Polymeric Ultrasound Contrast Agents Targeted to Integrins: Importance of Process Methods and Surface Density of Ligands

Margaret A. Wheatley,* Justin D. Lathia,† and Kelleny L. Oum

School of Biomedical Engineering, Science and Health Systems, Drexel University, Philadelphia, Pennsylvania, 19104

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The use of injectable gas-filled microbubbles during ultrasound imaging is accepted as a good method to increase image contrast. Site-targeted microbubbles are expected to provide higher sensitivity and specificity than blood pool contrast agents (CAs). We have shown that covalent attachment of GRGDS peptide fragments to the surface of poly(lactic acid) CAs facilitates attachment to MDA-MB-231 human breast cancer cells in vitro. This paper examines the effect of process conditions during microbubble fabrication and ligand attachment and also changes in ligand surface density and shows that they have important effects on in vitro acoustic response and CA adhesion to breast cancer and cell lines. Use of intermittent sonication in the emulsion step, shortening of reaction times, and increase in freeze-drying times allows for a reduction of 50% in the dose of GRGDS-modified capsules (from 0.16 to 0.012 mg/mL) required to achieve a maximum enhancement of 20 dB; signal loss after 15 min insonation of GRGDS-modified capsules is reduced from a loss of 60% to a loss of 40%, and cell attachment after 10 min contact time is increased from an average of 1.4 ± 0.86 to 1.8 ± 0.17 capsules/cell. Optimal attachment is achieved with a molar ratio of total −COOH groups to GRGDS of 1:0.5. The effect of process conditions during microcapsule fabrication, ligand attachment, and ligand surface density on in vitro acoustic response and CA adhesion to breast cancer cell lines in tissue culture are shown to be important parameters that can aid in the future design of an ultrasound CA that allows both cancer detection and treatment, potentially by targeted drug delivery.

Introduction

Diagnostic ultrasound offers certain advantages over other imaging modalities such as magnetic resonance imaging and X-ray, specifically safety, speed, portability, acquisition of real time images, and relatively low cost. However, even with recent advances in signal processing techniques, image quality is not optimal for imaging soft tissue malignancies. The use of injectable gas-filled microbubbles during ultrasound imaging has been accepted as a good method to increase image contrast.[1] Encapsulated gas presents a large impedance (the product of density of the medium and speed of sound in that medium) mismatch, which results in high reflection of the impinging ultrasound wave. The imaged bubbles also undergo volumetric oscillation due to pressure fluctuations resulting from the interrogating beam, and this enhances the backscatter when the resonant frequencies of the bubbles are achieved. Clinically, these microbubble contrast agents (CAs) are used to assess tissue perfusion in the heart and other organs. Over the past few years, there has been interest in the development of site-targeted microbubbles to allow for selective retention in regions of pathology, making the lesion distinguishable by ultrasound. The possibility of molecular imaging significantly increases the overall diagnostic potential of ultrasound as well as opens up the possibility of targeted drug delivery. Further, merging imaging with a microbubble drug delivery platform would allow local deposition of therapeutic agents with the ability to verify and quantify treatment. Recent reviews have outlined potential areas of application.[2−5]

Site-targeted microbubbles are expected to provide higher sensitivity and specificity than standard blood pool ultrasound CAs, allowing earlier and safer assessment of pathological tissue, for example, in breast cancer, to differentiate diseased from benign masses.[5−12] This active targeting usually requires attachment of ligands to the surfaces of microbubbles. Ligands belong to various classes of molecules including monoclonal antibodies and their fragments, polysaccharides, and specific peptide sequences that recognize disease antigens. Active targeting to inflammation sites has been demonstrated using perfluorocarbon (PFC) microbubbles having antibodies to endothelial cell adhesion molecules that are expressed on the surfaces of endothelial cells activated during the inflammatory response.[12] In vitro targeting and contrast enhancement of vascular thrombosis, another goal of active targeting, has been achieved with a lipid-coated PFC microbubble (MRX-408, ImaRx Pharmaceutical Corp., Tucson, AZ) having a peptide with a RGD (arginine, glycine, aspartic acid) sequence covalently attached via a poly(ethylene glycol) (PEG) spacer to a lipid membrane component.[13] Such agents may improve tumor imaging or help monitor promotion of angiogenesis in chronically ischemic tissue.[14]

Angiogenesis, the formation of new vasculature, is found in many in vivo processes, including embryogenesis, wound healing, and the female reproductive cycle, and is vital for tumor growth. Cellular adhesion, particularly in angiogenesis, has been shown to be dictated through heterodimeric cell surface integrin receptors. Integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ are important for the adhesion of the endothelium to the basal membrane and numerous other endothelial functions that are essential in

* Author to whom correspondence should be addressed. E-mail: wheatley@coe.drexel.edu.

† Current address: NIH−Cambridge Graduate Partnership Program, Laboratory of Neuroscience, National Institute on Aging, 5600 Nathan Shock Dr., Baltimore, MD 21224.

