10 Molecular Imaging with Targeted Ultrasound Contrast Microbubbles

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10.1 Introduction

Molecular imaging is rapidly gaining momentum as the next-generation approach to the general goal of personalized medicine (Pither 2003). The front-runners of molecular imaging are nuclear medicine and PET modalities, which allow noninvasive monitoring of metabolic pathways and help detect certain molecules (e.g., receptors) such as tumor-specific antigens, angiogenesis, or apoptosis markers in the disease tissues. However, the most widespread imaging equipment worldwide is not a gamma camera/SPECT/PET, but ultrasound. Ultrasound imaging systems are abundant (tens of thousands units), easy to use, and inexpensive; they are small (portable, even laptop devices are available commercially), so they are quite suitable for the doctor's private practice use, for ambulance, field, or emer-

gency room applications. Therefore, if worldwide molecular imaging application is desired, capabilities of ultrasound should not be overlooked. The use of parenteral contrast will be needed to enable ultrasound in the molecular imaging.

Ultrasound contrast materials were proposed several decades ago (Gramiak and Shah 1968; reviewed in Goldberg et al. 2001). Contrast particles are usually micron-sized gas-filled microbubbles, that are injected intravenously and circulate with the flow of blood, gradually losing gas as it diffuses out of the bubble, dissolves in the surrounding biological fluids, and is eventually exhaled. Some such agents are already approved for clinical use in the United States, Canada, European countries, and Japan. Typically, up to several billions of contrast particles (with the combined particle mass of less than a milligram, overall gas volume of several microliters, dispersed in several ml of aqueous medium) are administered to the patient. Ultrasound contrast is well tolerated and could be applied for aiding organ delineation (e.g., myocardial border delineation and left ventricle opacification) or in radiology (e.g., to aid in imaging of liver malignancies; Dill-Macky et al. 2002). Successful studies of myocardial perfusion imaging have been reported (Lindner et al. 1999). The mechanism of action of ultrasound contrast is as follows. The contrast particle, being filled with gas, is much more compressible than surrounding aqueous biological fluid. Therefore, medical imaging ultrasound pressure waves (in MHz range) result in the compression and expansion of microbubbles, and effective ultrasound backscatter (Leighton 1997). These scattered ultrasound waves are detected by the imaging system transducer.

The general idea for using ultrasound contrast in molecular imaging is very straightforward. A targeting ligand (a protein, antibody, peptide, etc.) is attached to the surface of the contrast particles. Particles are administered in the circulation, where they selectively bind to the molecular receptors on the diseased tissues and accumulate there. Excess circulating particles are cleared from the bloodstream, and the target/normal tissue ratio of the ultrasound signal becomes high enough for selective imaging of the upregulated receptors distribution.

In order to design a successful ultrasound contrast material capable of targeted molecular imaging, the following criteria should be

applied. The contrast agent is harmless to the patient and generates no undesirable or toxic side effects. The agent is easily detected by ultrasound imaging equipment in small quantities. The ligand is attached to the contrast particles in a manner that does not impede its binding to the target receptors. The contrast material circulates in the bloodstream for the time sufficient to reach the target and bind to it (i.e., contrast has a chance and ability to reach the target). Targeted particles are attached firmly onto the surface of target tissue; particles are stable for the duration of the imaging exam (i.e., contrast on the target is stable enough so that diagnostic imaging could be performed). Contrast material should be inexpensive and easy to make, stable on storage, robust, and easy to handle.

In the following sections of the review we demonstrate how to design such ultrasound contrast materials, and attach targeting ligands to them. We describe targeting experiments performed with these contrast agents in vitro and in animal models in vivo. In a limited review setting it is not possible to discuss in detail all the research that takes place in the exciting area of targeted ultrasound imaging, and only some of the representative experimental studies and approaches will be discussed. The general aim is to show that targeted ultrasound contrast imaging may provide a viable alternative to nuclear medicine approaches, and bring molecular imaging into widespread clinical use.

10.2 Microbubble-Based Ultrasound Contrast Agents: General Design and Preparation

There are several types of ultrasound contrast agents, such as liquid emulsions (Andre et al. 1993; Lanza et al. 1996), liposomes (Alkan-Onyuksel et al. 1996), and gas-filled microbubbles (Fritz et al. 1997; Skyba et al. 1996; Schutt et al. 2003). We will focus this review on the microbubble-based contrast agents, due to their excellent ultrasound response, combined with good stability and targetability.

