The most obvious example is intravascular ultrasound (IVUS). Since acquisition of IVPA signals requires an ultrasound transducer, IVUS images are virtually always collected with IVPA images. This provides anatomical images that are automatically co-registered with the photoacoustic image. Researchers have also combined

IVPA imaging with other modalities. In one example, researchers combined IVUS, IVPA, and near infrared fluorescence (NIRF) imaging into a single catheter (Abran *et al* 2014). Catheters combining photoacoustic imaging and optical coherence tomography (OCT) have also been reported (Dai *et al* 2017, Mathews *et al* 2018). In this review, contrast agents used for these modalities will also be included in the discussion due to their potential to be used in combination with IVPA.

The focus of this review will be on the use of nanoparticles for IVPA imaging. The first section will discuss fundamental requirements a nanoparticle must meet before it could be applied *in vivo* for IVPA imaging. Next, applications of contrast agents for diagnostic imaging in IVPA will be discussed. The third section will cover therapeutic applications of nanoparticles in IVPA. The fourth section will discuss clinical translation of these particles, with a focus on clinical studies. The final section will specify our conclusions about the current state of research in this area, and suggest possible directions of future research.

Required properties of diagnostic and therapeutic agents for atherosclerosis

Imaging contrast and therapeutic agents for IVPA imaging have the potential to provide significant benefits for atherosclerotic patients. Successful therapeutic agents could reduce mortality and other disease symptoms, while contrast agents can significantly enhance signal from atherosclerotic plaques, improving detection. For example, depending on the characteristics and local concentration of a specific contrast agent, signal enhancements of over 10 fold have been obtained (Pan *et al* 2009). Such a signal increase is particularly valuable in IVPA imaging, since light is strongly attenuated at 1210 (due to hemoglobin and, primarily, water) and 1720 (due to water) nm before reaching the vessel wall.

Thus, the wavelengths at which a diagnostic or therapeutic agent absorbs or is activated is an important consideration during agent design or selection. Commonly, the first (650–950 nm) and second (1000–1300 nm) biological windows are considered ideal wavelength ranges for *in vivo* light penetration due to the lower absorption of hemoglobin and water (Weissleder 2001, Smith *et al* 2009). Agents should have strong absorption bands in these windows to be effective agents for IVPA imaging. However, agents with strong absorption near the lipid wavelengths should be avoided, since simultaneous imaging of endogenous chromophores would have diagnostic value. Finally, agents that absorb at 1064 nm can be imaged using the widely available Nd:YAG laser, making their translation simpler and more cost effective. This requirement is unique to IVPA imaging compared to photoacoustic imaging generally, because IVPA imaging requires higher power lasers with PRFs in the kHz range to achieve real-time imaging (Hui *et al* 2017, VanderLaan *et al* 2017, Wu *et al* 2017).

Wavelength selection is an important consideration when designing or selecting a contrast agent for IVPA imaging. However, diagnostic and therapeutic agents must first meet several other requirements before being useful for IVPA imaging *in vivo*. These include being deliverable to the imaging target, stability to irradiation, and safety. Each of these requirements will now be addressed separately.

Delivery

Nanoscale particles have been shown to enter the vessel wall through diseased endothelium as a result of increased permeability (Kim *et al* 2014). While the mechanisms involved in this method of delivery have not been well studied (Lobatto *et al* 2011), general conclusions about the optimal size of nanoparticles for atherosclerosis can be inferred from several studies. Lobatto *et al* synthesized liposomes carrying prednisolone (a therapeutic agent) (Lobatto *et al* 2015), and van der Valk *et al* showed that the particles propagate to human plaques after intravenous injection (van der Valk *et al* 2015). These particles were 100 nm $\pm$ 10 nm in diameter. Zhang *et al* (2011) were able to deliver particles with a diameter of 250 nm to plaques in cholesterol-fed rabbits. However, the particles were delivered efficiently to rabbits fed on a high-fat diet for seven months but not three months, indicating that less developed plaques are less permeable. The authors also showed delivery *ex vivo* using excised rabbit arteries and confirmed the results using human samples from patients who underwent carotid endarterectomy, strongly indicating that the mechanism of particle delivery was passive diffusion. Further, superficial delivery of 2 μm particles to the intimal portion of arteries in rabbits fed a cholesterol diet for 12 months was shown by histology. Ultimately, the authors conclude that the size of particles that penetrate the endothelial barrier may be a marker of disease severity. These studies suggest that contrast agents for IVPA should be 100–300 nm in diameter, although particles with a diameter up to 2 μm may have useful applications.

The lifetime of particles in the blood stream will also affect delivery to atherosclerotic plaques. An obvious result of this is that particles must be stable in the blood stream to be delivered to plaque. In addition, the renal system will rapidly clear very small particles (<5–10 nm) from the blood. Intuitively, studies have shown that shorter circulation lifetimes result in less particle accumulation at sites of arterial disease (Im *et al* 2016). However, one benefit of rapid clearance is reduced interaction with the body, which would improve particle safety. Particles have been designed to circumvent this tradeoff by using a larger carrier for smaller gold nanoparticles to increase the particle circulation time with the idea that the gold nanoparticles would be rapidly cleared by the

renal system after the carrier was metabolized (Pan *et al* 2009). Although very small molecules are rapidly cleared from the blood stream, this does not unequivocally preclude their accumulation in atherosclerotic plaques. For example, the dye indocyanine green (ICG) has been shown to collect in atherosclerotic plaques in sufficient quantity for imaging (Vinegoni *et al* 2011, Verjans *et al* 2016). In addition, particles with negative or neutral surface charge will have increased circulation time; this can be achieved, for example, by surface coating with polyethylene glycol (PEG) (Alexis *et al* 2008). Optimal contrast agents for IVPA imaging will have longer circulation times and have a small enough profile to diffuse across the disrupted endothelial layer into diseased vessel.

Another common technique to enhance delivery of particles to plaques is targeting the particles to molecular markers of atherosclerosis. This is achieved by conjugating targeting ligands, such as antibodies, to the surface of the nanoparticles. A multitude of markers of AS are widely known and can be used as targets. These include cell adhesion molecules (e.g. P-selectin, VCAM-1), macrophages, matrix metalloproteinases, angiogenesis (VEGF), and many others. However, targeting does have drawbacks including more complex synthesis, increased costs, and reduced stability in storage. Although targeting is not a guaranteed method for improving collection of particles in plaques (von zur Muhlen *et al* 2010), its clear benefit is to provide specific information about the disease state at the molecular level. Targeting contrast agents to an imaging agent is a common technique in the studies that will be discussed in this review.

Stability

IVPA imaging differs from other photoacoustic applications in that it requires many more light pulses to acquire a cross sectional PA image of the vessel. This is because such an image must be constructed by combining the multiple photoacoustic signals acquired while rotating and pulling back the catheter in the vessel. It is common to use over 100 photoacoustic signals to construct one IVPA frame. Since the laser pulses during rotation and pullback will overlap, constructing a 3D image of a vessel can easily result in a single location (or a small cluster of contrast agents) being irradiated hundreds of times.

Many contrast agents degrade after repeated irradiation. Dyes, such as ICG and fluorescin (Jonak *et al* 2011), are known to photobleach even in ambient light. Gold nanorods will deform after repeated irradiation at high fluence, which can change their absorption properties (Chang *et al* 1999). This is a major concern for IVPA imaging, since damaged contrast agents may lose their photoacoustic properties partway through the imaging session. Contrast agents for IVPA imaging will have to be tested more rigorously than other photoacoustic imaging agents to ensure their stability during real time clinical imaging.

One way to address this is to carefully select contrast agents that are known to have high stability. However, researchers have found various methods that improve the stability of some contrast agents. For example, the stability of ICG was found to improve when in close proximity to gold nanoparticles (Geddes *et al* 2003). Similarly, encapsulating ICG in calcium phosphate nanoparticles improved the half-life of ICG during continuous irradiation by 500% (Altinoglu *et al* 2008). Coating gold nanorods in a thin (~20 nm) layer of silica causes them to maintain their optical properties and shape when irradiated at fluences up to 20 mJ cm^{-2} (Chen *et al* 2010). Similar methods may be found in future to improve the stability of other contrast agents. Otherwise, only contrast agents verified to have high stability will be viable for IVPA imaging.

