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Review

Application of Nanoparticles in Diagnostic Imaging *via* Ultrasonography

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ABSTRACT: The effectiveness of an imaging technique not only depends on its ability to image quantitatively both morphological and physiological functions of the tissue, but also on the contrast agent used to communicate with biomolecules. Several types of contrast media are used in medical imaging and they can roughly be cataloged based on the imaging modalities where they are used. More importantly, the use of contrast agent with their size ranging in nanometer scale has become general practice in medical diagnosis. As the matter of fact, nanoparticles have fascinated scientist for over a century and are now heavily utilized in biomedical sciences and engineering as they are long known to communicate effectively with the biomolecules. Today these materials can be synthesized and modified with various chemical functional groups which allow them to be conjugated with antibodies, ligands, and drugs of interest and thus opening a wide range of potential applications in biotechnology, and more importantly in diagnostic medical imaging *via* ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET). These imaging modalities differ not only in resolution, but also in the instrumentation and the type of nanoparticle that can be employed as its assistant. Of these imaging techniques, ultrasound is one of the oldest imaging modality which is still widely used to examine internal organs of the body and diagnose potential disease states such as cancer, plague, clots, and swelling. Various articles have been published over the period of years detailing the instrumentation and the applications of ultrasonography, but very few have emphasized the importance of particle size in developing a successful contrast agent for ultrasonography. Thus in the present review article we aim to present the basic principles involved in developing successful contrast agent for Ultrasound imaging. Furthermore, we have also discussed the experimental and physical aspects of various types of nanoparticles including its fabrication and design of targeted contrast agents. Finally, we have cited some of the best biomedical and clinical applications of the developed nanoprobes and their use for Ultrasound imaging.

KEY WORDS: ***Utrasonography; Nanoparticles; Solid nanoparticles; Diagnostic imaging; Medical imaging***

INTRODUCTION

The ultrasound (US) imaging also called ultrasound scanning, sonorgaphy, or ultrasonography uses the sound waves to form images of soft tissues in the body. These sound waves typically generated by a quartz crystal are reflected at the interfaces between different tissues due to differences in the mechanical properties of the tissues. The transmission and reflection of these high-frequency waves are then displayed with different types of US modes. The speed at which these reflected waves propagates in tissues and its time of reflection can be converted into distance of reflection. Hence with the higher frequencies of the sound waves, the absorption of the sound beam by the medium is also higher and better is the image resolution. Higher frequency sound waves are used to provide good detail of superficial organs such as the breast,

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whereas the lower frequency sound waves are used for examinations of the abdomen. In general, there are various types of US techniques that are typically used for biomedical imaging some of which are, brightness-mode (B mode), spectral doppler, color doppler and 3D US. Even with these advances in the imaging techniques, the US is unable to differentiate between tissues with similar acoustical properties. To address these challenges, US contrast agents (USCA) have been developed to artificially change the reflection coefficient between the two tissues; thus enhancing the backscattered signal and improving the image contrast.

The field of diagnostic ultrasound is again on the cusp of major change with the advent of contrast agents available for variety of clinical applications. In the last decade, major pharmaceutical companies, ultrasound scanner manufacturers, and biomedical investigators have invested manpower and funding in developing efficacious ultrasound contrast agents and new contrast-specific imaging modalities. They have made extensive use of solids, gases and liquids contrast agents in an attempt to discover novel USCA suitable for particular diagnostic purpose. The contrast agents based on solids, liquids or gases can improve the image quality of sonography either by decreasing the reflectivity of the undesired interfaces or by increasing the backscattered echoes from the desired regions.[1] In the former approach, the contrast agents are taken orally, and in the latter one, the agent is introduced vascularly. However, the contrast enhancing effect of the type of materials is regulated via three main phenomena namely, backscattering, beam attenuation, and difference in speed of the sound.