One would not expect that unprotected gas-filled bubbles would possess any reasonable degree of stability. The lifespan of a bubble in an aqueous medium is usually defined by the time it takes for the particle to float to the top of the solution and fuse with the atmo-

sphere or vial headspace (it may take up to an hour for a small micron-size bubble to reach the surface even in a high-viscosity medium, but long-term storage stability is a problem). Furthermore, if a gas microbubble is placed (or newly generated) in an aqueous medium, Laplace pressure is produced by surface tension, resulting in high internal pressure inside the bubble, which squeezes gas out of the bubble with its rapid dissolution in the surrounding medium (Epstein and Plesset 1950). Therefore, microbubble particles require a protective shell that will prevent gas loss and particle fusion; the shell will also aid in the improvement of the circulation time and stability in vivo. This protective shell may consist of lipid (Fritz et al. 1997), protein (Skyba et al. 1996), polymer (Villanueva et al. 2001), or combinations of these materials.

Obviously, one of the most convenient shell designs is based on lipid monolayers (Fig. 1). Lipid monolayer structures are usually prepared by self-assembly techniques. Surfactants from the aqueous medium are transferred to the newly generated gas-liquid interface as gas is dispersed in the liquid and position themselves at the interface, with the hydrophobic lipid tails facing towards the gas phase,

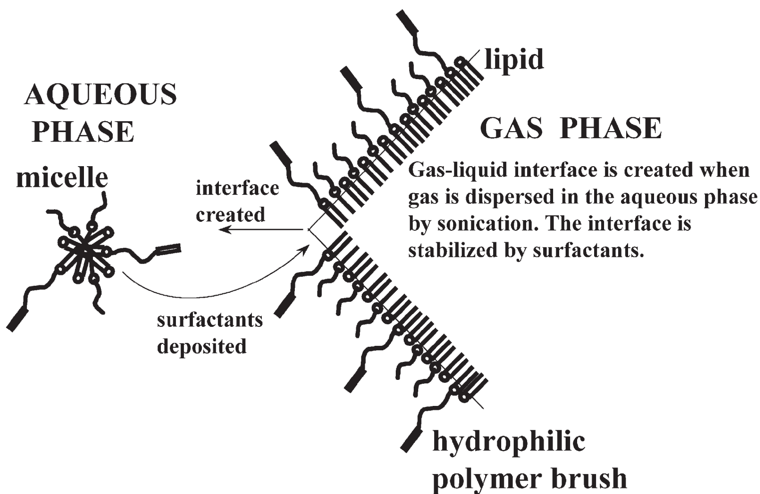

Fig. 1. Preparation of a microbubble coated with a lipid monolayer

and hydrophilic polar residues in the aqueous medium. If the monolayer is made of the molecules that do not possess a high level of cohesion, surfactant lateral diffusion would be high. This type of coating would not be able to serve as a good protective barrier. Storage stability of microbubbles prepared with such a coating will be quite poor. It is required to have a solid shell with little or no lateral diffusion or surface defects, so that resulting microbubbles will not be able to fuse with their neighbors upon storage. To eliminate gas loss due to Laplace pressure effect (Epstein and Plesset 1950), surface tension on such a bubble shell should be close to zero. One would expect that only thicker shells, such as those made of denatured proteins (albumin; Skyba et al. 1996) or organic polymers, like polylactide/glycolide (Yao et al. 2002), would be able to achieve this goal. However, solid phospholipid monolayer-coated microbubbles offer very reasonable stability at temperatures below the main transition temperature of the main lipid component (Lee et al. 2001; Borden and Longo 2002). Introduction of a grafted brush of hydrophilic poly(ethylene glycol) (PEG) polymer into the microbubble shell provides an additional degree of steric protection; in our experience, bubbles manufactured from distearoyl phosphatidylcholine (Tc $\sim 55\,^{\circ}$C) and PEG stearate were stable for months in refrigerated storage (Klibanov et al. 1999 a).

Actual preparation of such microbubbles is performed by simply dispersing gas (air, or preferably a poorly soluble gas, decafluorobutane) that is bubbled through the aqueous micellar solution (typically, several mg/ml) of a phospholipid, e.g., phosphatidylcholine, and PEG-containing surfactant, e.g., PEG stearate. Dispersion is performed with the aid of the probe-type sonicator (Klibanov et al. 1999 a), or other types of high-shear mixing (e.g., Fritz et al. 1997). As the new bubbles of gas are generated by high shear in the aqueous medium, they are immediately stabilized by deposition of the surfactant/phospholipid mixture on the bubble surface, with a monolayer self-assembly (Fig. 1). Gas is locked inside the bubble shell, and bubbles become protected from fusion with their neighbors and the atmosphere by the combination of “solid” phospholipid shell and a grafted PEG brush. The major advantage of this approach is in its flexibility: if one wants to introduce another lipid or surfactant in the bubble shell, it can be added to the aqueous solution at the mi-

celle preparation stage, usually at several mol %, prior to the bubble preparation stage (Klibanov et al. 1999 a). This way, targeting ligands (outfitted with lipid anchors) or hydrophobic fluorescent probes can be introduced in the microbubble coating.