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angiogenesis.[15,16] These integrin receptors have been shown to bind to particular peptides containing the RGD sequence.[16] This sequence acts as a good model for proof of concept studies for a site-specific CAs. In particular, it has been suggested that when considering ligand attachment to CA surfaces the spatial arrangement, spacing distance, and density on the surface should be taken into consideration.[17]

In related studies regarding cell adhesion and proliferation, cell attachment has been shown to be a function of RGD concentration in a dose-dependent manner.[17] This suggests a critical minimum RGD density for cell response. Massia and Hubbell found a minimum as low as 1 fmol RGD peptide/cm^2 sufficient for cell spreading and as low as 10 fmol/cm^2 sufficient for formation of focal contacts and stress fibers on a RGD-functionalized glycophase glass surface.[18]

We have reported on a CA based on a bioerodible shell composed of poly(lactic acid) (PLA) encapsulating a volatile core that can be removed by freeze-drying.[19−21] The process results in porous, hollow capsules that can be filled with the gas of choice such as air or the dense, insoluble gases of the PFC family or SF_6. In proof of principle studies, we have shown that covalent attachment of GRGDS peptide fragments to the surfaces of these PLA microcapsules facilitates attachment to MDA-MB-231 human breast cancer cells in vitro.[19] This paper examines the effect of process conditions during microcapsule fabrication, ligand attachment, and ligand surface density on in vitro acoustic response and CA adhesion to breast cancer cell lines in tissue culture. These studies provide important information toward the future design of an ultrasound CA that allows both initial cancer detection and treatment, potentially by targeted drug delivery.

Experimental Section

Materials. Poly(D,L-lactic acid) (Medisorb 100 DL low intrinsic viscosity (I.V.), molecular weight (M_w) 84.5 kDa, lots 7240-344 and W2297-587, both with transition temperature 49.9 °C) and poly(D,L-lactide-*co*-glycolide) (PLGA, Medisorb 8515 DL high I.V. lot 9097-403, molecular weight 121.0 kDa, with transition temperature 49.8 °C) were purchased from Alkermes, Cincinnati, OH (now Lakeshore Biomaterials, Birmingham, AL). Poly(vinyl alcohol) (PVA), 88% mole hydrolyzed, with a M_w of 25 kDa, was from Polysciences, Inc., Warrington, PA. The GRGDS (Gly-Arg-Gly-Asp-Ser) peptide complex, 1-ethyl-3,-3-dimethylamino-propyl carbodiimide (EDC), *N*-hydroxysulfosuccinimide (NHS), and ammonium carbonate were purchased from Sigma, St. Louis, MO. Fetal bovine serum (FBS), DMEM low glucose with L-glutamine media, antibiotics (penicillin and streptomycin), methylene chloride, isopropyl alcohol, and 2-[*N*-morpholino]-ethanesulfonic acid sodium salt (MES) were from Fisher Scientific, Springfield, NJ. MDA-MB-231 human breast cancer cells were from ATCC.

Preparation of Microcapsule CAs. Microcapsules of ~1 μm were initially prepared by a double water in oil in water ((W/O)/W) emulsion solvent evaporation process using camphor and ammonium carbonate as removable cores, previously described in El-Sherif et al.[21] Briefly, camphor (0.05 g) and PLGA (0.5 g) were dissolved in 10 mL of methylene chloride. To generate the first water in oil ((W/O)) emulsion, 1.0 mL of 4% ammonium carbonate solution was added to the polymer solution and probe-sonicated at 115 W for 30 s. The (W/O) emulsion was then poured into a 5 wt % PVA solution and homogenized for 5 min at 9500 rpm. The (W/O)/W emulsion was then poured into a 2% isopropyl alcohol solution and stirred for 1 h with a magnetic stirrer on a magnetic stir plate at a speed fast enough to create a vortex that spanned the entire solution. The microcapsules were collected by centrifugation for 5 min at 5000*g*, washed three times with hexane, then once with deionized water, and lyophilized, using a Virtis benchtop freeze-dryer, to remove the camphor and ammonium carbonate core.

The preparation conditions were optimized by independently varying three parameters: the temperature control during sonication (air at room temperature or a water bath at 25 or 4 °C), the sonication conditions (continuous versus pulsed sonication), and the length of time over which samples were freeze-dried (freeze-drying until a constant weight was achieved).

Peptide Conjugation. Peptide was immobilized on the CA surface by an adaptation of the dicarbodiimide method.[19,22] Briefly, the dried microcapsules (100 mg) were combined with 5 mg of EDC (1:1 molar ratio of −COOH groups in the microspheres to EDC), 2.7 mg of NHS (1:2 molar ratio to EDC), and 10 mL of buffer (0.1 M MES, 0.3 M NaCl, pH 6.5) and shaken on a rotary shaker (C1 Platform Shaker, New Brunswick Scientific, Edison, NJ) for 15 min. GRGDS peptide (150 μg, 1:10 molar ratio of −COOH groups in microcapsules to GRGDS peptide) was then added and shaken for 3 h. The microcapsules were then washed with deionized (DI) water (18 MΩ, Barnstead E-pure system, Dubuque, IA) and lyophilized using a Virtis benchtop freeze-dryer. The conjugation conditions were optimized by varying the reaction time from 3 to 2 to 1 h, while placing the reaction mixture on the stirrer on a thick styrofoam pad to shield the solution from heat generated by the stirrer.