The term "backscattering" refers to the phenomenon in which energy (ultrasound energy in this case) is scattered back towards the source by a scatterer with certain physical properties. Based on the physical characteristics of the scatterer, the scatterer with larger diameter will scatter a greater amount of the ultrasound wave than a smaller substance. Furthermore, the capability of a substance to backscatter ultrasound energy also depends upon its compressibility (κ_s). These two physical properties; the particle size and κ_s, can be considered in tandem when examining different substances to measure its ability as a good scatterer. In other words, it is useful to compare "scattering cross-section of the scatterer" which is one particular measure of the ability of a substance to cause backscattering.

The scattering cross-section of a small scatterer, s, can be determined by a known equation:

$$\sigma = \left[\frac{4}{9}\pi a^2 (ka)^4\right]\left[\left|\frac{\kappa_s - \kappa}{\kappa}\right|^2 + \frac{1}{3}\left|\frac{3(\rho_s - \rho)}{2(\rho_s - \rho)}\right|^2\right]$$

Where, $K = 2\pi/\lambda$, where λ is the wavelength; a = the radius of the scatterer; κ_s = adiabatic compressibility of the scatterer; κ = adiabatic compressibility of the medium in which the scatterer exists, ρ_s = density of the scatterer and, ρ = the density of the medium in which the scatterer exists.

This equation can serve as a valuable tool in evaluating the utility of different substances as image contrasting agents. The first bracketed quantity in the above equation can be assumed to be constant for the purpose of comparing solid, liquid and gaseous scatterers. Now for the purpose of medical imaging, it can be assumed that the compressibility of a solid particle is much less than that of the surrounding medium, whereas, the density of the particle is greater. Similarly, for a "pure liquid" scatterer, the adiabatic compressibility and density of the scatterer ρ_s are likely to be approximately equal to the surrounding medium ρ. Hence, from the above equation, the liquids would have a scattering cross-section of zero. In reference to the above equation and the following idea, pure liquids are relatively inefficient scatterers.

Moreover, the scattering cross-section of a gas is substantially different than a liquid or solid, in part, because a gas bubble can be compressed to a greater degree than a liquid or solid. To add, free gas bubbles in a liquid exhibit oscillatory motion at a frequency near that of the ultrasound waves commonly used in medical imaging. As a result, the scattering cross-section of a gas bubble can be over a thousand times larger than its physical size. Therefore, it is recognized that gas bubbles are superior scatterers of ultrasound energy than solid and liquid scatters.

In spite of the known advantages, the rapid dissolution of free gas bubbles in solutions such as blood or many aqueous intravenous solutions, severely limits their use as an ultrasound contrast-enhancing agent. The most important limitations are the size of the microbubble and the length of time for which a microbubble will exist before dissolving into the solution. Examining the size requirements for microbubbles more closely, the gas bubbles must, of course, be sufficiently small that a suspension of the bubbles does not carry the risk of embolism to the organism in which they are infused. At the same time, extremely small free gas bubbles composed of the gases generally used in ultrasound contrast imaging, dissolve into solution so rapidly that their image-enhancing capability exists only in immediate proximity to the infusion site.

Ideally, the contrast agents circulating in the vasculature should be less than 8□m in diameter so that they can pass through the pulmonary bed of the lungs, allowing for imaging in both the venous and arterial vessels.[2] To penetrate beyond the

vasculature, the agents need to be over an order of magnitude smaller, in the nm range. If this can be achieved, it opens up the possibility of targeting areas such as tumors and inflammation. The tumors will not survive unless supported by blood supply. The blood vessels in tumors are characterized by irregular diameters and ill defined structures with the reported pore cut-off size between 380-780nm for depending on the type of tumor being analyzed, its location and relative diameter. In fact, nanoparticles up to 700 nm have been observed passing through capillary walls in inflamed vessels and in tumors.[3] Although the imaging and drug delivery aspects of a nanosize USCA are evident, it is less clear that a nano agent is acoustically realistic. According to the equation the scattering cross-section for a single bubble, σ, is proportional to the sixth power of the radius. A drop in diameter with an order of magnitude would lower σ by six orders of magnitude. In addition, the bubble resonance frequency is inversely proportional to the diameter; this would require the use of unusually high frequency transducers for imaging applications with nanobubbles. Although recently a 40 MHz intravascular transducer with lateral resolution of 0.5 mm has been manufactured, the commercial applications of the nanosized bubbles as USCA have still to be evaluated.[4, 5]