10.3 Targeting Ligand Attachment to Microbubbles

In order to achieve targetability of ultrasound contrast, selective ligands have to be attached to the microbubble shell. General ligand conjugation chemistry has been developed quite extensively during the past two decades, mostly for targeted nuclear scintigraphy agents and MRI contrast. Similar coupling schemes can be applied for the attachment of ligands to microbubbles. The major points to pay attention to in the established procedures are (a) the necessity to retain ligand ability to bind to the target upon coupling, (b) avoidance of reagents undesirable for parenteral applications, and (c) the ability to achieve high coupling yield and high surface density of the ligand on the bubble shell.

The most convenient technique, especially suitable for the early research stage, is the avidin-biotin coupling method (Fig. 2; Lindner et al. 2001). Biotinylated phospholipid derivative (preferably with a long PEG spacer arm) is synthesized and incorporated in the microbubble shell during bubble preparation as described above. After the bubbles are prepared, they need to be washed from the excess of the aqueous micellar lipid; this is usually accomplished by multiple centrifugal flotation. Streptavidin is then added to the microbubble preparation in excess to avoid homo- and heterocrosslinking of biotins on the bubble surface. Excess of free streptavidin is also removed by centrifugal flotation, and biotinylated ligand is added to the dispersion; it binds to the unoccupied biotin-binding moieties on streptavidin molecules. The main advantage of this approach lies in its ability to quickly add to the bubbles various new ligands for early research-stage testing; a large variety of targeting ligands, such as monoclonal antibodies, are available commercially, often in biotinylated form. Another advantage of this technique is that only a small amount of precious biotinylated ligand is needed for the preparation, and a ligand-to-bubble initial ratio can be chosen so that most of the

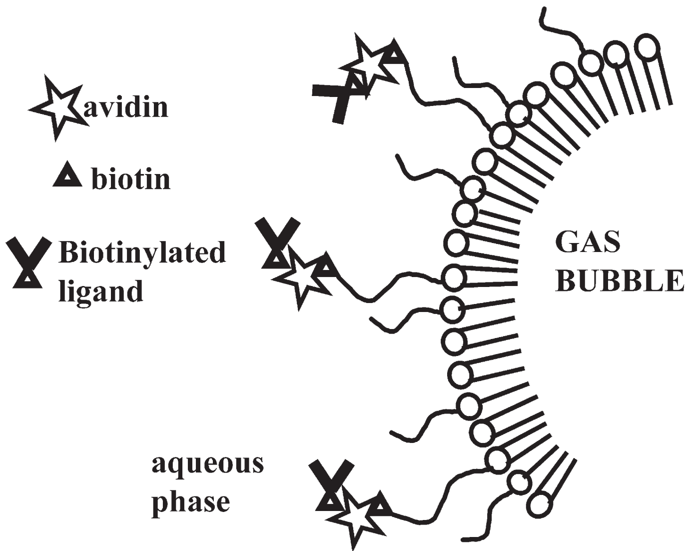

Fig. 2. Attachment of targeting ligands to microbubble surface via biotin-streptavidin bridge

added ligand is bound to bubbles. Two major disadvantages make it difficult to consider this technique for clinical diagnostic imaging use: first is the fact that streptavidin is a foreign protein and a possible immunogen, and second is the need for multiple centrifugal washes required for this multistep procedure. A preferred clinically applicable technique would be the covalent attachment of the ligand to the microbubble shell.

The obvious first choice of the covalent coupling method is the formation of an amide bond between the activated carboxyl of the lipid (or PEG-lipid) derivative and a primary amino group on the ligand molecule. In research settings, a monoclonal IgG antibody is usually applied as a first-choice ligand, because antibodies are available commercially against a wide variety of antigens. The IgG molecule has several dozen lysines in its sequence, and random attachment of one of these residues to the surface of a microbubble would not result in the loss of the antigen-binding capability. Coupling is typically performed by the active ester technique, where the carboxy-

