

This article discusses the fundamentals of ultrasound contrast agents including new innovations like targeted imaging.

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Surface Modification of Polymeric Contrast Agents for Cancer Targeting

by Justin D. Lathia, Dalia El-Sherif, Nikhil O. Dhoot, and Margaret A. Wheatley

Background

Ultrasound Imaging

Ultrasound is a popular medical imaging technique that has gained increased usage over the past 10 years. Some well-known uses of ultrasound include the in utero imaging of a fetus and the imaging of the heart to evaluate heart function. However, the applications and potential of ultrasound imaging extend well beyond pre-natal and cardiovascular imaging, including major human health issues such as tumor detection.¹

Ultrasound is a non-invasive medical imaging technique that relies on high-frequency sound waves to produce an image. Since most tissues are heterogeneous in structure, a sound wave sent via a transducer acting as both a transmitter and a receiver will be scattered or reflected.² It is this scattered energy, or backscatter, that returns to the transducer and from which an image can be produced. Ultrasound was first used for medical imaging of soft tissues in the 1970s when the technology was capable of capturing and displaying backscatter.² The first images that were captured were static, but as the technology improved, real time images were possible, as seen today in the imaging of a moving fetus in the uterus.

Ultrasound energy can produce many types of images depending on the mode by which the sound wave is sent and received. The first images that were produced used line of sight displays, such as in radar and sonar, and are referred to as A-mode. A-mode images are displayed as the amplitudes of the signals received and could initially be seen only on oscilloscopes. A-mode images recorded side by side to show motion are referred to as M-mode, which were produced on special thermal paper. The sweeping of the transducer, either electronically or mechanically, over an area to

produce a two-dimensional image is referred to as B-mode imaging.² B-mode images are different than A-mode images because they display images that vary in brightness instead of amplitude, in relation of the strength of the signal received. B-mode images could be seen on television monitors as a result of the build up of multiple scans of static images. Using more sophisticated equipment and image analysis techniques, it is now possible to see all three imaging modes on television monitors.

The first uses of ultrasound imaging were to study motion, such as in the heart. A-mode and M-mode imaging techniques were used to study many changes in the heart that correlated to clinical conditions. For example, motion of heart valves, thickening of heart chamber walls, and heart motion in relation to pressure. Many of these analyses are still used today for cardiac monitoring. B-mode imaging is two-dimensional and was first used in the imaging of soft tissue and obstetrics.² More recent applications of ultrasound imaging include three-dimensional imaging and Doppler imaging, used for flow analysis. With modern ultrasound, blood flow can be imaged to determine abnormalities in blood vessels and cancers can be detected through the vascular changes caused by tumor development. Research is being done on drugs that can be delivered locally using ultrasound energy as a release mechanism.³

Ultrasound Contrast Agents

Ultrasound relies on an interface between two different types of tissues to produce an image. At the interface of the two different tissues, there is a difference in properties, which is referred to as an impedance mismatch. Impedance is related to the product of density and the speed of sound through a material. Thus, skin and bone have different impedances and an image of bone can easily be produced. In a