Image contrast has also been observed in conventional imaging due to localized beam attenuation of the sound waves owing to the differences between certain tissues. Measurement of the attenuation contrast caused by microspheres containing gas microbubbles and solid particles has been accomplished. The contrast enhancement from the use of these particles is also attributed to the attenuation of the ultrasound wave generated from the presence of dense particles in a soft medium which absorbs energy by a mechanism referred to as "relative motion." The change in attenuation caused by relative motion can be shown to increase linearly with particle concentration and as the square of the density difference between the particles and the surrounding medium. Therefore, where substantial accumulation of solid particles occurs, attenuation contrast may be a viable mechanism for observing image contrast enhancement. This phenomenon acts as the basic principle behind the nano sized targeted USCA. However, techniques based on attenuation contrast as a means to measure the contrast enhancement of a liquid agent are not well-developed and, even if fully developed, may suffer from limitations as to the internal organs or structures with which this technique can be used. For example, it is unlikely that a loss of attenuation due to liquid contrast agents could be observed in the image of the cardiovascular system because of the high volume of liquid contrast agent that would need to be present in a given vessel before a substantial difference in attenuation could be measured.

An additional possible technique to enhance contrast in an ultrasound image has been proposed based on the fact that the speed of sound varies depending on the media through which it travels. Therefore, if a large enough volume of an agent is infused into a target area then the speed of sound is into the infused area different than the surrounding tissue. This difference in the speed of sound through the target area may be measurable. Presently, this technique is only experimental.

Therefore, considering the three techniques described above for contrast enhancement in an ultrasound image, the marked increase in backscatter caused by free gas microbubbles is the most dramatic effect and contrast-enhancing agents that take advantage of this phenomenon would be the most desirable if the obstacle of their limited stability in solution could be overcome.

GAS BUBBLES AS USCA

As discussed in the previous section small bubbles are readily detected in an image produced using standard ultrasound imaging techniques. But the history of small bubbles as USCA can be traced back to 1968, when Dr Charles Joiner, a cardiologist, made an accidental observation while he was performing M-mode echocardiogram by injecting a patient with indocyanine green through a left ventricular catheter, to measure cardiac output.[6] During his measurements he observed an increase in the US signal after each injection. Consequent research showed that the increase was caused by small bubbles forming at the catheter tip. However, the use of these contrast agents was seriously hindered as their effects were transient and could not be successfully repeated. These features limited their clinical use but subsequent research permitted the use of small bubble contrast agents in clinical practice. Since then various filling gas such as nitrogen, perfluorocarbons, and sulfur hexafluoride has been used as microbubble which carried a particle size of 1-5 microns. In practical, a small amount of the suspended contrast agent (ranging from microliters to milliliters) is injected intravenously during an ultrasonic exam.[7] However, the collapse of the microbubble and the rapid formation (due to the coalesce of smaller particles) when exposed to US are some of the other side affects observed after on the usage of these contrast agents. This has also led to various innovative systems such as shell coated microbubble which can increase the stability of microbubble under the operating condition. The shell loaded with microbubble is designed to reduce diffusion of the bubble into the blood and increase it stability and the dissociation from strong ultrasonic waves. The outer coating of shell can be