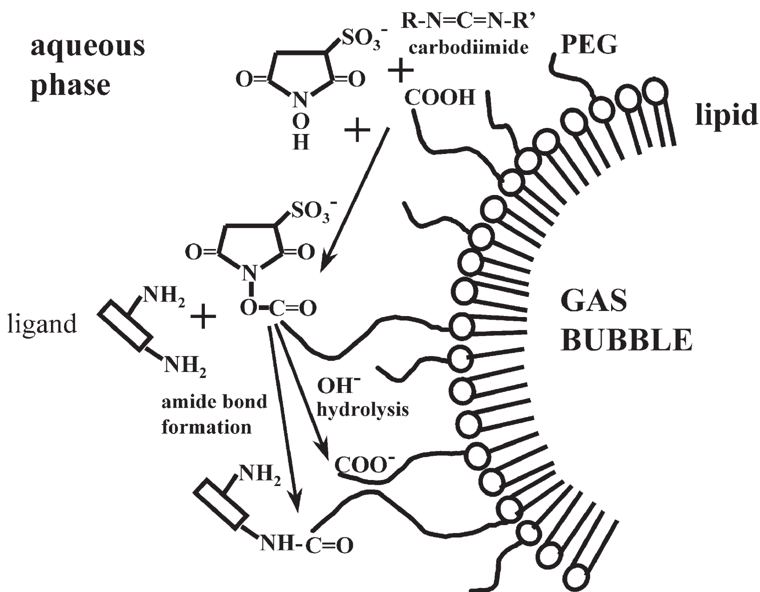

Fig. 3. Attachment of targeting ligands to microbubble via active ester coupling

late of the microbubble-associated PEG-lipid is first activated by water-soluble carbodiimide and converted to sulfo-*N*-hydroxysuccinimide (NHS) ester by the addition of sulfo-*N*-hydroxysuccinimide (at pH ∼4–5; Fig. 3; Villanueva et al. 1998). Activated microbubble preparation is then added to the antibody (or other aminoligand) solution (at pH ∼7–8), and coupling of ligand to the bubble occurs with the formation of the amide bond. In the mild alkaline conditions that are required for the successful progression of the amide bond formation, most of the active ester is hydrolyzed (an undesirable side reaction), and does not have a chance to bind to the antibody. Therefore, in order to couple a reasonably high amount (tens of thousands of molecules) of antibodies per microbubble to achieve successful targeting, one would require large excess of antibody, most of which would not be bound to the bubbles and wasted/removed in the subsequent bubble purification washes.

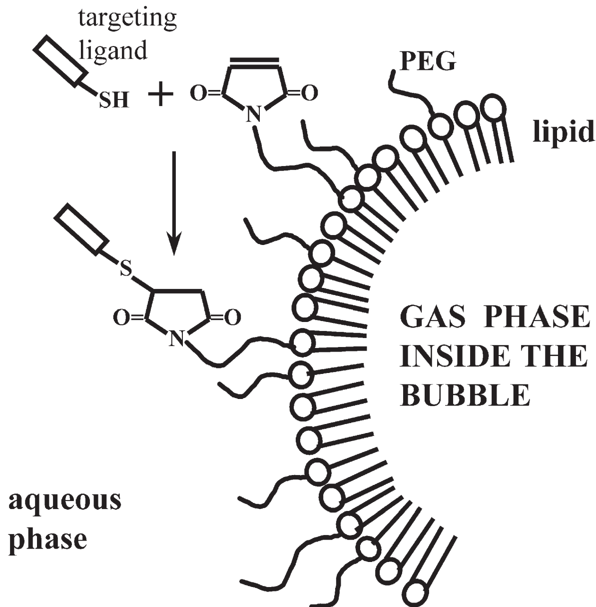

Fig. 4. Attachment of targeting ligands to microbubble via thiol-maleimide coupling

The most suitable technique for coupling ligands onto the microbubble surface seems to be thiol-maleimide chemistry, as developed for targeted liposome applications (Kirpotin et al. 1997). In this case, active maleimide lipid derivative (available commercially) would be incorporated into the microbubble shell, and after the flotation wash to remove free lipids not associated with the bubbles, microbubbles would be added to the solution of thiol ligand derivative (Fig. 4). Maleimide residue on the bubble surface is stable for days; however, its reaction with thiol is completed within less than an hour. Consequently, reaction yield is improved. Large excesses of the ligand would not be required for the preparation, so loss of this expensive material can be avoided. Another advantage of this approach is the ability to generate a single thiol in a ligand molecule, either by synthesis or by controlled cleavage (such as Fab′-fragments generation from IgG antibodies); the ligand will then be

attached to the microbubbles in a controlled, oriented fashion, so that all of the ligands will be available for target binding without inactivation.

10.4 In Vitro Targeting: Binding Studies in Model Systems

Testing of targeted microbubble functionality was initially performed in vitro in model systems, such as plastic or glass surfaces coated with the model receptor, with ligand attached to the surface of microbubbles as described. Microbubbles were first placed into the dish that contained the model target, with the dish inverted so that the bubbles would float to the target surface and touch it; after several minutes of incubation, free nonattached bubbles would be washed off the dish, and detection of targeted bubbles would be performed by microscopy and/or ultrasound imaging. The first model targeting receptor tested was avidin, with the targeting ligand being biotin, attached to the microbubble surface via a phosphatidylethanolamine anchor (preferably with a PEG spacer arm; Klibanov et al. 1997, 1999 b). When biotinylated microbubbles had an opportunity to touch avidin-coated target and bind, their hold to the target surface was quite firm (aqueous medium flow rates up to 0.6 m/s were required to dislodge the bubbles, depending on the amount of biotin on the bubble surface; Klibanov et al. 1999 b). When an antigen-antibody ligand-receptor system was tested instead of avidin-biotin, the strength of the bubble-target bond was not as high, and detachment occurred at slower flows (Klibanov et al. 1999 b).