Morphology. Capsules were viewed using a Phillips (Amsterdam, Netherlands) XL-30 environmental scanning electron microscope. Briefly, the samples were secured using carbon tape and platinum sputter-coated for 100 s in a Denton Desk sputter-coater prior to imaging. The samples were placed in the microscope in a round sample holder and put under vacuum. High-definition photographs were taken at an imaging distance of approximately 10 mm on the *z*-axis.

Acoustic Testing. Acoustic properties of microcapsule preparations were characterized as described previously.[21] Briefly, a pulse−echo setup was used for in vitro studies, employing a single-element, broadband, 12.7 mm element diameter, 50.8 mm spherically focused transducer with a center frequency of 5 MHz (Panametrics, Inc., Waltham, MA). This frequency was chosen because it was shown to give the maximum enhancement for the previously developed microcapsules.[21] The −6 dB bandwidth of the transducer was 91%. The pulse lengths of the transducer were 1.2 mm, corresponding to 0.9 cycles/pulse length. A 0.5 mm diameter polyvinylidene fluoride needle hydrophone (Precision Acoustics) was used to measure the negative peak pressure at 0.838 MPa. The hydrophone was calibrated from 1 to 20 MHz in 1 MHz steps by the National Physics Laboratory (Dorchester, U. K.).

The transducer was inserted in a water bath ($0.5 \times 26.7 \times 25.4$ cm^3 custom-built acrylic tank filled with deionized water (37 °C)) and focused through an acoustic window of a custom-made sample vessel. During the insonation, the transducer was positioned within the water bath at a depth of 14 cm from the top of the liquid in the sample vessel, corresponding to a pressure of roughly 11 mmHg (similar to the 7 mmHg reported as a mean pulmonary artery pressure in rabbits). A pulser/receiver (model 5072 PR, Panametrics, Inc., Waltham, MA) was used to pulse the transducers at a pulse repetition frequency of 100 Hz. The received signals were amplified and fed to the digital oscilloscope (Lecroy 9350A, Lecroy, Chestnut Ridge, NY). The digitized data was stored and analyzed using LabView (National Instruments, Austin, TX).

The sample vessel containing 100 mL of phosphate buffered saline (PBS), pH 7.4, was held in the water bath with a stable mounting. The cover of the bath was fitted with an $x-y$ positioning system (Edmund Scientific, Barrington, NJ) to position the ultrasonic transducers. The microcapsules were weighed, suspended in 200 μL of PBS, and injected into the sample vessel in 10 μL doses, which was stirred continuously with a magnetic stirrer at a speed that prevented entrainment of atmospheric gas bubbles. The reference (PBS) was taken as an average of six values.

Cumulative dose−response curves were constructed for doses in the range of 0.0015−0.16 mg/mL at 37 °C. The exact amount varied depending on the history of the CA. After the dose−response curves

were established, stability (time−response) curves were performed for the lowest steady-state dose. Time−response curves record data points once a minute for 15 min at 37 °C. Results were then normalized to allow for comparison between samples with different starting decibel values.

Cell Culture. MDA-MB-231 human breast cancer cells were cultured using growth medium containing 90% RPMI 1640 with L-glutamine, 9% FBS, and 1% penicillin/streptomycin antibiotic in a humidified 5% CO_2 environment. Experiments were conducted between the third and fourth passage.

Attachment Studies. For static in vitro attachment studies, MDA-MB-231 cells were seeded on 12 well culture plates and permitted to grow to confluence. On the day of experimentation, growth media was prepared that contained either the microcapsule CA or peptide-modified CA at a concentration of 0.5 mg/mL. The cells were first washed with media and then incubated with the 2 mL of CA-containing media for the specified time points. All studies were performed in triplicate, and one sample set was preblocked for an hour prior to experimentation with the same peptide on the surface of the peptide-modified CA at a concentration of 10 μg/mL in 2 mL of medium. Counting was performed by a blinded observer. At the specified time point, the medium was removed, and the cells were washed with 2 mL of growth media solution twice. The zero time points involved contacting the capsules with the cells and at once washing them off. The cells were then viewed under a Wesco Verta 7000 series microscope. Digital pictures were taken with an Olympus DP11 digital camera interfaced with the microscope. Three pictures per group were assessed for microbubble attachment. Microbubble attachment was determined by visual inspection. Prism software (Graphpad Software, San Diego, CA) was used to analyze the data sets for CA attachment to cells. The parameter of microbubbles attached per cell was compared between each experimental group and between experimental and control groups. A two-way analysis of variation (ANOVA) was used to determine significance. For comparison between experimental groups, Newman−Keuls multiple comparison tests were performed, and *p*-values were obtained.