stiff (e.g., albumin) or more flexible (phospholipid), with the shell thickness in the range of 10-200 nm. In fact, Feinstein was the first to find that albumin was capable of improving microbubble stability and its size can be controlled by sonication. This leads to the first pharmaceutical echo-enhancer, Albunex™, by Molecular Biosystems (San Diego, CA, USA) but is no longer in production.[8, 9] These encapsulated microbubbles are highly echogenic due to differences in their compressibility (k) and density (ρ), compared to tissue or plasma. The compressibility of air is 7.65×10^{-6} m^2/N, in comparison with 4.5×10^{-11} m^2/N for water (on the same order of magnitude as tissue and plasma).[10] The compressibility for an encapsulated microbubble falls within this range, as *De Jong* predicts the compressibility of Albunex™ to be 5×10^{-7} m^2/N.[10, 11] This impedance mismatch results in a very high echogenicity, such that the echo from an individual contrast agent can be detected by a clinical ultrasound system. The research work over the period of year gave rise to various clinically used USCA such as Levovist™, Echovist™, EchoGen® (Abbot Laboratories, Chicago, IL, USA), Sonovue™ (BR 1 Bracco, Milan, Italy), Aerosomes™ (ImaRx, Tucson, AZ, USA), Sonovist™ (Schering AG, Berlin, Germany) are been currently marketed and clinically used. Two agents containing perfluoropropane are currently approved in the United States for use in cardiology: Optison® (Mallinckrodt Inc., St. Louis, MO), with a denatured albumin shell, and Definity® (Bristol-Myers Squibb Medical Imaging, Inc., Billerica, MA), with a phospholipid shell. However, this micron sized USCA limits their use as the intravascular contrast agent suited to targeting cells outside the capillary vasculature, such as cancer cells. This can be well achieved by nanosized USCA which are also likely to circulate within the human body for a reasonable length of time, providing a greater time window for imaging. This offers an advantage over larger sized contrast agents (>1 μm diameter), which are cleared rapidly by the body's reticuloendothelial system following injection into the bloodstream. Moreover, these tiny bubbles have short life time due to the high gas pressure caused by the surface tension. For the utilization of the nanobubbles they have to be some how stabilized so as to increase its lifetime. A surfactant, electrolyte or lipid which can cover the nanobubble could reduce the surface tension, and the diffusion of the nanobubble to the surrounding liquid. In addition to the stability of the nanobubbles, the decrease in the size of the bubble leads to decrease in its echogenicity which further decrease its use as the potential USCA. But many research articles have been proposed to increase its stability and its echogenicity so as to enable their use in medical imaging to its utmost potential.

Present nanomolecular ultrasonographic contrast agents are mostly encapsulated perfluorocarbon in a polymer or a liquid shell as shown in **figure 1.**[12] Being intravascular most of its potential applications are concentrated on diseases in which blood vessels are involved, such as thrombus generation, inflammation, and tumor growth. To increase the stability of these nanoformulations these intravascular USCA against dissolution and coalescence they are encapsulated with an elastic solid shell which can support a strain to counter the effect of surface tension. In other cases, the material is a surfactant, or a combination of two or more surfactants. They promote stability by greatly reducing the surface tension at the interface. Even though, sulfur hexafluoride, nitrogen, and perfluorochemicals are used as microbubble-filling gases, most newer nano sized USCA use perfluorochemicals because of their low solubility in blood and high vapor pressure. By substituting different types of perfluorocarbon gases for air, the stability and plasma longevity of the USCA have been markedly improved, usually lasting more than five minutes.[13]