Safety

All nanoparticles injected into the body must be safe. Ideally, particles will be inert with respect to the general biological environment in the body. In practice, this varies widely depending on the type of nanoparticle used. For example, several small molecular dyes are already clinically approved, including ICG and methylene blue. These particles are rapidly cleared from the body by the renal system due to their small size. On the other hand, the toxicity of metallic nanoparticles is more complex. Metallic nanoparticles that are smaller than the renal threshold will still be rapidly cleared. However, the toxicity of larger metallic nanoparticles is dependent on a variety of factors including the particle size, shape, coating, surface charge, and surface functionalization (Fratoddi *et al* 2015, Imanparast *et al* 2015, Valdiglesias *et al* 2016, Feng *et al* 2018). Studies using particle modifications, such as encapsulation in spherical organic carriers or using inert surface coatings, to improve the particle safety profile will be discussed. Further research will likely continue both to investigate the effect various nanoparticles have on the body and to find further modifications to improve their safety profile.

Contrast agents for diagnostic imaging

There are a variety of contrast agents that could be applied to IVPA imaging (Table 1). These contrast agents have been split into three categories. The first category consists of small (<2 nm), single molecule contrast agents. The second category includes inorganic nanoparticles. These are metallic or carbon-based particles that vary in size from several nanometers up to hundreds of nanometers. The third category consists of organic particles, meaning liposomes or micelles, which are often used as delivery vehicles for other imaging or therapeutic agents.

Small molecule contrast agents

Small molecule contrast agents for IVPA imaging consist of dyes. Examples of these dyes include ICG, IRDye800CW, AlexaFluor, and methylene blue. Their small size (<2 nm) causes them to be rapidly cleared from the body by the renal system, reducing the time they can interact with the body. The FDA has approved a number of dyes for human use, including ICG and methylene blue. These dyes each have absorption bands at near infrared and visible wavelengths. Free ICG has a primary absorption band at 790 nm (Benson and Kues 1978). However, this absorption peak will shift to 820–830 nm in biological solutions such as blood plasma (Landsman *et al* 1976). The absorption peaks for the other dyes mentioned above are 774 nm for IRDye800CW (Marshall *et al* 2010), 346–784 nm for AlexaFluor (ThermoFisher Scientific 2018), and 677 nm for methylene blue (Song *et al* 2008).

Another trait of many dyes is that they exhibit fluorescence (e.g. ICG). Inherently, there is a tradeoff between the quantum efficiency for fluorescence and the effectiveness of a contrast agent's PA signal generation, since the energy from an absorbed photon must be dissipated in one way or the other. However, this indicates the potential to use these particles for multimodal imaging. Imaging catheters capable of ultrasound, photoacoustic, and fluorescence imaging have already been mentioned that would be able to identify ICG using the combined modalities (Abran *et al* 2014). Finally, many of these dyes are prone to photobleaching after repeated irradiation. As mentioned earlier, researchers have determined several techniques to stabilize certain dyes (Geddes *et al* 2003, Altinoglu *et al* 2008).

Out of all the dyes mentioned, only ICG has been shown to naturally collect in atherosclerotic plaques, presumably due to its lipophilic properties (Vinegoni *et al* 2011). Using fluorescence imaging, ICG has been shown to propagate to atherosclerotic plaque in rabbit models (figures 2(A)–(E)) of atherosclerosis and in a small clinical study of 4 humans (Vinegoni *et al* 2011). Another study using fluorescence confirmed the tendency of ICG to accumulate in human plaque following coronary endarterectomy and *ex vivo* imaging (Verjans *et al* 2016). Intriguingly, ICG tended to accumulate in regions of macrophages, lipid, and even intraplaque hemorrhage in humans, indicating ICG's potential ability to identify plaques that may be prone to rupture. In this work, ICG was also injected into atherosclerotic models of swine and successfully imaged at plaque locations using NIRF-OCT *in vivo*, further illustrating the clinical viability of this technique.

However, PA imaging of ICG in the vessel wall must possible for it to be a useful contrast agent for IVPA imaging. This has been shown more recently (Bui *et al* 2017). Researchers loaded macrophages with ICG *in vitro*, and then injected them into the arterial wall of swine vasculature *ex vivo*. Using a catheter with simultaneous rotation and pullback, a 3D image of the vessel was constructed (figures 2(F)–(H)). The ICG-loaded macrophages were clearly identifiable.

An additional attribute of ICG is that in high concentrations it will form aggregates that shift its absorption peak to 900 nm (Landsman *et al* 1976). These aggregates are broken apart when they are phagocytosed, which shifts the absorption peak of the ICG back near 800 nm. This was leveraged by incorporating the ICG aggregates into lipophilic shells, which in turn were targeted to folate receptor beta (Harris *et al* 2017). The idea was that folate receptor beta is more highly expressed in activated macrophages, which are present in plaques prone to rupture. These particles were injected into apolipoprotein E knockout (apoE-/-) mice, which are a well-established murine model of atherosclerosis (Meir and Leitersdorf 2004), and normal mice. They had higher uptake in the heart and arteries of the apoE-/- mice than the normal mice. ICG's propensity to naturally collect in plaques, the potential to use ICG as a dual modality agent, and the ability for the absorption peak of ICG to shift while in different states makes it a versatile contrast agent for IVPA imaging.

Although only ICG was found to naturally collect in atherosclerotic plaques during the literature search for this review, it is possible that other dyes could also be shown to naturally collect in plaques. If not, then other dyes could still be used in IVPA imaging, for example, by incorporating them into liposomes or micelles. The fact that some dyes have received FDA approval for human use will continue to generate interest in their application as IVPA contrast agents.

Inorganic contrast agents

Gold contrast agents

Gold nanoparticles are widely used as contrast agents in biomedical imaging (Fratoddi *et al* 2015, Li and Chen 2015, Carneiro and Barbosa 2016, Spivak *et al* 2016, Amendola *et al* 2017, Varna *et al* 2018). A major advantage of these particles is their strong absorption and scattering properties. Like other plasmonic nanoparticles, their optical properties are the result of free electrons on their surface. These electrons oscillate in concert when stimulated by light. In the case of nanosized plasmonic nanoparticles, this oscillation is distributed over the entire volume of the particle and is referred to as localized surface plasmon resonance (Amendola *et al* 2017). This property, and the consequent strong optical absorption, has contributed to the popularity of gold nanoparticles as a contrast agent for IVPA imaging.

Gold nanoparticles can also be synthesized with absorption peaks across a wide wavelength range in the NIR. This 'tunability' is a result of changes to the size and shape of the gold nanoparticles. Spheres and rods are the