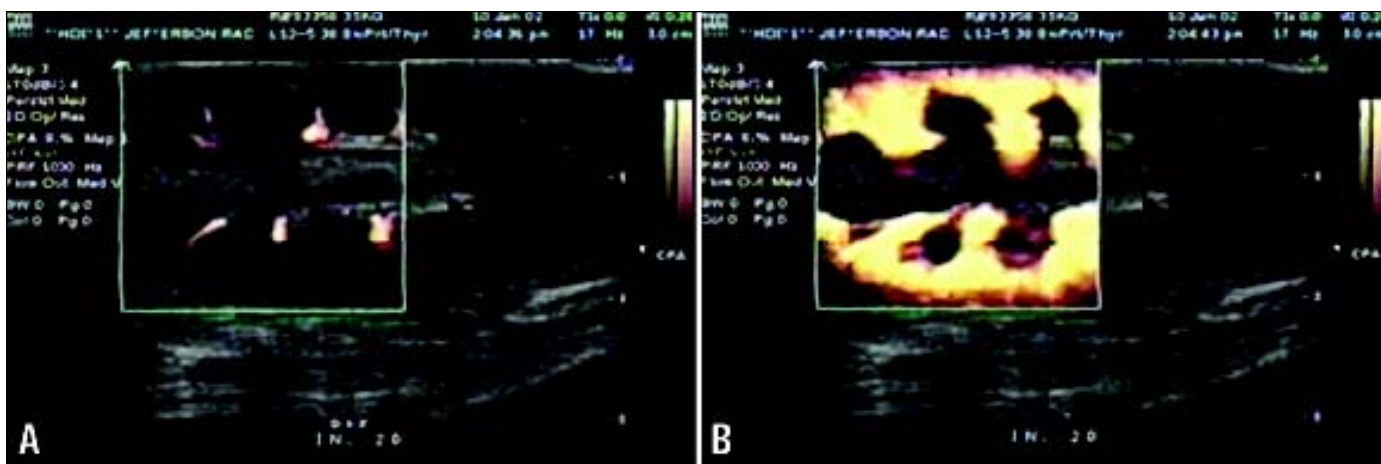


Figure 1. PLGA (50:50) contrast agent injected into a rabbit kidney and imaged in Power Doppler mode (Courtesy of Thomas Jefferson University, Department of Radiology). The Doppler image is inside the box and shows parenchymal enhancement. A) pre-injection, B) Post injection (0.15ml/kg (2.42 x 10⁻⁶ ft³)).

situation where the tissue is homogenous and there are no differences in impedances, such as the imaging of breast tumors, an ultrasound contrast agent is needed. A contrast agent provides a difference in impedance and makes imaging which was impossible now possible. Currently, contrast agents are used in two-dimensional imaging and color Doppler imaging, but they are under investigation of use in three-dimensional imaging as well as drug delivery.⁴

Most contrast agents utilize the impedance mismatch between gas and the suspending fluid, usually blood, to cause contrast that can be imaged. In most cases, the contrast agents are spherical, thin shelled, microbubbles. There are five types of systems which were noted to make good contrast agents: free gas bubbles, encapsulated gas bubbles, colloidal suspensions, emulsions, and aqueous solutions.⁴ Because contrast agents are injected into the body, there are several requirements which they must satisfy. The contrast agent should be biocompatible, not cause any hemodynamic effects, nor alter blood flow. The contrast agent should be smaller than a red blood cell so it will not block any smaller capillaries and should have similar a physiological transit time and velocity profile to that of a red blood cell. Additionally, the contrast agent should perfuse into the target tissue, stay in the circulation long enough to be imaged, and remain stable long enough for recirculation.⁴⁻⁵ The backscatter produced by the contrast agent is dependent on the physical characteristics of the microbubble. The reflectivity produced is proportional to the concentration of the microbubbles present at a given site and to the fourth power of microbubble diameter.⁶ The most important characteristic of the contrast agent is the resonating ability of the bubble, which can produce up to three orders of magnitude more enhancement. Fortunately, the resonance frequencies of microbubbles of 1 - 7 microns in diameter are in the range of frequency for medical imaging, 2 - 15 MHz.⁶

The first contrast agent was discovered by accident by Gramiac⁷ and Joiner in the later 1960s. During an M-mode echocardiogram, Joiner noticed a transient ultrasound signal, which was later discovered to be a result of small bubbles

that formed at the tip of the catheter.⁶ After this discovery, many hand agitated agents emerged, but their performance was plagued by transient effects and their results were not consistent. It was found that mixing a patient's blood with saline and injecting it back into the body by two syringes through a three-way tap produced bubbles that were stable.⁶ It was discovered that sonication of blood produced stable microbubbles of a controllable size. It was later found that the blood protein, albumin, was responsible for the stability. The first contrast agent, Albunex[™], was made from sonicated albumin. After Albunex[™] emerged onto the market, many other contrast agents based on different principles followed. Levovist[™] and Echovist[™] are made from galactose powder. By adding water, small air microbubbles form on the surface irregularities of the galactose crystals and when injected into the blood stream, the galactose crystals dissolve releasing the air microbubbles.⁶ Sonovue[™] and Definity[™] are biological membranes, phospholipid, filled with air.⁶ Sonovist[™] is made from a biodegradable synthetic capsule filled with sulphur hexafluoride.⁶ The newest FDA approved contrast agent is Definity[™]. It is used for echocardiographic examinations and consists of a perfluoropropane gas encapsulated within a lipid shell.⁸

Polymeric Contrast Agents

As research has advanced in the fields of biomedical engineering and materials science, polymers have emerged as a key biomaterial. Much of the processing technology for polymers is already available though similar manufacturing methods in plastic processing, so production is easily possible. The structure of polymers also is controllable and polymers can be made with a variety of different mechanical properties. Most importantly, many polymers are inert and biocompatible, thus they do not pose a large risk when implanted into the body. For these reasons, polymers have become a useful implant material with applications ranging from joint replacements to drug delivery devices.