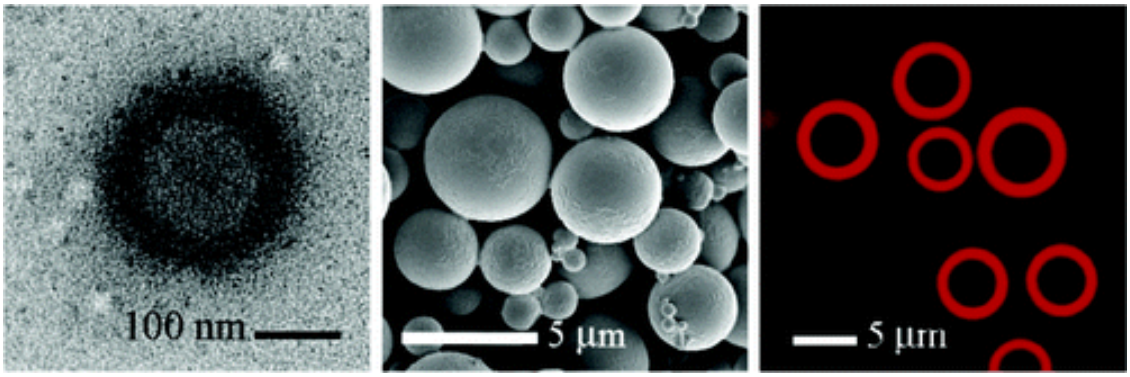

Figure 1: Nano/microcapsules with a single core of liquid perfluorocarbons within a biodegradable polymeric shell of homogeneous thickness. Copyrighted from reference[12].

DEVELOPMENT OF NANOBUBBLES

Of various methods available for the synthesis of nanobubbles, Masato and coworkers successfully developed nanobubbles using Shirasu-porous-glass (SPG) membranes with uniform pores in a system composed of dispersed gaseous and continuous water phases containing sodium dodecyl sulfate (SDS) as a surfactant. On pressuring air in SDS, monodispersed nanobubbles with a mean bubble diameter of 360–720 nm, were stably produced.[14] Also, stable nanobubbles with effective diameters of several hundreds nanometer were developed when an aqueous solutions were sonicated with a palladium electrode. This method is versatile in obtaining bubbles of different sizes, as the size of the nano bubbles could be varied with varying amount of the salts and surfactants.[15]

Even though the development of nanobubbles is long known, Oeffinger and Margaret pioneered the development of a surfactant stabilized nanosized USCA filled with perfluorocarbon gas. The ST68 surfactant (Span 60 and Tween 80) stabilized nanometer-sized, USCA were developed via differential centrifugation. The centrifugation at 300 rpm for 3 min produced USCA with a mean diameter of 450 nm as measured by dispersive light scattering. Further, while the imaging studies with these nanoshells produced an enhancement of 25.5 dB *in vitro*, they produced excellent power Doppler and grey-scale pulse inversion harmonic images at low acoustic power when administered in rabbits as shown in **figure 2**. Infact, *in vivo* dose-response curves (not shown) obtained in three rabbits also showed enhancement between 20 and 25 dB for dosages above 0.025 mL/kg.obtained with the particle Still, the echogenicity recorded for these USCA was quite low as compared to the 1.4□m sized ST68 (35 dB). This reduction in echogenicty is attributed to the smaller size of the USCA.[2, 16]

Similar to the surfactant stabilized nanoparticles, MRX-15 developed by ImaRx Therapeutics (Richmond, WA) is the next generation of lipid coated, perfluorocarbon filled nanobubbles which are being evaluated in clinical trials for the dissolution of blood clots in ischemic stroke and other vascular occlusions. Due to their small size and their relation to USCA their progress can be monitored on diagnostic ultrasound.