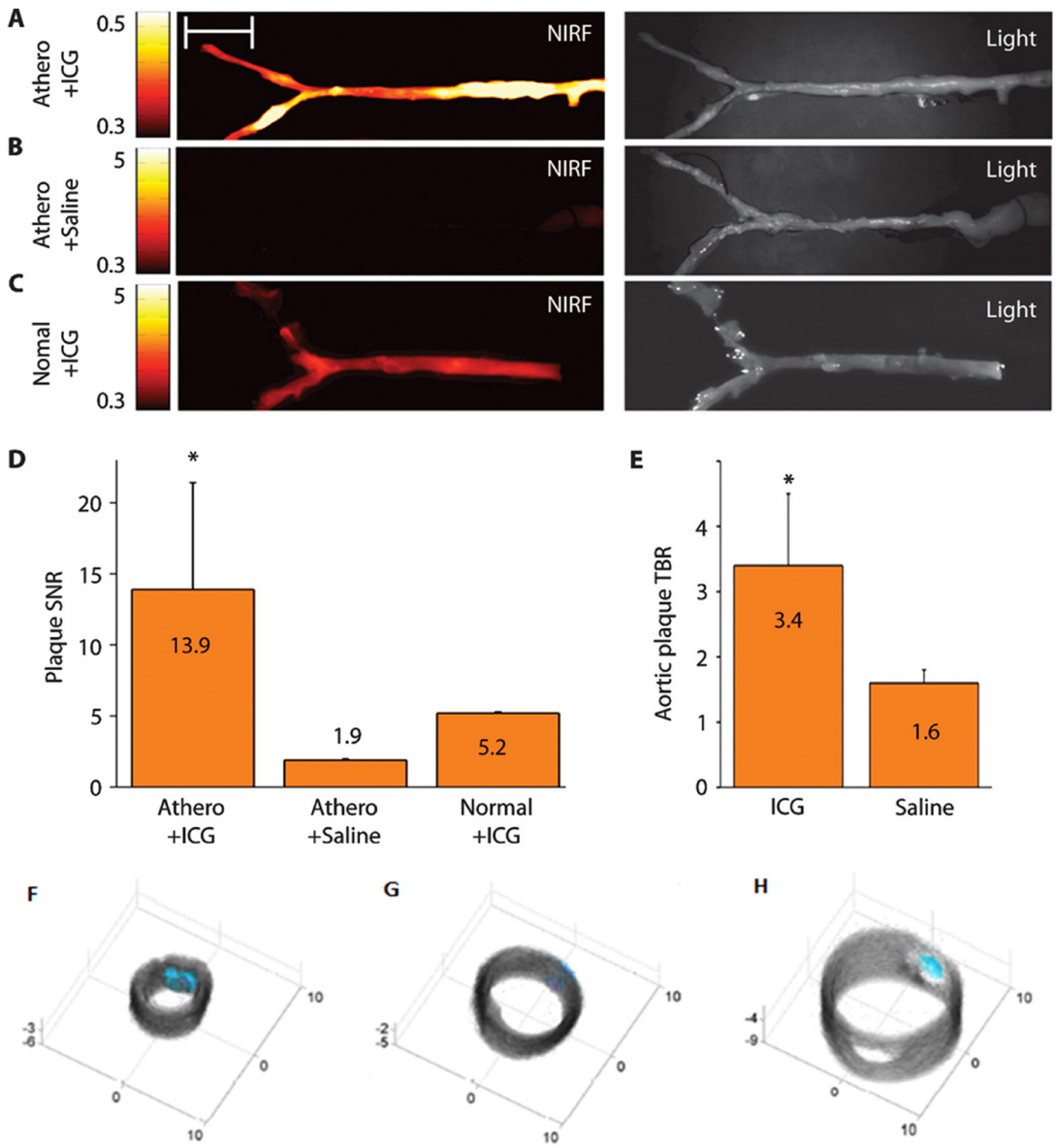

Figure 2. ICG rapidly targets atheroma in rabbit arteries and provides NIRF signal enhancement. Atheroma bearing or normal rabbits were injected with either ICG or saline and then killed after 45 min. (A)–(C) *Ex vivo* fluorescence reflectance images of aorta obtained at 800 nm (left) and white light images (right) for (A) atherosclerotic rabbits injected with ICG (B) atherosclerotic rabbits injected with saline (C) normal rabbits injected with ICG. Scale bar 2 cm. (D) signal to noise ratio in atheroma bearing ICG-injected animals was significantly higher than in control groups (E) Plaque target-to-background ratios were significantly higher than the saline control group. From Vinegoni *et al* (2011). Reprinted with permission from AAAS. (F)–(H) 3D volumetric IVUP images of atherosclerotic plaque-mimicking tissues with ICG-loaded macrophages injected in diameters of (F) 8 mm (200 μl injection), (G) 12 mm (50 μl injection), and (H) 16 mm (100 μl injection). Reproduced from Bui *et al* (2017).

simplest shapes. However, other shapes include shells, prisms, cages, stars, and vesicles (Li and Chen 2015). Most gold nanoparticles reported in the literature have absorption peaks within the biological windows. In addition, gold nanoparticles can be tuned to 1064 nm, at which the Nd:YAG laser can be used for real time imaging. This would make translation to real time imaging simpler and more cost effective. However, the full range of reported maximum absorption peaks is much wider (Li and Chen 2015), with peaks as high as 2200 nm being reported for the more complex shapes. Unfortunately, many of these particles have sizes over several hundred nanometers, which may preclude their use in IVPA imaging due to reduced delivery to disease sites.

As explained previously, delivery of the agent to disease sites is a significant issue in using contrast agents for IVPA imaging. One common technique is to use antibody targeting of molecular markers of atherosclerosis. A variety of examples of techniques with gold nanoparticles are in the literature. For example, gold nanorods were used to pinpoint inflamed cells in culture (Kim *et al* 2007). The particles were targeted to intracellular adhesion molecule (ICAM)-1, an inflammatory marker, and put in culture with human umbilical vein endothelial cells. A subset of cells was then exposed to the proinflammatory cytokine interferon gamma. Photoacoustic images

could differentiate between the inflamed and control (no cytokine treatment) cells based on the increased presence of gold nanoparticles in the former. These targeting techniques have also been applied to *in vivo* mouse models of atherosclerosis. In one example, nanoshells targeted to vascular cell adhesion molecule (VCAM)-1 were injected into apoE-/- mice (Rouleau *et al* 2013). PA imaging signal was higher in the atherosclerotic mice compared to control mice only minutes after injection. The authors also injected these gold nanospheres into mice at high concentration (OD 200) and checked for cytotoxicity after 24 h and 7 d. A veterinary pathologist was not able to detect visible signs of toxicity, which is a positive indicator of the safety of these particles. Due to its place in disease progression, molecular inflammation markers are a desirable target for imaging when looking for the presence of atherosclerosis.

The ability of researchers to tune gold nanoparticles to desired absorption bands has also made it possible to separately label multiple molecular markers. Bayer *et al* (2011) used gold nanorods with absorption peaks at different wavelengths to target different cell types. Their targets were A431 cells or MCF7 cells, two cancer cell lines. A431 cells were labeled using nanorods with an absorption peak at 830 nm and conjugated to antibodies targeted to EGFR. MCF7 cells were labeled using nanorods with an absorption peak at 780 nm and conjugated to antibodies targeted to HER2. Regions containing different types of cells in a phantom could be differentiated using multispectral photoacoustic imaging. In a study with closer application to IVPA imaging, Ha *et al* (2011) were able to target inflammatory markers in human umbilical vein endothelial cells. A set of cells were selectively inflamed with inflammatory cytokines which induced expression of ICAM-1 and E-selectin. Two sets of gold nanorods, tuned to different wavelengths and each conjugated to an antibody targeted to either ICAM-1 or E-selectin, were introduced to the cell culture. Photoacoustic imaging confirmed delivery of the nanorods to the inflamed cells. Further, the PA intensity was shown to track relatively well with rt-PCR quantification of the inflammatory markers in the cells across multiple time points, strengthening the argument for clinical translation of such techniques. Multispectral targeting could be particularly useful in determining patient risk with IVPA imaging in the future, since atherosclerosis is correlated with a large number of diverse molecular markers.

Another approach for improved delivery is to target inflammation at the cellular level by using macrophages as an imaging target. In one study (Wang *et al* 2009), macrophages loaded with gold nanopheres were imaged in a phantom using IVPA imaging. Successful imaging of the particles was also completed after direct injection of the loaded macrophages into the artery of a diseased rabbit *ex vivo*. This has also been done using a balloon injured rabbit model of atherosclerosis (Wang *et al* 2010b). Gold nanospheres with a diameter of 20 nm were shown to accumulate in macrophages, with delivery enhanced by conjugation with targeted antibodies. The gold nanospheres could be detected using IVPA imaging. Similarly, AuNRs were injected both in one balloon-injured and one Watanabe rabbit model of atherosclerosis (Yeager *et al* 2012). The presence of AuNRs was confirmed in both rabbits with photoacoustic imaging and spectral unmixing. Gold nanoparticles were also successfully detected through blood, which significantly attenuates PA signal (figure 3). Histological analysis indicated that gold nanoparticles propagated through regions of disrupted endothelium and then were consumed by macrophages. The direct delivery of untargeted nanorods shown in this study makes these contrast agents appealing, since as stated before it simplifies nanomaterial synthesis and improves nanomaterial stability in storage.

Hybrid Gadolinium(III)-gold nanorods were developed for dual use in magnetic resonance imaging (MRI) and PA (Qin *et al* 2013). The idea is that MRI could be used to initially locate plaque, and then IVPA imaging could be used to ascertain a more accurate characterization of plaque characteristics. The particles were detectable by both modalities *in vitro*. The authors then injected the particles into the back of the mice. Again, the particles were detectable using either PA or MRI. Finally, the particles were injected into an otherwise healthy rabbit vessel *ex vivo* and successfully imaged using both MRI and an intravascular catheter (figure 4). Multimodality contrast agents such as these would be particularly useful, since it makes it possible to use more cost-effective imaging techniques for prescreening of patients before imaging with IVPA, which is more invasive.

A primary concern about the use of gold nanoparticles is potential cytotoxicity. The ultimate safety of gold nanoparticles is unclear, with a variety of studies indicating conflicting results. In one example (Shukla *et al* 2005), it was found that gold nanoparticles were not toxic to macrophages, and in fact reduced the presence of reactive oxygen and nitrite species. Further, the expression of inflammatory cytokines tumor necrosis factor alpha and interleukin 1 beta were not detected. Conversely, another study (Falagan-Lotsch *et al* 2016) using both gold nanorods and nanospheres in human dermal fibroblasts found changes in gene expression even 20 weeks after only acute exposure to gold nanoparticles. These are just two examples of the types of conflicting indications in the literature.