Results

PLA−GRGDS CA Attachment to MBA-MB-231 Cells. The in vitro attachment of ligated PLA microcapsules to MDA-MB-231 cells at 0, 5, 10, and 15 min in tissue culture was quantified and statistically analyzed. GRGDS peptide-modified microcapsules demonstrated improved attachment over the unmodified microcapsules in all cases. Receptor specificity was demonstrated through the use of cells with receptors preblocked with the peptide sequences GRGDS or RGDS prior to incubation with GRGDS peptide-modified microcapsules. It was hypothesized that either sequence should have a similar effect since both have the serine (S) following the aspartic acid (D), which was shown to be vital in binding, and in the ligated capsules, the glycine (G) of the GRGDS sequence was bound to the polymer and thus potentially inactivated.[23] The quantified results for the entire study can be seen in Figure 1. There was no significance between the blocking peptides at a *p*-value of less than 0.01, and a post hoc unequal *N*-test showed no significant difference between the two different peptides at a *p*-value of less than 0.01 (results of RGDS not shown). Once this was established, results of blocking by the two peptide sequences were combined into the same group and a two-way ANOVA was performed on all three groups as well as time. This relatively high measure of confidence was chosen due to the complex nature of the visual examination and counting of capsules on the cells. The time points, groups, and interaction between the time points and the groups were all shown to be statistically significant at a *p*-value of less than 0.01. The post hoc Scheffe test showed no significance between the unmodified PLA microcapsules and the preblocked sample with peptide-modified microcapsules. However, there was statistical significance between the peptide-modified microcapsules and both the unmodified PLA microcapsules and the preblocked sample with peptide-modified microcapsules at a *p*-value of less than 0.01. Quantified results using PLGA 85:15 capsules were identical (results not shown). Light micrographs taken after only 5 min of contact time of PLGA 85:15 capsules ligated with GRGDS are shown in Figure 2. Note the cell rounding that occurs with the cells preblocked with GRGDS and also to the cells targeted with GRGDS-ligated capsules.

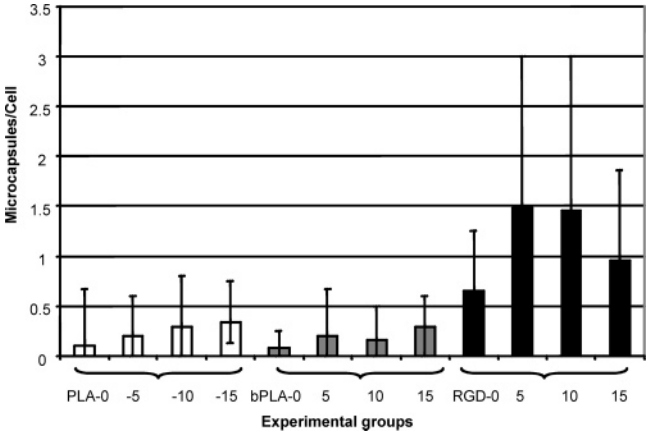

Figure 1. Quantification of microcapsules attached per cell (n = minimum of 9 fields, ± represents range). PLA represents blank capsules; bPLA indicates PLA−GRGDS capsules directed against cells preblocked with peptide. RGD represents PLA−GRGDS capsules directed against unblocked cells. Number after legend indicates time of exposure.

Acoustic Characterization. The dose−response curves for ligated CA (PLA−GRGDS) made by the original method were performed at physiological temperature (~37 °C) and are shown in Figure 3 (square symbols). Although the PLA−GRGDS is echogenic and with a high enough dose (0.16 mg/mL, open symbols) eventually reaches a maximum approaching 20 dB, in comparison to the dose−response curves for the unmodified microcapsules (closed symbols, maximum reached at 0.012 mg/mL), a substantially larger dose is required to reach this maximum. The acoustic stabilities of the CAs after the peptide conjugation process (that is, how well the CA retained its echogenicity during insonification) using an initial dose (0.036 mg/mL) close to the dose giving maximum enhancement are shown in Figure 4. The data were normalized to the initial acoustic enhancement at time 0 min to compare percent loss from the baseline. The time response indicates that, unlike the unmodified agent that is relatively stable (5% loss in 15 min), there is a substantial (60%) reduction in acoustic enhancement over 15 min for the modified agent. The peptide-modified contrast agent is more fragile. However, the reduced acoustic properties for the peptide-modified PLA are less than that reported by us for an unmodified contrast agent made from the related copolymer PLGA 50:50 at 37 °C.[20] The reduction in acoustic enhancement at 37 °C may be due to several factors. To attach the peptide to the surface of the PLA contrast agent, over 3 h of incubation in aqueous buffer is recommended, conditions that initiate CA degradation, resulting in fewer intact, gas-filled capsules to produce enhancement. Further, we have shown that ultrasound causes capsules to degrade at an accelerated rate, and the weakened microcapsules could be more susceptible due to constant insonation at 5 MHz over 15 min.[24]