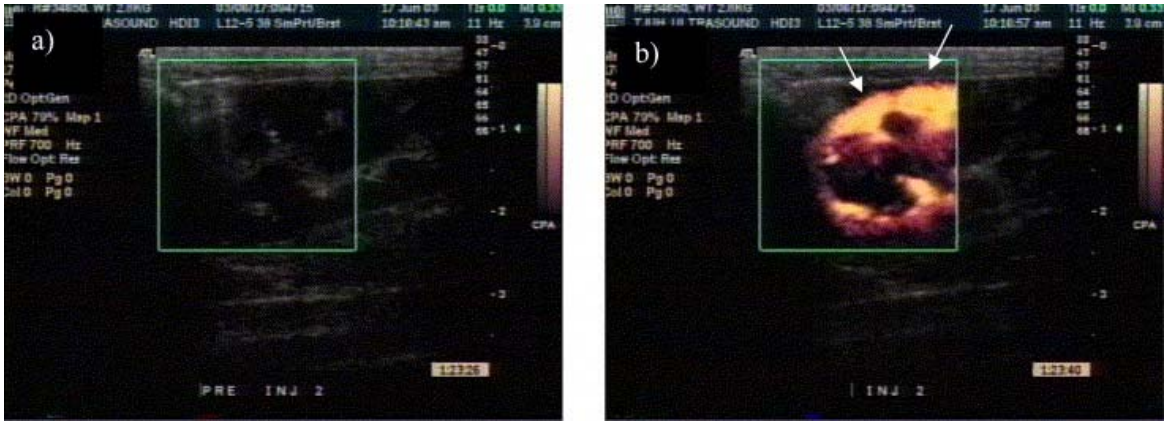

Figure 2: Power Doppler image of New Zealand rabbit right kidney, (a) preinjection; (b) postinjection of 0.1 mL/kg of ST68-N (3 min at 300 RPM). Arrows indicate kidney capsule. Copyrighted from reference[2]

In another study, *Wheatley et al* has also developed Poly (D,L-Lactic acid)/camphor based USCA which encapsulates sulfur hexafluoride gas (SF_6), a hydrophobic and biocompatible inert gas to enhance the backscattered signals. Camphor was used to render Poly(D,L-Lactic acid) nanoparticles hollow, also acting as the plasticizer for efficient gas introduction. While the camphor was removed during the lypholization, the SF_6 stayed encapsulated and hence enhancing the acoustic property. These particles obtained were spheroidal in shape with the mean diameter of 200 nm as measured by DLS. On isonating the nanoparticles encapsulated with SF_6, they showed an enhancements of 7.5dB over the background dose of 0.35 mg/mL.[17]

Another novel class of nano/microbubble irreversible switch is also developed by Natalya Rapoport and co workers. The phase state and sizes of these nanoparticles were sensitive to the copolymer/perfluorocarbon volume ratio. At physiological temperatures the nanodroplets coalesce and are converted into nano/microbubbles which can further act as better imaging probe due to increase in particle size. On injection into mice the nanobubbles extravasated from the blood vessels surrounding the tumors, selectively into the tumor interstitium, These nanoparticles then coalesce into larger microbubbles, which were visible using standard ultrasound imaging instruments. Focused ultrasound is then directed at the tumors, triggering drug release within the tumors for its therapeutic effect.[18, 19]

Even with the use of the nanobubbles the safety of microbubbles and nanomolecules needs to be considered briefly. USCA being foreign chemical the intrinsic side effects, and also the potential for enhanced bioeffects caused by the ultrasound wave itself have to be considered. Some mild side effects have been described for perfluorochemicals: mild allergy symptoms and increased liver enzymes[20] and trembling, mild fever, and low back pain.[21] In addition, the vascular damage has also been described in animals in vivo in the lungs,[22] the bowels,[23, 24] and the kidneys[25]. Therefore, extreme caution should be exercised before UCM are introduced in obstetric ultrasonography.

SOLID NANOPARTICLES AS USCAS

It has been reported that solid nano-sized particles could, in theory, be a better ultrasound contrast material than nanobubbles owing to the acoustic-impedance mismatch between solids and soft tissue.[26] Jun Liu and co workers have investigated the effect of solid silica nanospheres for enhancing ultrasonic grey scale images in tissue phantoms and mouse livers *in vivo*.[27] The image brightness of mouse livers increased following particle administration suggesting that it is feasible to use solid nanoparticles as contrast enhancing agents for ultrasonic imaging. Unfortunately, to date, the development of nanoparticulate contrast agents has concentrated mainly on MRI.