One confounding issue is that toxicity is dependent on a large array of variables, including the nanoparticle size, shape, coating, surface charge, and surface functionalization (Fratoddi *et al* 2015). Further studies will be necessary to determine if gold nanoparticles can be used safely in the clinic without long term side effects (Fratoddi *et al* 2015, Carneiro and Barbosa 2016). Researchers will continue to have a variety of tools (e.g. modifications to size or shape, the use of nanoshell carriers, surface functionalization) at their disposal to improve

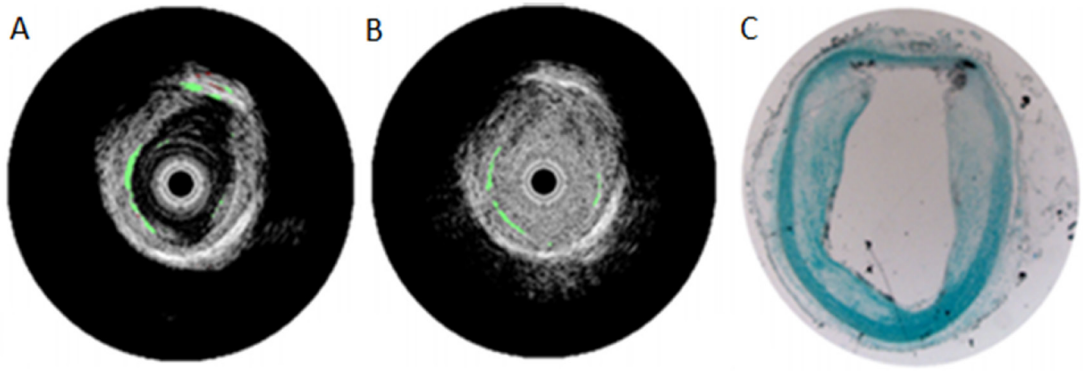

Figure 3. Intravascular photoacoustic imaging of gold nanorods (AUNR) labeled atherosclerotic plaque cross section. (A) and (B) Intravascular ultrasound (gray) and photoacoustic (green) signal obtained from imaging at the AUNRs peak absorbance wavelength through saline (A) or blood (B). (C) Corresponding silver stain histology revealing distribution of AUNRs within the plaque. Reproduced with permission from Yeager *et al* (2012). © 2012 Society of Photo-Optical Instrumentation Engineers (SPIE).

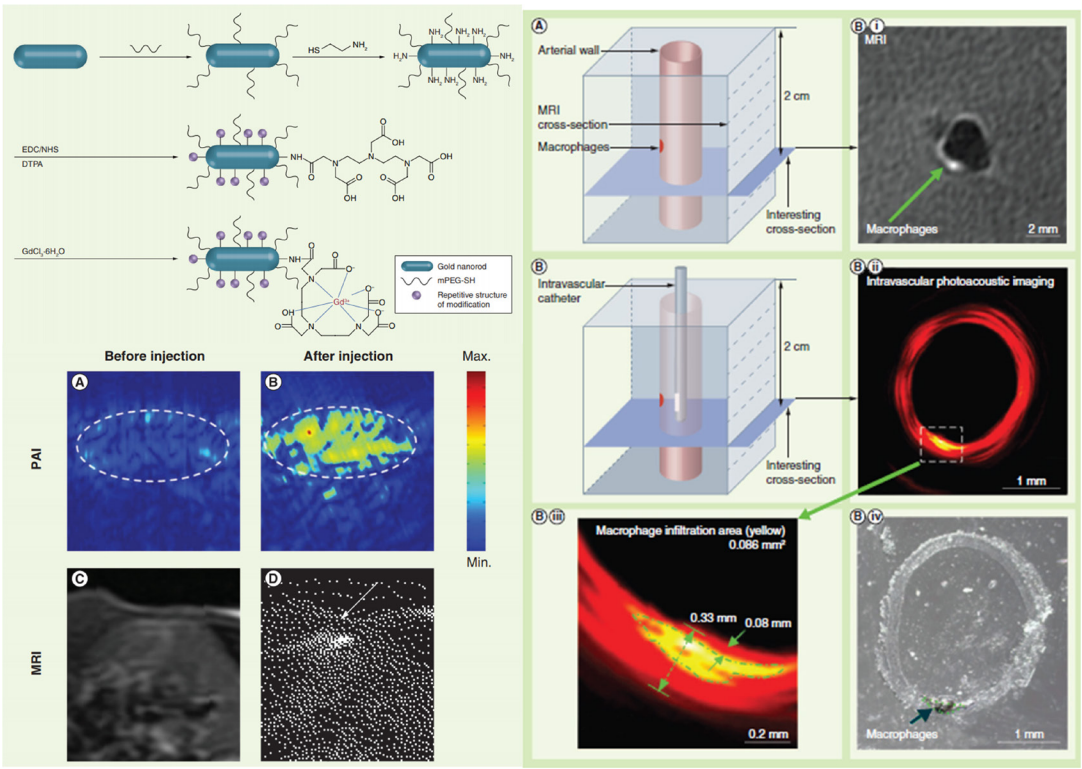

Figure 4. (top left) Fabrication procedure of GD^{III}-gold nanorods. (bottom left) *In vivo* dual modality imaging of macrophages labeled with gadolinium(III)-gold nanorods in a mouse. (right) Quantification of the extent of macrophage infiltration/invasion in a macrophage-rich model by combined MRI and intravascular photoacoustic imaging. Reproduced with permission from Qin *et al* (2013). © Future Medicine Ltd.

nanoparticle safety. Nonetheless, this topic will be a continued area of interest for researchers throughout the biomedical imaging field due to the wide use of gold nanoparticles.

Gold nanoparticles have significant advantages as contrast agents in IVPA imaging. These advantages include large increases in PA signal, natural accumulation in plaques without targeting, easy synthesis, simple targeting to molecular markers, and tunable absorption peaks. Further innovations in their use, such as silica coating to improve stability (Chen *et al* 2010), will likely be discovered due to their wide use in biomedical imaging research. Papers which transparently describe their synthesis also make them increasingly accessible (Manohar *et al* 2011, Kinnear *et al* 2013, Xu *et al* 2014, Scarabelli *et al* 2015). In sum, the advantages of using gold nanoparticles indicates that research using these particles as IVPA contrast agents will continue.

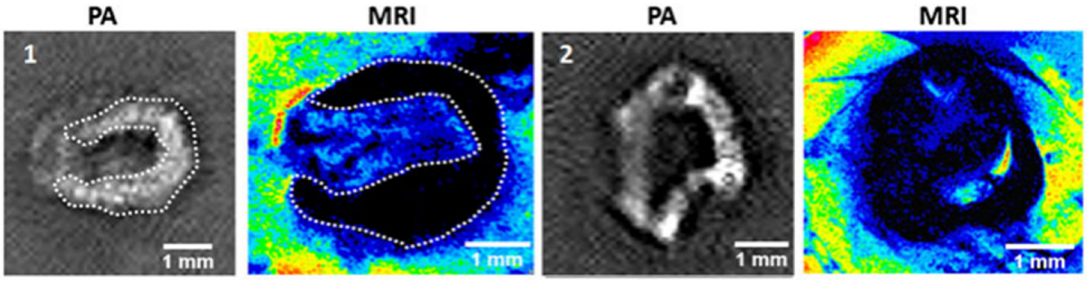

Figure 5. Photoacoustic and MR image comparison of all resected lymph nodes with contrast injection. As shown in lymph node 1 (white dotted line), the PA response pattern is comparable with the location of MRI signal decrease. Grootendorst *et al* (2013). John Wiley & Sons. Copyright © 2012 John Wiley & Sons, Ltd. CC BY 4.0.

Iron oxide contrast agents

Nanoparticles of iron oxide also have potential as contrast agents for IVPA imaging. They generally range in size from a few nanometers to 150 nanometers and have strong absorption properties (Imanparast *et al* 2015). Iron oxide nanoparticles are widely used in MRI. Indeed, some commercial products, such as Endorem®, have already been approved for clinical use (Wang 2011).