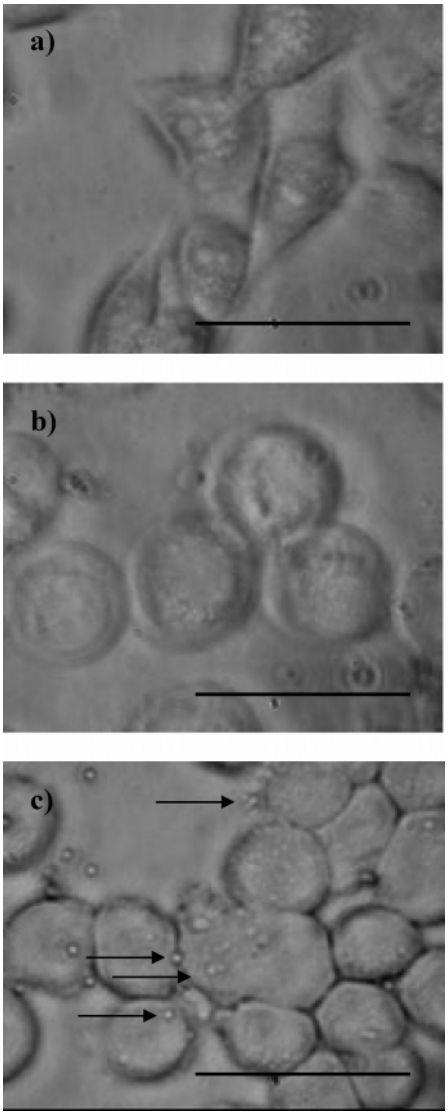

Figure 2. Light micrograph of MDA-MB-231 cells grown in tissue culture and targeted with different types of PLGA 85:15, GRGDS-ligated capsules with a contact time of only 5 min. (a) Control, cells contacted with PLGA 85:15 capsules with no ligand attached, (b) cells preblocked with free GRGDS, then treated with ligated contrast agent, (c) cells treated with GRGDS-ligated contrast agent made from PLGA 85:15. Bar represents 50 μm. Arrows indicate attached capsules.

Process Improvement. The approach to improving the production and use of PLA−GRGDS was in three steps. First, the initial CA fabrication methods were modified to improve the morphology and echogenicity of the standard microcapsules. Second, the conjugation process was modified to reduce the loss of echogenicity that occurred after the aqueous peptide conjugation process. Finally we addressed attachment of the CA to the target receptor and determined the effect of the surface density of peptide on the CA, since a relationship between cell attachment and RGD surface density has been demonstrated.[18,25−28]

During fabrication of the normal PLA microbubbles, sonication using a Misonix sonicator XL continuously for 30 s caused the solution to become hot, an increase of 8 °C over room temperature (usually 24 °C). This higher temperature accelerates hydrolysis of the polymer, which at this point is in an aqueous environment, and also brings it closer to the glass transition temperature. The reaction rate approximately doubles for every 10 °C temperature rise. Therefore the procedure was modified to include sonication with the vessel standing in an ice bath and using a regime of 3 s on and 1 s off for a total of 30 s instead of continuous sonication. Through the use of this procedure (arrived at by systematic trial as described in methods) the temperature of the solution dropped 6 °C by the end of sonication. The freeze-drying time was also increased from 24 to 48 h (this change was employed both for CA fabrication and after peptide modification). This was done to ensure the complete sublimation of the encapsulated water, ammonium carbonate, and the camphor in the shell as well as to ensure that the microcapsules were sufficiently hardened from drying.

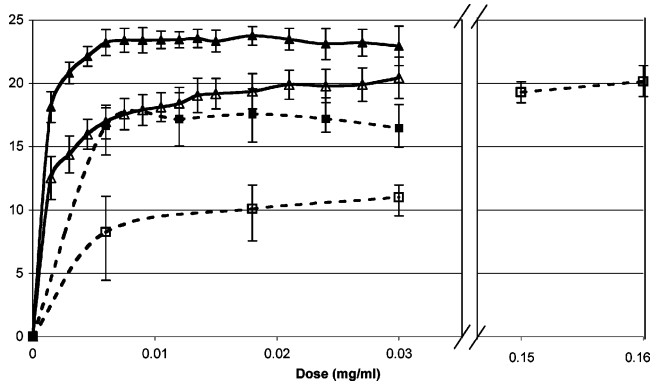

Figure 3. In vitro dose−response curve (acoustic enhancement as a function of dose) of PLA (closed symbols) and PLA−GRGDS (open symbols) CA, original method (■, □) and improved method (▲, △), insonated with a 5 MHz transducer ($n = 3$, ± represents range).

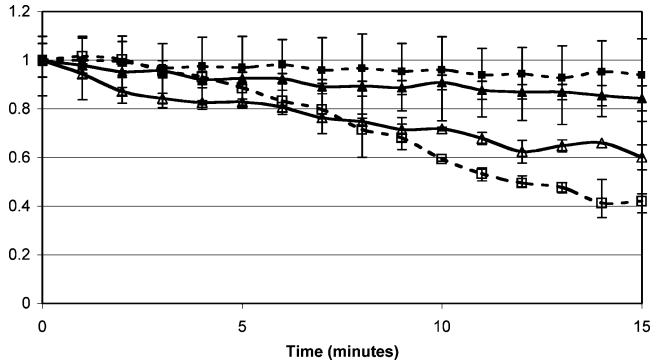

Figure 4. Normalized time response of PLA (closed symbols) and PLA−GRGDS (open symbols) at 37 °C, original method, (■, □), improved method (▲, △), insonated with a 5 MHz transducer ($n = 3$, ± represents range).