TARGETED CONTRAST AGENT

Various tissue targeted USCA has been developed over the period of time using both gas encapsulated nanoparticles and solid nanoparticles. Moreover, one of the first ultrasonographic applications of the targeted USCA was detection of fibrin thrombi. Herein, the USCA were composed of the lipid encapsulated non gaseous, perfluorocarbon emulsion that utilizes a triphasic targeting approach based upon the interaction of avidin and biotin molecules as shown in **figure 3**.[28-30] These acoustic particles were approximately 250 nm in diameter with inherently low acoustic reflectivity when free in suspension, but significantly increase the acoustic backscatter when bound to a surface. The ligand-avidin- contrast targeted contrast system used in conjunction with high-frequency ultrasound (30 to 50 MHz) demonstrated the feasibility of the novel USCA for the detection of targeted pathology with intravascular ultrasonic catheters. While, the increases in backscattered power of approximately 6 dB were found for the biotinylated, the intravascular ultrasonic images (30 MHz nominal center frequency) of plasma clots after exposure to the targeted contrast agent were brighter 0, < 0.05) than controls. These results demonstrated the first high-frequency acoustic enhancement with a novel targeted contrast agent. [31]

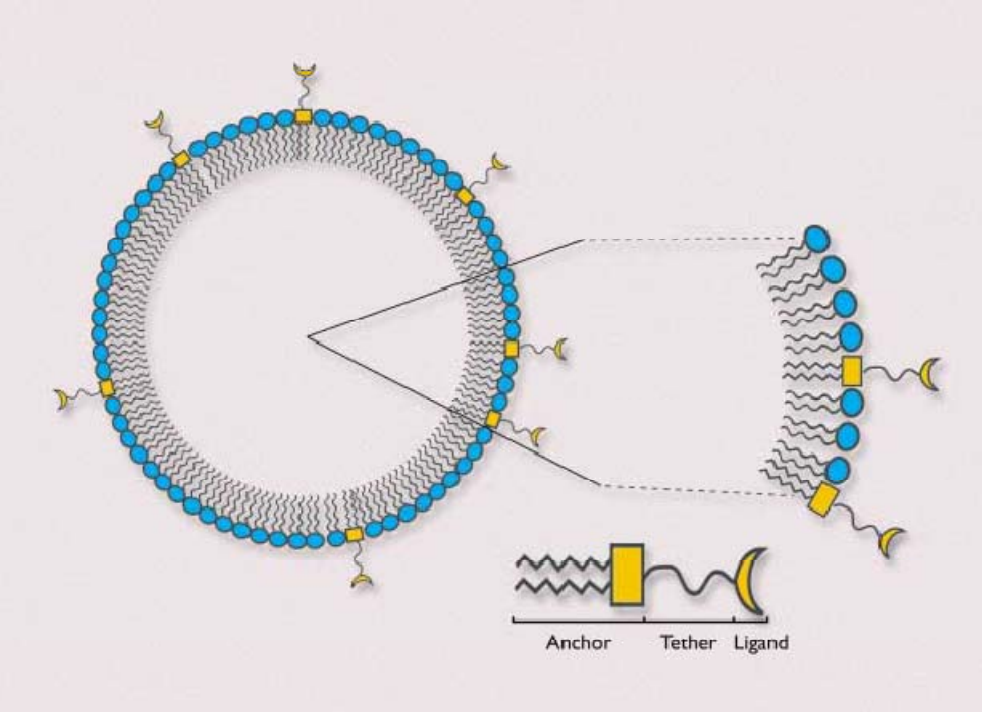

Figure 3: Microbubble with bioconjugates attached. The enlarged view shows the anchor, tether and ligand. Copyrighted from reference [32]