The use of iron oxide nanoparticles as photoacoustic contrast agents has been proven. One of these products, Endorem®, was shown to be an effective contrast agent for PA imaging (Grootendorst *et al* 2013). Iron oxide nanoparticles could be detected using photoacoustic imaging in non-metastatic lymph nodes of mice, but not in metastatic lymph nodes (figure 5). The presence of the iron oxide nanoparticles was confirmed with both MRI and histology. Recent work confirmed the viability of dual modality nanoparticles by using iron oxide nanoparticles surrounded with carboxy-terminated poly(D,L-lactide-co-glycolide)-*block*-poly(ethylene glycol) and loaded with a near infrared (NIR) fluorescent dye (Armanetti *et al* 2018).

The natural collection of iron oxide nanoparticles to sites of atherosclerosis has already been demonstrated. For example, preferential iron oxide nanoparticle deposition to areas of inflammation was shown using histology (Litovsky *et al* 2003). Three mouse models were used: apoE-/- knockout mice that had been injected with inflammatory cytokines, apoE-/- knockout mice that received a sham injection of saline, and healthy wild-type mice. Mice treated with inflammatory cytokines had nearly 4 times as many iron containing macrophages as the sham group. No iron was detected in the wild-type healthy mice. More importantly, preferential collection in plaques has also been shown in pilot clinical studies with small numbers of patients. Trivedi *et al* (2004) completed a pilot study on 8 patients undergoing carotid endarterectomy. Ultrasmall superparamagnetic iron oxide nanoparticles (USPION) were injected and shown to accumulate in macrophages in seven out of eight of the patients using MRI. In a similar study, Kooi *et al* (2003) tested USPIOs on eleven patients undergoing the same procedure. Arteries were analyzed on a segment by segment basis, with USPION detection also being achieved using MRI. USPION detection was not as high in this study, although histology revealed USPIONs in 27 of 36 ruptured and rupture prone plaques and in only 1 out of 14 stable lesions. The photacoustic response of iron oxide agents combined with their natural tendency to accumulate in plaque make them viable contrast agents for IVPA imaging.

Like gold nanoparticles, iron oxide nanoparticles have also been targeted to inflammation using conjugated antibodies. Recently, USPIONs targeted to VCAM-1 were used to identify inflammation in apoE-/- mice (Michalska *et al* 2012). The increased presence of the USPIONs was detected using MRI. An increase in nanoparticle presence relative to both untargeted USPIONs in apoE-/- mice and both targeted and untargeted USPIONs in healthy mice was shown. This result was confirmed with histology and stokes raman scattering microscopy. Besides molecular or cellular markers of atherosclerosis, iron oxide has been shown to propagate and adhere preferentially to cholesterol crystals when bound to β-Cyclodextrin (Li *et al* 2012). The idea was validated *in vitro* and then shown to occur in Watanabe arteries *ex vivo*.

One unique property of iron oxide nanoparticles is their ability to be selectively heated by the application of electromagnetic fields, which results in enhanced photoacoustic contrast (Feng *et al* 2014). Referred to by the authors as thermally modulated photoacoustic imaging, this gives researchers an additional tool for increasing sensitivity to iron oxide nanoparticle presence using photoacoustic imaging. However, the safety of this method would need to be evaluated before clinical use.

The toxicity of iron oxide nanoparticles can be dependent on a variety of factors including size, coating, dosage, cell type, and exposure time (Valdiglesias *et al* 2016, Feng *et al* 2018). Reviews in the literature discuss this issue in more depth (Imanparast *et al* 2015, Valdiglesias *et al* 2016, Feng *et al* 2018). However, the use of iron oxide nanoparticles in clinical applications, primarily for MRI imaging, (Wang 2011, Iv *et al* 2015) indicates that toxicity concerns can likely be overcome when using iron oxide for IVPA imaging.

Iron oxide nanoparticles have similar benefits to gold nanoparticles. These are a strong increase in PA signal, natural propagation to sites of atherosclerosis, and the availability of multiple modifications to improve safety. Iron oxide nanoparticles have the additional advantages of being multimodal contrast agents, and being available in clinically approved formulations. As a result, iron oxide nanoparticles have strong potential as contrast agents for IVPA.

Single walled carbon nanotubes

Single walled carbon nanotubes (SWNTs) have a large and broad absorption spectrum, which makes them useful photoacoustic contrast agents (Moore *et al* 2003). This includes absorption in the 1064 nm range, which would make them directly usable in real time imaging with widely available Nd:YAG lasers. In addition, hybrid SWNTs have been shown to collect in the tumor microvasculature in mice (De La Zerda *et al* 2008). The tubes were 50–300 nanometers in length and 1–2 nm in diameter. Nanoparticles penetrate tumor microvascular through enhanced permeability (Greish 2010) in the vessel wall, so it is possible that these particles would also penetrate atherosclerotic vessels. In addition, SWNTs could be incorporated into carrier particles for delivery to plaque.

Hybrid nanoparticles have been reported in which the SWNTs were coated in a gold layer (Kim *et al* 2009), and used for photoacoustic imaging. This enhanced the SWNT absorption properties while also enhancing the ability of the SWNT to be conjugated with targeting antibodies. In addition, ICG has been conjugated to SWNTs which resulted in a 20 fold increase in optical absorption (Zanganeh *et al* 2013). If SWNTs are shown to be deliverable to atherosclerotic plaques, they could also be a viable contrast agent for IVPA imaging.

Copper sulfide contrast agents

Copper sulfide nanomaterials also have strong absorption bands and can be used for photoacoustic imaging. They have an absorption band near 970 nm, although this can be altered by changing the ratio between $CuCl_2$ and Na_2S during synthesis (Ku *et al* 2012). The resultant absorption peaks vary from 1000 to 1500 nm. However, these absorption peaks are very broad so photoacoustic imaging could realistically be achieved using wavelengths from 800 to 2000 nm or wider. This also means copper sulfide agents are one of the few types of agents that can be imaged with an Nd:YAG laser.

The size of many copper sulfide particles used for photoacoustic imaging is near 11 nm, which is close to the renal clearance threshold. Thus, it is not clear how easily these particles would collect in atherosclerotic plaques. Literature was not found in which this was tested directly. However, delivery by encapsulation in other nanoscale particles, such as nanodroplets (Santiesteban *et al* 2017), is likely to be a successful approach based on past success with this method using other cargo (van der Valk *et al* 2015).

Organic contrast agents

Liposomes and micelles

Liposomal and micelle like structures are spherical organic contrast agents. Liposomes have a double layer of phospholipids enclosing a hydrophilic core. They vary in size from fifty to thousands of nanometers. Micelles have a single layer of phospholipids and so have a hydrophobic core. Micelles are smaller than liposomes, with sizes from 2 to 20 nm. These agents are highly customizable. They can be used as carriers for strong photoacoustic absorbers. Hydrophobic cargo can be encapsulated in the bilayer of liposomes or the internal shell of micelles. Hydrophilic cargo can be encapsulated at the center of the liposome. In addition, these agents can be surface modified, for example with PEG, to improve biocompatibility. Conjugation of the PEG layer with a targeting antibody is also possible. Encapsulation of very small, highly absorbing nanoparticles that would otherwise be rapidly cleared by the renal system to improve delivery or to reduce toxicity can be used effectively with these structures.

One application of these structures to atherosclerosis is the liposomal agent Visudyne®. Visudyne injected into apoE-/- mice naturally collects in the vessel wall (Jain *et al* 2016). This study indicates that liposomal structures with a variety of potential cargos could be successfully delivered to atherosclerotic plaques.

Microbubbles for intravascular ultrasound imaging

A common contrast agent based technique is contrast enhanced ultrasound. This technique uses injections of microbubbles into the vasculature to improve image quality during IVUS imaging (Lindner 2009, Schinkel *et al* 2016). Since IVPA imaging requires the presence of an ultrasound transducer for collection of the photoacoustic signal, contrast enhanced ultrasound could be used synergistically with IVPA imaging. The advantage of these agents is that IVUS is already used in the clinic. However, the particles are generally too large to diffuse into plaque, and instead adhere to the lumen wall.

As with other contrast agents, researchers have used inflammation targeted ultrasound-based contrast agents to enhance imaging of atherosclerosis. A variety of results have been achieved in apoE-/- mice. These include preferential binding to plaque regions using VCAM-1 targeted microbubbles (Kaufmann *et al* 2007) or P-selectin targeted microbubbles (Kaufmann *et al* 2010) and detection of a decrease in VCAM-1 targeted microbubbles in

Table 1. List of photoacoustic nanoparticles with applications to IVPA imaging and their properties.