A more realistic experimental approach would be not to allow microbubbles to bind to the target in the static conditions first, but to let them flow through the target area like it would occur in the actual targeted imaging application setting (Fig. 5). A parallel plate flow chamber has been used to characterize particle and cell-targeted binding in the flow conditions (Lawrence et al. 1987); it is easily applied to targeted microbubble testing (it only needs to be turned upside down so that bubbles would have a chance to roll by the target surface). Targeted bubbles carrying fluorescein ligand attached to the antifluorescein scFv receptor-coated flow chamber deck from the flowing aqueous medium at $\sim 140\ s^{-1}$ shear rate;

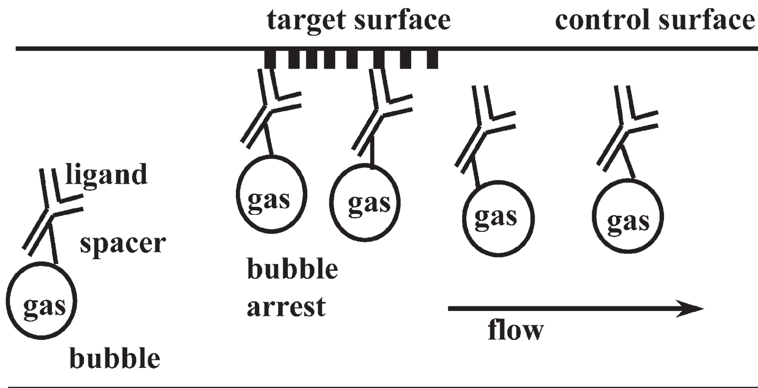

Fig. 5. Targeting of microbubbles in the flow of liquid

control nontargeted bubbles floated by without attachment (Klibanov et al. 1999 b).

Direct measurement of the bubble-target bond strength was performed with the use of a micromanipulation system (Kim et al. 2000). An avidin-coated glass microbead, held by micropipette under suction pressure, was brought in contact with the biotinylated bubble held by another micropipette, and binding allowed to occur. The critical pull manometric pressure required for the detachment of the target bead from the bubble was then determined. The detachment force was calculated based on the cross-section surface area. A force of up to tens of nanoNewtons (saturated at biotin concentration over ∼ 3 mol% of total lipid present) was required for bubble-target detachment. Interestingly, comparison of biotin-PEG-lipid anchor (Fig. 6 a) and shorter biotinamidocaproyl-lipid derivative (Fig. 6 b) revealed nearly complete lack of ability of the short-arm anchor to produce targeted binding, probably due to the steric hindrance for the access of avidin target surface to biotin through the dense PEG brush that covered the surface of both types of bubbles.

Once selective targeting of microbubbles to model receptors was demonstrated, the issue of the sensitivity of ultrasound imaging and its ability to detect contrast particles was evaluated. Surprisingly, even with the older, fundamental imaging ultrasound systems, a very