Improvements in the processing conditions produce a CA that is highly echogenic but less robust under insonation conditions (Figures 3 and 4, solid symbols). The maximum reflected signal increased from 17 to 23 dB, and the required dose to achieve this maximum is reduced by half from 0.012 to 0.006 mg/mL. Through the use of ANOVA with a Newman−Keuls comparison test the improvements in contrast agent manufacture with respect to echogenicity were found to be significant ($p < 0.05$).

The loss of signal after 15 min of insonation (a more than adequate time frame for a diagnostic ultrasound scan) for the "improved" fabrication method was 16% (Figure 4, solid triangular symbols) compared to 7% loss with the original preparation methods (solid square symbols). Through the use of ANOVA with a Newman−Keuls comparison test, however, the improvements in contrast agent manufacture with respect to stability, unlike the improvements in intrinsic echogenicity, were not found to be significant ($p < 0.05$).

The peptide ligation procedure for these improved CA capsules was modified to prevent any increase in solution temperature that had been noted during the incubation period,

Table 1. Comparison of Acoustic Properties of CAs PLA and PLA−GRGDS before (Original) and after (Improved) Process Improvements[a]

	minimum dose for maximum dB (mg/mL)		maximum enhancement (dB)		dose used for stability test (mg/mL)		signal loss insonated 15 min @ 5 MHz	
	original	improved	original	improved	original	improved	original	improved
Pre-GRGDS	0.012	0.006	17 ± 2.2	23 ± 1.8	0.036	0.006	0.9%	15%
Post-GRGDS	0.16	0.02	20 ± 3.9	20 ± 2.4	0.036	0.006	60%	40%

[a] All values quoted for 37 °C.

and the speed of shaking was also decreased to reduce shear damage to the microbubbles during incubation. A 1.75 in. thick styrofoam insulation platform was introduced on the shaker. Finally, the reaction time was reduced from 3 to 1 h in MES buffer, a procedure that had been shown to be effective elsewhere.[17] These changes to the peptide modification procedure in combination with the improvements in the CA manufacturing resulted in a reduction in the dose required to achieve maximum in vitro acoustic response (20 dB in each case) from 0.16 mg/mL before improvements were implemented down to 0.03 mg/mL (Figure 3, open symbols), PLA−GRGDS original versus PLA−GRGDS improved ($p < 0.01$). In both cases, as would be expected, the dose required was significantly higher ($p < 0.01$) after peptide modification in an aqueous environment, compared to premodification CAs (open vs closed symbols).

The acoustic stability of the GRGDS-modified agents made by the improved methods compared to that of modified CAs made by the original methods was not significantly different ($p > 0.05$), with the capsules losing 40% and 60% of their enhancement activity, respectively, under constant insonation for 15 min (Figure 4, open symbols). Table 1 lists the key properties of the original method of CA preparation and ligation and the new methods.

The morphology of the ligated contrast agent changed from capsules that were broken, or with large indentations that gave the capsules the appearance of being collapsed upon themselves (Figure 5c), to capsules that were spherical and intact (Figure 5b) by adopting these modifications. As can be seen in Figures 5a and 5b, before peptide ligation the capsules are smooth and unbroken. After ligation (shown here for a reaction time of 1.25 h, Figure 5b), some capsules are broken, mostly the larger ones, and if the modification reaction is continued for 3.25 h, then most of the capsules appear broken (Figure 5c).

Effect of Surface Density of Peptide. To investigate the potential of an optimum surface density of ligands for binding to the cells, total carboxyl/peptide molar ratios of 1:0.33, 1:0.5, 1:0.66, or 1:0.8 were used in the peptide attachment step. Total carboxyl was used as a convenient measure since the exact surface concentration is not known. MDA-MB-231 cells grown in tissue culture were challenged with the modified capsules, and the total number of capsules adhering per cell was estimated under a microscope. As seen in Figure 6, the optimal carboxyl/peptide group ratio was 1:0.5, giving a maximum attachment of approximately 1.8 ± 0.17 microcapsules/cell after 10 min of contact. This compares with 1.4 ± 0.86 capsules/cell prior to modification. Through the use of ANOVA (Newman−Keuls multiple comparison test), the microcapsules conjugated with a 1:0.5 ratio had statistically significant greater attachment ($p < 0.001$) compared to the two controls, one of unmodified PLA CA contacted with the cells and the other of GRGDS-modified CA contacted with preblocked cells. The 1:0.66 and 1:0.8 ratios were also significantly different from both controls with *p*-values of <0.01 while the 1:0.33 ratio was statistically significant from those of control groups with a *p*-value of <0.05.

Discussion

The initial cell adhesion study (Figure 1) indicates that the polymer CA can be modified to promote cell adhesion. Second, this adhesion is significantly more than the control ($p < 0.01$) and thus is likely to be through the integrin sequences targeted through the GRGDS peptide. The observed drop between the 10 and the 15 min numbers is probably the influence of experimental conditions. It is possible to rule out receptor cleaving being the cause of the observed 15 min drop because the receptors that were targeted were integrins, membrane spanning receptors not known to be easily cleaved. Receptor shedding is more likely in targeting a receptor such as p-selectin since these receptors are not membrane bound and are activated in leukocyte rolling. The drop is more likely the result of the experimental protocol in which the culture plate was exposed to changing environments, namely, temperature and moving growth medium, as a result of coming in and out of the incubator. Current studies in which the cell environment is under tighter control do not show this drop.