	Size	Absorption peak	Natural plaque delivery	Other attributes	Refs
Indocyanine green	<2 nm	790–820 free; 900 J-aggregates	Y, in humans	Clinically approved products; fluorescent	Landsman *et al* (1976) and Benson and Kues (1978)
IRDye800CW	<2 nm	774 nm	U	Fluorescent	Marshall *et al* (2010)
AlexaFluor	<2 nm	346–784 nm	U	Fluorescent	ThermoFisher Scientific (2018)
Methylene blue	<2 nm	677 nm	U	Fluorescent	Song *et al* (2008)
Gold spheres	2–100 nm	520–600	Y, rabbit *in vivo*	—	Wang *et al* (2010b)
Gold nanorods	Tens to hundred nm length; several to tens of nm diameter	650–1400	Y, rabbit *in vivo*	—	Kim *et al* (2007), Bayer *et al* (2011), Ha *et al* (2011) and Xu *et al* (2014)
Gold nanoshells	45 nm	710 nm	U	—	Rouleau *et al* (2013)
Gadolinium(III) gold nanorods	60 nm length; 20 nm width	700 nm	U	Dual modality; PA + MRI	Qin *et al* (2013)
Iron oxide nanoparticles	2–150 nm	500–750 nm	Y, in humans	Clinically approved products	Wang *et al* (2001), Grootendorst *et al* (2013) and Armanetti *et al* (2018)
SWNT-gold coated particles	100 nm length; 11 nm width	520–530 nm	U	—	Kim *et al* (2009)
SWNT + ICG particles	100–1000 nm	750 nm	U	—	Zanganeh *et al* (2013)
CuS particles	~11 nm	1000–1500 nm	U	Wide absorption band	Zanganeh *et al* (2013)

Y: Yes; U: Unknown.

mice treated with statins (Khanicheh *et al* 2013). These techniques have also been applied to higher animal models of atherosclerosis. Microbubbles have also been used to detect the early expression of P-selectin and VCAM-1 in adult male rhesus monkeys fed on a high-fat diet over two years (Chadderdon *et al* 2014), to simultaneously target two disease markers (ICAM-1 and antifibrinogen) in atherosclerotic swine models (Demos *et al* 1999), and to detect plaques with greater neovascularization in atherosclerotic rabbits using microbubbles targeted to VEGFR-2 (Liu *et al* 2011).

Therapy

One exciting area of research for contrast agents in IVPA is therapeutics (Tang *et al* 2012). The primary goal is to arrest or regress the disease state in a targeted manner. Using theranostic IVPA-compatible agents, sites of atherosclerotic disease could be simultaneously imaged and treated in one session. Further, the effectiveness of therapy could be monitored with IVPA imaging, either in the clinic or in studies that evaluate the efficacy of new therapeutics. Multiple studies have explored the use of therapeutic contrast agents that would be compatible with IVPA imaging. Generally, these can be split into two larger categories: photodynamic and photothermal therapy.

Photodynamic therapy

Photodynamic therapy involves the delivery and stimulation of a light activated drug, referred to as a photosensitizer. Since IVPA imaging already requires delivery of light, photodynamic therapy is highly compatible with IVPA imaging. In photodynamic therapy, the photosensitizer is stimulated at a unique wavelength of light (Lucky *et al* 2015). Upon stimulation, some of the excited elections will enter a triplet state (Castano *et al* 2004). At this point, the excited electron can damage the surrounding biological material by the production of reactive oxygen species (Lucky *et al* 2015).

Photodynamic therapy is an accepted form of cancer treatment (Castano et al 2004, Huang 2005, Lucky *et al* 2015). Its advantages include no long-term side effects, that it is less invasive than surgery, takes a short amount of time, can be precisely targeted, and can be repeated at the same site. Its disadvantages include that it can only be used to treat locations where light will penetrate, it leaves people light sensitive shortly after treatment, and it cannot be used in patients with certain blood diseases. However, these disadvantages are not as applicable to IVPA imaging. For one, light penetration is not an issue since the light will be delivered intravenously via the catheter. In addition, it is reasonable to expect that light sensitivity will be less of an issue to patients if the photosensitizer

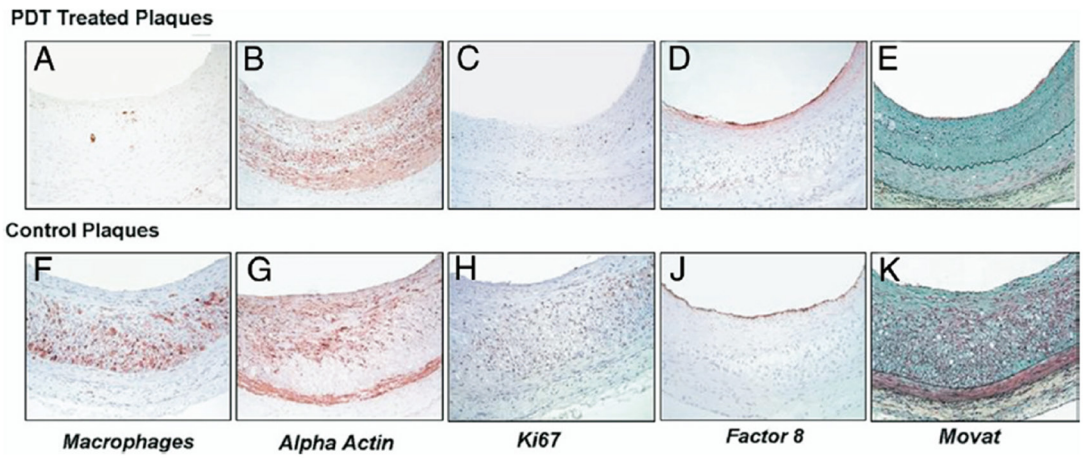

Figure 6. Photodynamic therapy (PDT) simultaneously reduced macrophages (A) and (F) and replaced existing plaque matrix with quiescent smooth muscle cells (B) and (G) in the non-G2 or S phases (C) and (H) as indicated by the Ki67 cell proliferation marker. After endothelial denudation of all rabbit arteries, staining with Factor VIII revealed the presence of endothelium in PDT-treated arteries and controls (D) and (J) indicative of arterial repair by 28 d. Panels (E) and (K) show the presence of the internal elastic membrane and external elastic membrane with Movat's stain. Note that plaque area is significantly less than controls at 28 days after PDT. Magnification ×200. Adapted from Waksman *et al* (2008), Copyright 2008, with permission from American College of Cardiology Foundation. Published by Elsevier Inc. All rights reserved.

is efficiently delivered only to sites of atherosclerosis. This is because these sites will be deeper in the body where light will not penetrate as effectively, as opposed to current treatments sites which are often located near body surfaces. Multiple studies have investigated the use of photosensitizers for the treatment of atherosclerosis.

As with contrast agents for imaging, researchers have used molecular targeting of inflammation to treat atherosclerosis, for example by killing inflammatory murine macrophages (McCarthy *et al* 2010). Vascular smooth muscle cells, which have been shown to be relevant to atherosclerosis pathogenesis (Chistiakov *et al* 2015, Bennett *et al* 2016, Grootaert *et al* 2018), have been killed *in vitro* using chlorin e6 (Wawrzynska *et al* 2010) and liposomal Zn(II)-phthalocyanine (Magaraggia *et al* 2006). Photosensitizers have also been used successfully in mice to induce macrophage apoptosis using Visudyne®, which consists of the photosensitizer verteporfin contained in a liposome (Jain *et al* 2016). More recently, ICG has been successfully used for photodynamic therapy in rats (Lin *et al* 2018), illustrating its potential both as an imaging and therapeutic agent. This is different from the other photosensitizers discussed in this section, which would need to be combined with IVPA imaging using either endogenous or exogenous chromophores to identify areas of the artery which required treatment.

Photodynamic therapy has also been applied successfully in larger animal models of atherosclerosis, such as rabbits and swine. In one example, lutetium texaphyrin was able to eradicate plaques in rabbits with diet induced atherosclerosis (Woodburn *et al* 1996). Similarly, using photodynamic therapy with the photosensitizer PhotoPoint, balloon-denuded rabbits fed on a high fat diet were treated. Macrophage content was shown to have dropped 92% a full 28 days later (figure 6), which had been repopulated with smooth muscle cells (Waksman *et al* 2008). Similarly, photoactivation of motexafin lutetium in balloon-denuded rabbits showed a reduction of macrophage content in treated plaques after two weeks (Hayase *et al* 2001). Finally, a long-term study used photofrin with irradiation at 630 nm using 120 J cm^{-2} on swine (Cheung *et al* 2004). One group received both photofrin and light treatment, another group received only the drug, while a third was exposed only to light. The pigs were sacrificed at either three or six months. The first group showed a 30% reduction in intimal area compared to only 6% in control group, indicating a long term positive effect on the vessel wall structure. These studies in animals show the promise of photodynamic therapy. However, successful translation of these results to clinical trials in humans has not yet occurred.