Similarly, solid nanoparticulate USCA developed from a biodegradable polymer, polylactic acid (PLA) (mean diameter = 250 nm) surface conjugated to an anti-Her2 antibody (i.e., Herceptin) for specific binding to breast cancer cells that overexpress Her2 receptors has been developed by Jun et al.[27] **Figure 4**, shows the synthetic scheme for the preparation of USCA from biodgradable PLA and surface conjugated to anti-Her2 antibodies. These synthesized particles were examined for target specific binding and the resultant ultrasound enhancement in both Her2-positive and negative cells. The *in vitro* experiments on the Her2-positive cells demonstrated substantial staining after incubation with nanoparticle/antibody conjugates, while minimal staining was found in Her2-negative cells, indicating receptor-specific binding of the conjugated PLA nanoparticles. Correspondingly, the high-resolution ultrasound B-mode images of the Her2-positive cells were more gray after nanoparticle treatment (133 ± 4 in treated cells versus 109 ± 4 in control, $p < 0.001$, $n = 5$), while no difference was detected in the cells that did not overexpress the receptors (117 ± 3 in treated cells versus 118 ± 5 in control). Hence the authors demonstrated the feasibility of using targeted nanoparticles to enhance ultrasonic images *in vitro*. This may be a promising approach to target cancer biomarkers for site-specific ultrasound imaging.[27]

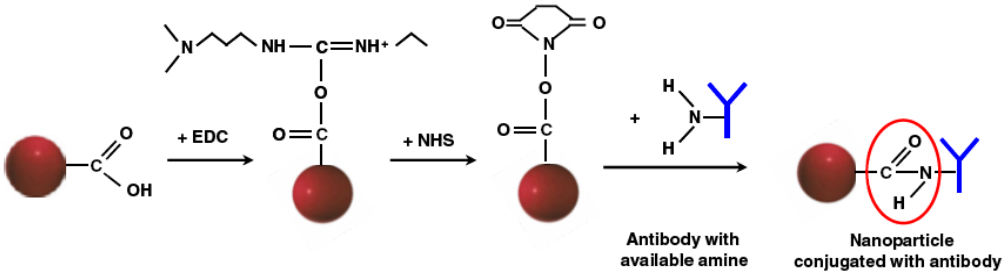

Figure 4: Synthetic scheme for the preparation of USCA developed from biodgradable PLA and surface conjugated to anti-Her2 antibodies. Copyrighted from reference[27]

In general, Ultrasound imaging offers high spatial resolution (<1 mm) and anatomical information. [33] Even though, the nanosize of the USCA does not contribute much to the imaging, recent instrumental advances have been accomplished to overcome some of the limitations. An important limitation to the use of US imaging is the need of relatively large size contrast agent (>500-700 nm), that restricts the tissue penetration and thus limit applications to vascular targets. Its interobserver dependability and the inability to differentiate between surrounding and the abnormal tissue are some other major limitations of US.

CONCLUSION

An abnormality at a molecular level is extremely essential to diagnose at an asymptomatic stage. This task now heavily rely on nanoparticles who are able to communicate with biomolecules and hence found tremendous applications as a diagnostic agent.[34] This communication between nanoparticles and tissue when decoded can find useful applications to understand the anatomical aspects of the tissue where the molecular changes have occurred. Existing noninvasive, non-ionizing diagnostic imaging techniques such as ultrasound (US) can serve as a mode to decode these communications. Even though the stability of nanoparticles for ultrasound imaging is the matter of concern, technological advances has enabled the use of these unstable nanoparticles for the period of time which allows decoding the molecular information. However, a relatively large size contrast agent for US imaging restricts the tissue penetration and thus limit applications to vascular targets. Moreover, the development of multifunctional particles in which magnetic materials can be incorporated into a nanoparticle along with the nanobubbles can also assist in the use of multimodal techniques such as US and MRI.[35] These hybrid forms of imaging via incorporating magnetic and optical properties with microbubbles may be exploited to increase sensitivity and therapeutic potential. Such hybrid imaging techniques may improve sensitivity for molecular imaging and our ability to characterize disease All in all, scientist across the world has developed these nanorobots which can, not only, evade body's immune system, but also, assist in the diagnosis of various disease states at its onset.

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