Similar cell attachment studies were conducted using CA prepared using PLGA 85:15, in which the polymer backbone is composed of 15% glycolic acid residues.[25] Almost identical results were obtained. The glycolic acid residues in the PLGA are less hydrophobic than the lactic acid residues, since they do not contain the methyl group. However, these results compared with the results outlined here indicate that at least up to 15% of the glycolic acid residues do not alter the affinity of the capsules for the cells. This is desirable since future studies will combine targeting and drug delivery, where the hydrophobicity of the CA shell does have a large influence on the amount of drug that can be loaded into the agent, a result that is highly dependent on the hydrophobicity of the drug in question.

In all in vitro targeting studies it was noted that the cells became round when contacted with free GRGDS peptide, as noted in Figure 2. This has been reported previously in studies involving synthetic extracellular matrix (ECM) development. The loss of attachment of cells to the ECM can be induced in the presence of free RGD peptides.[17] Often this loss of attachment can cause apoptosis, a process known by the Greek word for homelessness, anoikis.[31] Other researchers have reported an active integrin-mediated death pathway in which the cells do remain attached to the substrate. In this mechanism unligated integrins recruit capsase 8 to the membrane and activate an apoptotic pathway.[32] In either case, anoikis or some other cell-mediated death, it would in certain instances be helpful should a ligated CA also induce a cell cascade that initiated death. In this way the agent targeted against a cancer would both image it and lead to its destruction.

The acoustic characterization of the capsules ligated to GRGDS showed that, after modification with ligand, a higher dose of CA was required to achieve maximum acoustic enhancement (Figure 3). The most likely cause of this is the fact that some of the microcapsules are being damaged during the conjugation process, which is carried out under aqueous conditions, conditions that promote polymer hydrolysis. The

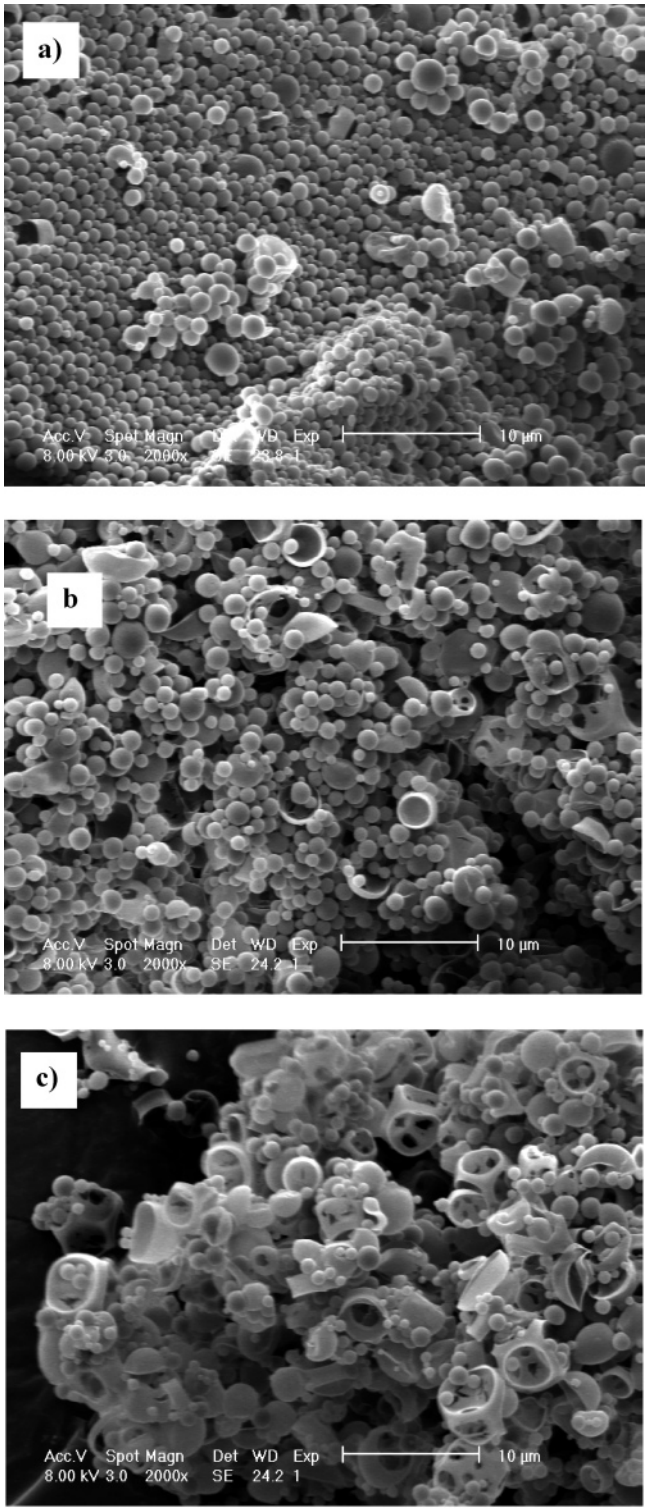

Figure 5. Scanning electron microscopy images of PLA microcapsules: bar = 10 μm; magnification, 2000×. (a) Unmodified microcapsules, (b) capsules after a 1.25 h ligation procedure, (c) capsules after a 3.25 h ligation procedure. Capsules were made with the improved method.