In addition, so called second and third generation photosensitizers have been developed for photodynamic therapy which have improved therapeutic potential (Lucky *et al* 2015). Research now focuses on third generation photosensitizers which have higher wavelength absorption peaks, reduce the side effect of light sensitivity in patients following treatment, and have improved delivery. Improved delivery could also be achieved by direct modification of the photosensitizer or by combining the photosensitizer with a delivery agent by either conjugation or encapsulation. Continued developments of photoacoustic sensitizers, particularly in effective delivery to atherosclerotic plaque, could make them an effective complimentary treatment to IVPA imaging.

Photothermal therapy

In photothermal therapy, the local biological environment is irreversibly damaged by excessive heating. Effective contrast agents for this therapy are molecules with very high absorption coefficients. A few studies on photothermal therapy have been conducted that involve atherosclerosis. Kosuge *et al* (Kosuge *et al* 2012)

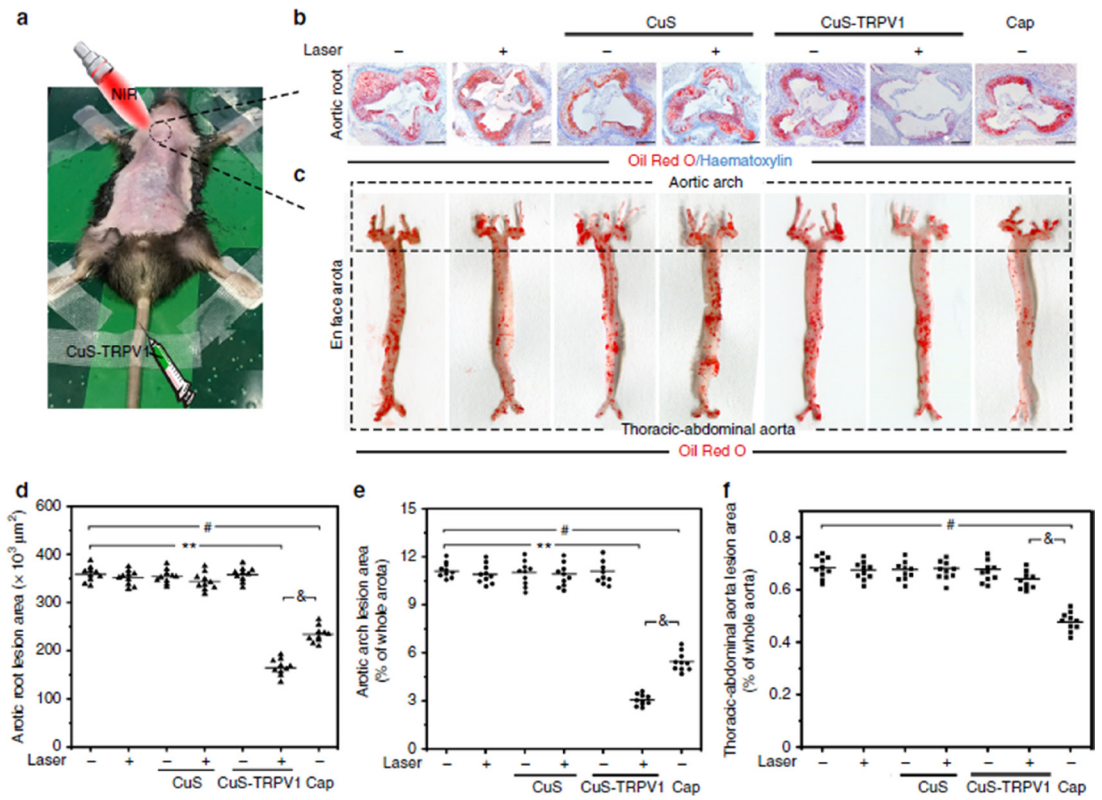

Figure 7. *In vivo* photothermal activation of TRPV1 reduces atherosclerotic lesions in apoE-/- mice on a high-fat diet. (a) NIR laser treatment. (b) Representative images of Oil Red O-stained aortic root sections. Haematoxylin was used as a counterstain. Scale bar = 250 μm. (c) Representative images of Oil Red O-stained en face aortic preparations. Within the dashed box above is the aortic arch and below it is the thoracic-abdominal aorta. (d)–(f) Quantification of Oil Red O-stained area. Data are shown as mean ± S.D. ($n = 10$), and analyzed by Student's *t*-test. $^{**}P < 0.01$ for CuS-TRPV1 + Laser versus PBS, $^{\#}P < 0.05$ for Cap versus PBS, $^{\&}P < 0.05$ for CuS-TRPV1 + Laser versus Cap. Gao *et al* (2018). Copyright © 2018, Springer Nature. With permission of Springer. CC BY 4.0.

used ligated mice carotid arteries as a model for atherosclerosis and injected them with single-walled carbon nanotubes *in vivo*. After sacrifice, arteries were removed and imaged *ex vivo*, which showed macrophage death in the ligated arteries that had been subjected to photothermal therapy. Copper sulfide nanoparticles have also been used in apoE-/- mice to similar effect (Gao *et al* 2018). The particles were targeted to transient receptor potential vanilloid subfamily 1 cation channels, which when activated allow increased Ca^{2+} transport. This in turn activates autophagy in vascular smooth muscle cells, preventing foam cell formation. This treatment was shown to reduce plaque formation and lipid storage in apoE-/- mice (figure 7). Photothermal therapy has potential as a treatment for atherosclerotic plaque, although the long term side effects of such treatment must be investigated further.

It has also been shown that the temperature of gold nanoparticles could be monitored due to a dependence between temperature and photoacoustic signal intensity (Yeager *et al* 2013). This would allow for increased precision in choosing light dosages when applying photothermal therapy to plaques, resulting in a reduction of potentially harmful heating in surrounding healthy tissue. Photothermal therapy could be a treatment for AS in future, although many aspects of its application and safety are yet to be explored in detail.

Photodynamic and photothermal therapy have potential as viable treatments for atherosclerotic disease. One limitation in the development of therapeutic strategies for AS using IVPA is the shortcomings of animal models. Many of the balloon injured models have macrophage rich plaques which may not have the high lipid content found in humans. In addition, the larger animals required for deployment of the IVPA imaging catheter require higher costs. Significant space for advancement in the field exists, including the use of different contrast agents, improvements in delivery, and testing on more advanced animal models over longer time periods. Further, several clinical studies on imaging and treatment of AS have implications for the use of nanoparticles in IVPA imaging.

Clinical translation

Several clinical studies have investigated the use of therapeutic treatments of AS that are closely related to IVPA imaging. A few involve applications of photodynamic therapy. The photosensitizer motexafin lutetium was tested in a phase I drug and light escalation trial in humans (Kereiakes *et al* 2003) after the treatment was shown to be effective in rabbits (Hayase *et al* 2001). The results indicated that the treatment method was safe in humans at six month follow up, with no clear side effects. However, no further clinical trials were conducted. In 2013, a