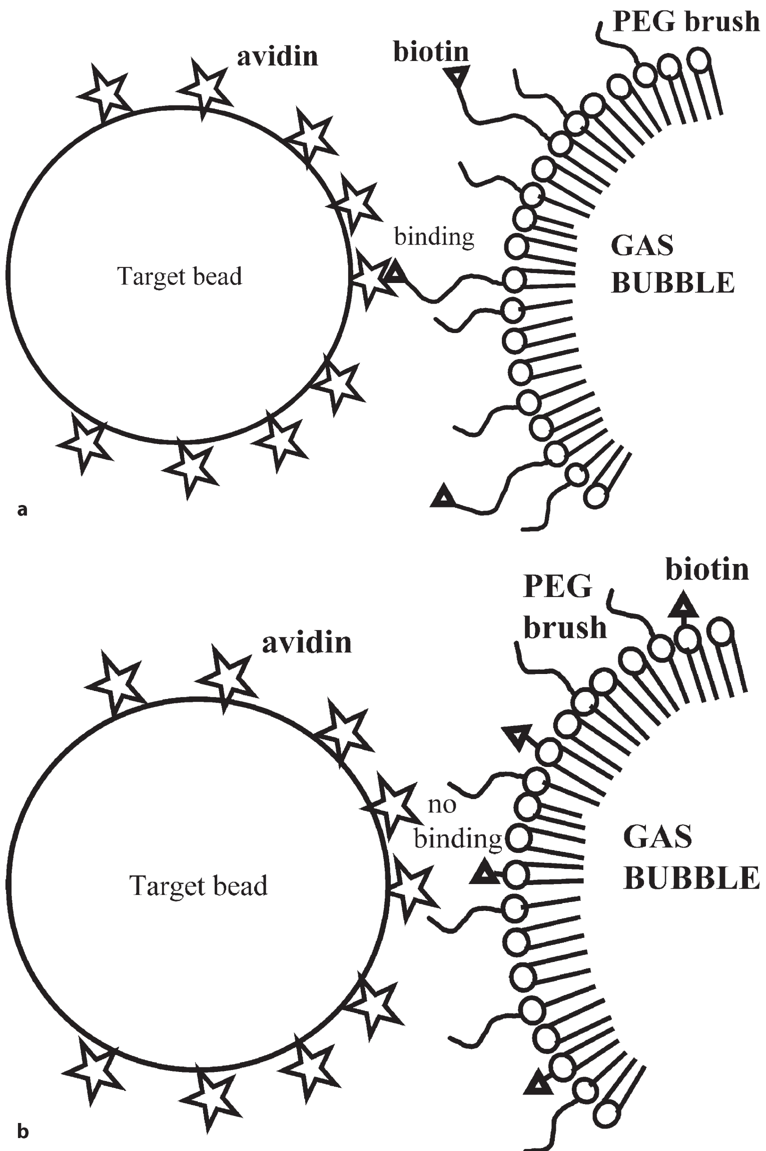

Fig. 6. a Advantage of long PEG spacer arm for microbubble targeting. **b** Disadvantage of short spacer arm for microbubble targeting

small number of microbubble contrast particles targeted to the surface of the Petri dish were visualized (Klibanov et al. 1997). Bubble patches as small as 1×2 mm on the flat, receptor-coated plastic surface were visualized as bright spots by ultrasound imaging, i.e., several thousands of bubble particles with the overall mass in the nanogram range were detectable (Klibanov et al. 1997, 1999 b). Technically, the modern contrast ultrasound imaging modalities, such as pulse inversion, ultraharmonic, or power modulation imaging techniques, are very sensitive to the presence of microbubbles, and allow detection of individual microbubbles in the aqueous dispersion (each microbubble has a mass in the picogram range; Klibanov et al. 2002). Furthermore, it was recently shown (Klibanov et al. 2004) that an individually targeted microbubble in vitro, attached to the Petri dish surface, could also be detected by ultrasound imaging in the pulse inversion mode. All these results imply that detectable doses of microbubbles can be easily achieved during in vivo accumulation in the target tissues.

10.5 In Vivo Microbubble Targeting: Biodistribution, Nonspecific and Targeted Accumulation and Ultrasound Imaging

Two basic techniques are in use to evaluate in vivo behavior of targeted microbubbles. One is intravital microscopy, which is normally performed in the cremaster muscle model setting, aided by the fact that fluorescence lipid probes can be easily incorporated in the microbubble shell and visualized by fluorescence epi-illumination imaging in real time. The other imaging technique is actual ultrasound, usually performed with the clinically oriented systems, where the presence of microbubbles in the particular tissue or organ is evaluated by echo response.

Prior to describing results of targeted accumulation of ligand-carrying microbubbles in the tissues of experimental animals, we should first evaluate the nonspecific uptake of the microbubble contrast agents into various organs, tissues, and cells. This information will help to assess the ability of targeted microbubble contrast to

achieve high target-to-normal tissue accumulation ratios, which is the main prerequisite for success of targeted imaging.

Microbubbles after intravenous administration are freely circulating in the bloodstream, not exiting the vasculature. It has been shown (Keller et al. 1989) that the rheological behavior of microbubbles in the flow of blood is quite comparable to the behavior of red blood cells. Depending on the shell composition, such as inclusion of apoptosis marker phosphatidylserine (Christiansen et al. 2002), microbubbles may demonstrate accelerated uptake by leukocytes deposited in the postcapillary venules or, possibly, by Kupffer cells in the liver. Surface charge and composition may also have an influence on the interaction of the microbubbles with leukocytes on the endothelium lining of the vasculature, either directly (Lindner et al. 2000) or via complement activation and complement C3 shell deposition mechanisms (Lindner et al. 2000; Fisher et al. 2002). In the presence of a strong negative surface charge, nonspecific adhesion and accumulation of microbubbles on the vascular endothelium has been noted both in the cremaster muscle model setting by microscopy and in the echocardiography setting in the large animal model (Fisher et al. 2002). Even the presence on the bubble surface of PEG brush, which is often used as a steric protective layer on pharmaceutical nanoparticles, only partially reduced this charge-related microbubble retention. Therefore, in order to reduce undesirable nonspecific deposition of targeted microbubbles on endothelium and uptake by leukocytes, microbubble surface charge should be kept close to neutral.