peptide-modified contrast agent is more fragile (Figure 4, showing loss of echogenicity with time). However, even the peptide-modified PLA has superior acoustic properties compared to those reported by us for an unmodified CA made from the related copolymer PLGA 50:50 at 37 °C, for which the signal drops close to baseline within the first 5 min, with a half-life of less than 3 min.[20] To attach the peptide to the surface of the PLA contrast agent, over 3 h of incubation in aqueous buffer is

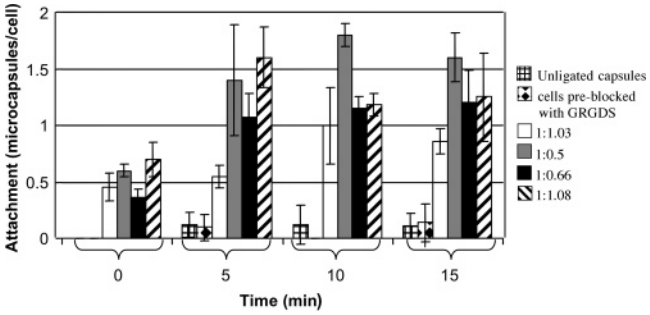

Figure 6. Effect of surface density of GRGDS ligands on the static attachment of capsules to MDA-MB-231 cells in tissue culture (n = 3, ± standard error from the mean). Numbers indicate ratio of −COOH groups to GRGDS.

recommended in the standard conjugation protocols, conditions that initiate CA degradation, resulting in fewer intact, gas-filled capsules to produce enhancement. Further, we have shown that ultrasound causes capsules to degrade at an accelerated rate, and the weakened microcapsules could be more susceptible to constant insonation at 5 MHz over 15 min.[24] This is born out in the scanning electron microscopy results (Figure 5). For capsules prepared by the improved fabrication methods, this loss of integrity was especially evident in preparations that were incubated with ligand for over 3 h. The changes in both microcencapsulation and conjugation methods produced a PLA−GRGDS agent that was several fold more echogenic even than the original, unligated capsules. However, the new agent demonstrated an increase in fragility even prior to ligation, which is probably due to the production of an agent with a thinner wall. This is balanced against the fact that a much smaller dose is required to achieve a higher acoustic enhancement. The results emphasize the need to carefully tailor microencapsulation and conjugation methods to the application at hand and highlight the fact that compromises need to be reached between optimal contrast properties and high targeting abilities. Choosing a targeted agent that does not have the highest affinity for the target receptors may not be severely detrimental; for example, Adams et al. have shown that intrinsic affinity properties regulate the quantitative delivery of antitumor single-chain molecules to solid tumors and the penetration from the vasculature into tumor masses with an upper limit.[33]

The surface density of conjugated ligand must also be considered, as confirmed by the fact that we observed a concentration-dependent optimum in capsule adhesion (Figure 6). The most recent results from the related field of cell surface attachment and reported by Massia et al.[18] give the most conservative ligand density that is required for cell attachment to a ligated surface (in this case glycophase glass) as 1 fmol/cm^2 required for maximal spreading. This corresponds to 6 ligands/μm^2, or an average peptide-to-peptide spacing of 440 nm. Earlier studies by other groups using less rigorous systems quoted as high as 400 fmol/cm^2, corresponding to 2400 ligands/μm^2, or an average peptide-to-peptide spacing of 22 nm.[34] Our microcapsules of 1.2 μm in diameter have a total surface area of 4.5 μm^2, of which only a small faction will be in contact with the target cell. It is probably unreasonable to expect that cell-accessible peptide densities comparable to 2400 ligands/μm^2 [34] are achievable, but certainly cell-accessible densities of 6 ligands/μm^2 [18] could be expected. The fact that we see capsule adherence to MDA-MB-231 cells (Figures 1, 2, and 6) adds credence to the more conservative values quoted by Massia et al. Studies are ongoing to quantify ligand density.

Finally it is acknowledged that the RGD sequence targets many integrin receptors and that the studies described here are

exploratory. The final choice of ligand will need to render the targeting CA highly specific to the desired cell population. Peptide sequences are preferred over larger potential ligands such as antibodies for many reasons, including their greater stability toward enzymatic degradation, their lower immunogenicity, and lower cost. Fortunately it has been found that even minor changes to the RGD sequence, including strategies which add a D-amino acid in flanking sequences or presenting the RGD as part of a cyclic peptide, have shown great promise in increasing target specificity.[23]

In conclusion, we have shown that in the development of targeted ultrasound CAs composed of a biodegradable polymer, processing parameters have a significant influence on the echogenicity, stability during insonation, and targeting ability of the resulting agent. The end use must be carefully considered during development, and the various factors contributing to these properties must be balanced against each other to achieve an agent that is optimized for the required situation.

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