With the emergence of polymers as a premier biomaterial, ultrasound contrast agents made from polymers also have

been explored. The novel polymeric contrast agent developed in our laboratory is based on an emulsion and freeze-drying technique principle and uses poly (lactic - co - glycolic) acid (PLGA). PLGA has been FDA approved for use in sutures and drug delivery devices. PLGA is also biodegradable and biocompatible. It degrades into lactic and glycolic acid by non-enzymatic hydrolysis of the ester backbone. Both lactic and glycolic acid are able to be processed by the body.⁹ Our PLGA contrast agent is formed by a patented¹⁰ double emulsion technique in which camphor and ammonium carbonate are encapsulated and then later sublimed, creating an echogenic microsphere.¹¹ The PLGA contrast agent developed by our group has shown good enhancement of the blood flow in a rabbit model - *Figure 1*.

Angiogenesis

One emerging application is the use of ultrasound to detect, diagnose, and monitor angiogenesis. Angiogenesis is the formation of blood vessels from existing vessels and is an early sign in many types of developing tumors. Angiogenesis is also involved in wound repair and inflammation.¹² In the first step in angiogenesis, the parental vessels vasodilate and the basement membrane of the vessels begin to degrade via proteolytic enzymes. Endothelial cells begin to migrate into the extracellular space and proliferate to form a leading edge. The endothelial cells form tubules with lumen and begin to synthesize basement membrane. Finally, the tubules anastomose and recruit smooth muscle cells and pericytes to complete the vessel structure. This process can also be seen in the second half of embryonic vascular development where the immature vessels are stabilized through growth factors such as Vascular Endothelial Growth Factor (VEGF) as well as angiopoietins.

In the 1960s, Dr. Judah Folkman saw the correlation between this process and the development of vasculature during the early stages of cancer. Tumors implanted into isolated organs showed limited growth while tumors implanted into mice grew rapidly and eventually killed the host.¹³ These results sparked the idea that for a solid tumor to develop, growing vasculature is necessary. The angiogenesis in tumors is similar to the angiogenesis that occurs in developing vessels, however tumor angiogenesis is not as tightly regulated and is more chaotic.¹⁴ While it is possible to detect a tumor using various techniques, confidently determining if the tumor is malignant is more difficult. In this area, ultrasound can be used in a non-invasive way to assess the state of the tumor. By using the Doppler technique, it is possible to measure blood flow and correlate the results back to the functionality of the developing vascularity.¹ Since Dr. Folkman's observation, the understanding of angiogenesis in relation to cancer has been heavily studied through various techniques such as Doppler imaging, and inhibition of angiogenesis is being researched as an alternative therapy to chemotherapy.

Angiogenesis Targeting

The start of angiogenesis occurs when a signaling molecule,

in this case a angiogenic growth factor, binds to the cell via a cell surface receptor and initiates a sequence, which results in new blood vessel formation. Integrins, a type of cell surface receptor, directly associate with the receptors that bind these growth factors and have the capacity to affect the outcome of the cell's behavior.¹⁵ The endothelial cells that line the blood vessels also express receptors for angiogenic growth factors. As a tumor grows and develops it requires vasculature and it has been shown that chemotherapeutic agents bound to the growth factor receptors resulted in localized cytotoxic effects.¹⁶ This observation suggests that if these receptors can be targeted, angiogenesis may be affected and thus, the growth of the developing tumor can be slowed down or even stopped.

Through much experimental evidence, the link between angiogenic growth factors and angiogenesis has been determined to be modulated through the cell surface integrins, $\alpha_v\beta_3$ and $\alpha_v\beta_5$.^{17,18} Using an accepted angiogenesis assay, the chick chorioallantoic membrane (CAM) which sprouts vessel during its development and can be used to either induce or inhibit angiogenesis, it was determined that molecules blocking $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors (anti- $\alpha_v\beta_3$ and anti- $\alpha_v\beta_5$) can slow down the progression of angiogenesis.¹⁷ Since these findings, efforts have been made to find antagonists for these receptors or use the receptors to target angiogenesis in developing tumors.

Cell surface integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, among many others, have been found to bind to a generic peptide sequence arginine-glycine-aspartic acid (RGD), which is found in the extracellular matrix protein (ECM) fibronectin.¹⁹ The RGD sequence is a versatile peptide sequence and has been used as both a coating to encourage cells to bind to materials as well as a coating to bind smaller, free floating particles to target cells. In a mouse model, an RGD peptide sequence specific to α_v integrins was shown to bind specifically to the blood vessels in human tumors and less in the surrounding normal vasculature.²⁰ As seen in the literature, RGD containing peptide sequences specific to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins have been used to target angiogenesis with good success.