Selective targeting with microbubbles is generally directed to the specific receptors of pathological conditions on the surface of the endothelium. Because of the size of microbubbles (typically, several microns), it is presumed that only the intravascular structures can be within reach for contact with the ligands on the microbubble surface, so the imaging targets are limited to thrombi (Schumann et al. 2002) or the molecules located on the luminal surface of the endothelium, especially the molecules that are overexpressed in certain pathological conditions, such as inflammation, ischemia/reperfusion injury, or angiogenesis (Lindner et al. 2001; Villanueva et al. 1998). Via these markers, vascular endothelium provides information to the immune system about the status of the underlying tissues. P-selectin,

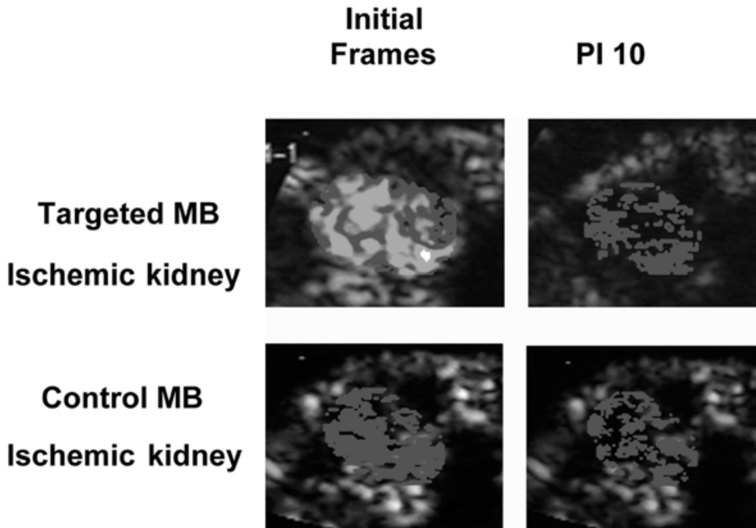

Fig. 7. Background-subtracted color-coded ultrasound images of kidneys 1 h after ischemia-reperfusion injury in wild-type mice. Initial images acquired 8 min after intravenous injection of microbubbles and images subsequently obtained at PI of 10 s are shown (reprinted with permission from Lippincott, copyright 2001, from Lindner et al. Color images not available here)

an endothelial cell adhesion molecule, is an obvious object of interest for targeted imaging. In the areas of inflammation it is substantially upregulated on the surface of the vascular endothelium. When a monoclonal antibody (RB40.34 against mouse P-selectin) was attached to microbubbles via a biotin-streptavidin scheme, such microbubbles showed a statistically significant increase of accumulation in the cremaster muscle tissue that was treated by proinflammatory stimuli (TNF-α), as compared with control, nontargeted bubbles (Lindner et al. 2001). In addition to inflammation caused by TNF, upregulation of P-selectin expression is caused by ischemia-reperfusion injury. Hence, when RB40.34 antibody-carrying bubbles were administered to mice in which one of the kidneys was subjected to transient (30 min) ischemia followed by 1 h reperfusion, selective accumulation of targeted microbubbles in the target kidney was demonstrated (Fig. 7; Lindner et al. 2001).

Background subtraction image processing is a powerful technique crucial for the analysis of these contrasted targeted images. First, a control precontrast image was obtained, then contrast was administered and microbubbles were allowed to circulate for 8–15 min, so that target accumulation could occur. Then, contrast ultrasound imaging was performed and the contrast image was subtracted from the background and color-coded (Fig. 7, left panel). Finally, high-intensity ultrasound pulses were used to destroy all the bubbles in the tissue, and after a 10-s interval contrast imaging was performed again, to evaluate the residual amount of circulating microbubbles (Fig. 7, right panel). In a separate study, the same biotinylated microbubbles carrying biotinylated monoclonal antibody against ICAM-1 integrin were prepared by streptavidin bridging and tested in the rejected heart transplant rat model (Weller et al. 2003). ICAM-1 is upregulated on the endothelium of rejected hearts. Respectively, a statistically significant increase of contrast ultrasound video intensity was demonstrated in the rejecting heart with the injection of targeted bubbles over control, nonrejecting hearts (with little accumulation of nontargeted control bubbles in either; Weller et al. 2003).