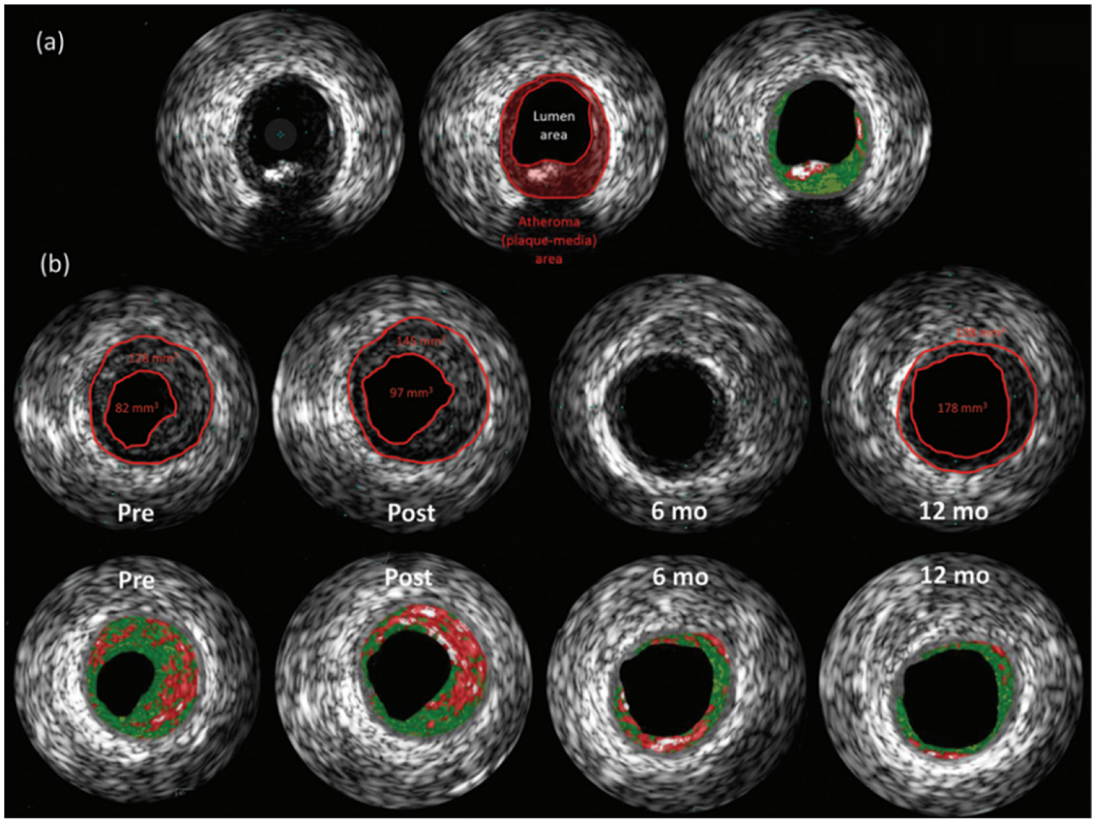

Figure 8. Imaging results of the NANOM-FIM trial. Panel (a) shows the methodology of IVUS measurements. The cross-section for analysis is identified. The external elastic membrane (EEM) and the boundary of the lumen are marked by red lines. The atheroma (plaque media) area is planimetered as a product of subtraction of the cross-sectional area of the lumen from the area of the EEM. Panel (b) documents an example of atheroma regression with grey-scale and virtual histology IVUS analysis pre-, post-procedure at 6 and 12 month follow-up in the case of the micro-bubbles infusion with the incorporated NP. Red lines indicate the EEM and the luminal boundary of the vessel wall. The atheroma area decreased from 178 to 108 mm^3 (sic) with proportional changes in the lumen area. Adapted from Kharlamov *et al* (2015) with permission from the Royal Society of Chemistry.

multicenter study called CosmoPHOS-nano was started (Letourneur and Trohopoulos 2014). The main aim of the study is to use near-infrared fluorescence molecular imaging and targeted photodynamic therapy for real-time treatment and follow-up therapy monitoring of coronary artery disease. The details of the study, such as specifics of the system and details of the therapeutic agent, do not appear to be publically reported as of the submission date of this manuscript. Nonetheless, the study is scheduled to end in 2018. The results of this study may strongly indicate future directions of research in photodynamic therapy.

NanoAthero is another multicenter study that is evaluating nanoscale particles for treatment of atherosclerosis (Letourneur and Trohopoulos 2014). Although it is not IVPA specific, nanoparticles used include iron oxide nanoparticles as well as liposomal constructs (Lobatto *et al* 2015, Suzuki *et al* 2013, van der Valk *et al* 2015). Thus, its results are relevant to applications of IVPA imaging. This study also began in 2013. The stability, synthesis, toxicity, delivery, and therapeutic attributes of a variety of nanoparticles will be evaluated starting *in vitro* and concluding with testing in humans. The first clinical trial validated delivery of prednisolone, a therapeutic agent to sites of plaques in humans, although the therapeutic effect was not significant (van der Valk *et al* 2015). Other clinical trials are planned in future.

Another promising use of nanotherapeutics for atherosclerosis is the NANOM-FIM trial (Kharlamov *et al* 2015) (figure 8). This study used nanoparticles and photothermal therapy to treat atherosclerotic plaques, with three treatment groups. In the first group, silica-gold nanoparticles in a bioengineered patch were surgically placed on the arteries of patients. In the second group, silica-gold iron bearing nanoparticles were delivered using targeted micro-bubbles and stem cells using a magnetic delivery system. After delivery, members of both nanoparticle groups were subjected to irradiation. The third group was a control group which received stent implantation. Total atheroma volume was measured at 12 months, with the silica-gold nano patch group having an event free survival rate of 91.7%, compared to 81.7% and 80% for the stem cell and stent control group, respectively ($p < 0.05$). At a five year follow up, the gold nano patch group had lower mortality (six versus nine versus ten, $p < 0.05$) and fewer instances of late thrombosis (two versus four versus six, $p < 0.05$) (Kharlamov *et al* 2017). This result is a strong indicator of the safety of gold nanoparticles for atherosclerosis imaging and treatment, although the gold nanoparticles were not delivered intravascularly in this study.

The use of nanoparticles as contrast agents and therapeutic agents in IVPA imaging has received significant attention at the preclinical level. The few clinical studies mentioned are a promising start despite sometimes limited success. The results of ongoing clinical studies should be followed closely to determine directions for future research.

Conclusions

The use of nanoparticles for photoacoustic imaging can significantly increase signal from imaging targets. Thus, nanoparticles can play a significant role in improving the diagnostic capability of photoacoustic imaging modalities. This is particularly useful in IVPA imaging, since blood and water in the vessel lumen will significantly absorb light at lipid relevant wavelengths. As a result, we have reviewed the state of current research that involves using nanoparticles for IVPA imaging, including potential therapeutic applications. From this, there are several major observations.

First, there is potential to improve existing nanoparticles or synthesize new particles. Many forms of modifications are available to develop improvements to current agents. This includes changes in the size and shape of metallic particles, changes in chemical structures of dyes, the development of hybrid contrast agents, the development of multimodal agents, or the development of switchable probes. Effective nanoparticles for IVPA imaging will require several attributes. These include safety, stability, effective delivery to plaques, and the ability to give valuable insight into the disease state in a patient. To be used clinically, particle synthesis must also be scalable and reproducible.

In addition, targeting of nanoparticles to antibodies has shown to be effective, although not full proof, in a variety of preclinical and clinical studies. Targeted agents could be a significant source of information about the disease state in patients, particularly if molecular markers are found that are more predictive of imminent plaque rupture or erosion. We expect researchers to continue using molecular targeting as a method of delivery. Potentially, it could even be used as a method for monitoring the efficacy of therapy.

An issue that will always be relevant to contrast agent development is safety. As a result, contrast agents that have already received FDA approval will receive continued interest. However, modifications to the size, structure, and surface coating of non-approved contrast agents, as well as encapsulation in more biologically friendly carriers, provide researchers with a number of methods to improve the safety of nanoparticles.

Using nanoparticles for imaging and treatment of atherosclerosis is still an open and active research field. Several directions for future research are apparent. These include improvements to nanoparticles, including their stability, ease of synthesis, effectiveness (diffusion to plaque and degree of signal enhancement), ability to target molecular markers, and the ability to be multimodal. Further, real time imaging of contrast agents in animal models of atherosclerosis is desired yet not readily available. In addition, direct comparison of the efficacy of different contrast agents is needed. Such studies could illuminate particles that would work most effectively in the clinic. Finally, fundamental studies to understand the mechanism of nanoparticle diffusion to locations of atherosclerotic disease are currently lacking. A better understanding of this phenomenon would speed up development of new particles.

IVPA imaging has been developed into a promising tool for the diagnosis of atherosclerosis. However, translating preclinical results into clinical practice remains a hurdle. The use of exogenous contrast and therapeutic agents would provide significant benefits, including enhanced plaque characterization and the possibility of therapy. A variety of preclinical studies have illustrated the advantages of this technique. More recently, clinical studies have shown limited but promising results. Further long term animal studies and clinical trials will be necessary to advance IVPA imaging and therapeutic agents to the clinic, resulting in the potential for improved diagnosis and treatment of atherosclerosis for patients.

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Conflict of interests

Dr Emelianov has a financial interest in DecisIV Interventions, LLC.

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