The application of targeted ultrasound contrast is not limited to visualization of inflammation or transplant rejection: an important potential application of this technology is the imaging of angiogenesis. Angiogenic endothelium is known to overexpress $\alpha_v\beta_3$ integrin (Brooks et al. 1994); this molecule may be a sensible target for the evaluation of the status of the endothelium (i.e., development of neovasculature or blood supply) in tumors (Winter et al. 2003; Sipkins et al. 1998), or perhaps a marker for the monitoring of proangiogenic therapy in ischemic tissues. Antibodies against $\alpha_v\beta_3$ are available, as well as smaller targeting ligands, e.g., disintegrin peptide echistatin (Yahalom et al. 2002); both molecules could be biotinylated and attached to the bubbles by the above-mentioned streptavidin scheme.

The angiogenesis animal model first used to test this targeted contrast agent was an FGF-2 matrigel plug in a mouse setting; both confocal microscopy and ultrasound imaging revealed selective accumulation of targeted microbubbles in the angiogenic area (Fig. 8), which was confirmed by intravital microscopy of the FGF-2-treated

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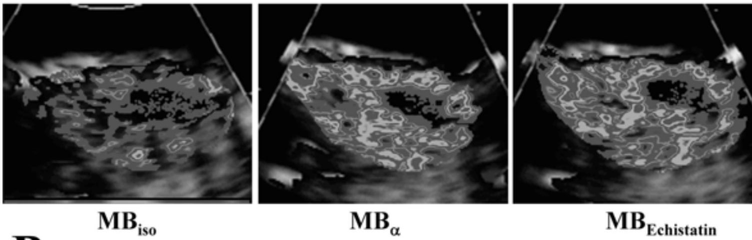

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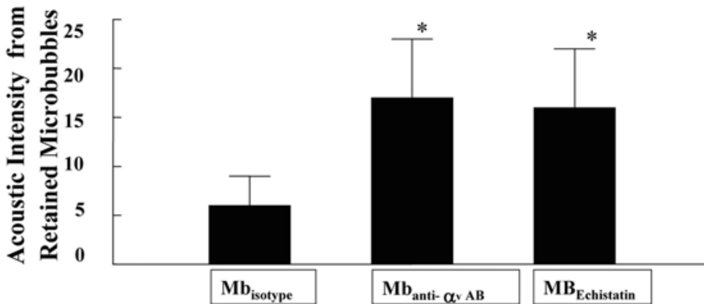

Fig. 8 A, B. CEU assessment of microbubble retention in matrigel. **A** Examples of background-subtracted color-coded CEU images reflecting signal from microbubbles retained in matrigel plugs 15 min after intravenous injection of microbubbles. **B** Mean (+SD) background-subtracted acoustic intensity for retained microbubbles. $*P<0.01$ compared with MBc (reprinted with permission from Lippincott, copyright 2003, from Leong-Poi et al. Color images not available here)

cremaster (Leong-Poi et al. 2003). Further studies were performed in a rat glioma setting, where angiogenesis during the time course of tumor development was evaluated both by nontargeted, circulating ultrasound contrast microbubbles and echistatin-coated targeted microbubbles (Ellegala et al. 2003). A statistically significant increase of targeted microbubble accumulation in the areas of tumor angiogenesis was reported, as compared with the control tumor-free hemisphere, or with the administration of control ligand-free microbubbles. Selective targeting of echistatin-carrying bubbles to the angio-

genic endothelium was confirmed by confocal microscopy. Tumor intravascular blood volume showed good correlation with the targeted microbubbles' acoustic intensity in the tissue. Overall, this technique offers a noninvasive method to evaluate the level of upregulation of an angiogenesis marker in the tumor-related vasculature, which is one of the major goals of molecular imaging.

10.6 Conclusions

Microbubbles were prepared from insoluble perfluorocarbon gas, stabilized with a monolayer of phospholipid with a grafted PEG brush and decorated with targeting ligands, such as monoclonal antibodies or peptides on a flexible PEG spacer arm. Such targeted bubbles selectively and firmly bind to the surfaces coated with the specific receptors in vitro. Bubble deposition areas can be detected by ultrasound imaging. Single bubble detection sensitivity is possible.

Targeting of microbubbles to areas of inflammation, ischemia-reperfusion injury, and angiogenesis (including tumors) was achieved in vivo in experimental animal studies, by the use of microbubbles directed towards P-selectin or $\alpha_v\beta_3$, respectively. Selective accumulation of ligand-carrying bubbles in the tissues of interest was confirmed by fluorescence microscopy (intravital and confocal) and ultrasound imaging.

Acknowledgements. The author thanks the Cardiovascular Imaging Center, Cardiovascular Research Center, University of Virginia Cardiovascular Division, Sanjiv Kaul, Jonathan Lindner and Klaus Ley for help and support. Donation of laboratory equipment by Mallinckrodt Inc. (St. Louis, MO) to A. Klibanov's laboratory at UVA Cardiovascular Imaging Center is gratefully acknowledged.

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