Ultrasound Targeting

With recent advances in cell and molecular biology as well as ultrasound imaging, research is being done to identify changes that occur in pathologic condition using targeted imaging. Such imaging can be used to detect a change in tumor vasculature and then correlated back to the malignancy of the tumor. This type of imaging requires that ultrasound contrast agents be modified with molecules, such as antibodies or peptides, which target the pathology and then are viewed using ultrasound imaging. In recent studies, such targeted contrast agents have been shown to be successful at targeting larger pathologies such as clots. Using a monoclonal antibody that targets thrombi formation, a targeted perfluorocarbon emulsion was made to detect thrombi in the vasculature.²¹ The detection of thrombus formation could be indicative of pathologic conditions such as coronary artery disease and peripheral vascular disease. In the early stages,

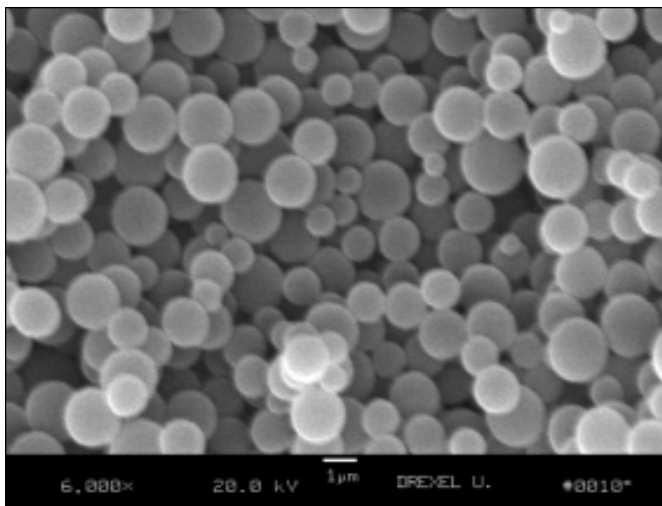


Figure 2. PLGA (50/50) microcapsules imaged with a Scanning Electron Microscope (Amray model 1830D), size bar indicates 1 μ m (3.94 x 10⁻⁵ in).

atherosclerosis is marked by an increase in endothelial cell expression of leukocyte adhesion molecule (LAM). Based on this principle, monoclonal antibodies were coupled to lipid-based perfluorocarbon microbubbles and successfully shown to target overexpression of LAM *in vitro*.²² These studies show the possibility of coupling a marker and a contrast agent to create a targeted contrast agent, which can ultimately be used in the early detection, diagnosis, and treatment of a variety of pathologies.

In this study, our objective was to create a targeted contrast agent by the surface modification of a polymeric contrast agent with the RGD peptide. The eventual project goal is two fold. First, to create a drug-loaded targeted contrast agent that binds to the vasculature of a developing tumor, which can be imaged to evaluate the vascularity of the tumor. Second, once the drug loaded contrast agent is bound to the tumor vasculature, ultrasonic energy is used to release the drug to the local area. Our strategy is aimed towards the

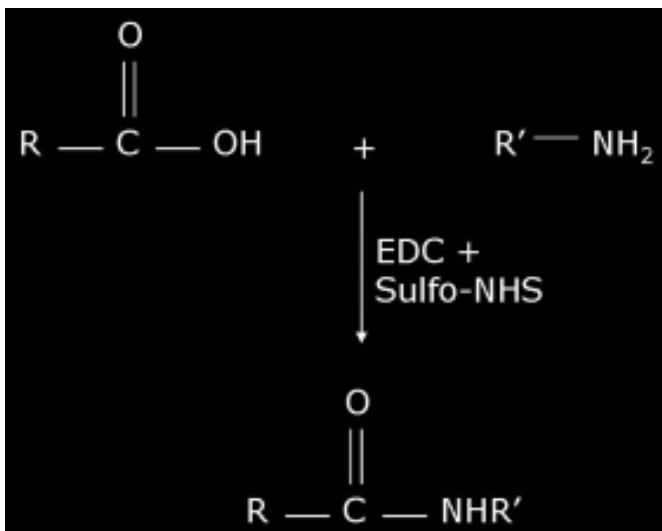


Figure 3. Carbodiimide conjugation of the carboxylic acid group (R-COOH) group of the microspheres to the amine terminal group of the peptide (R'-NH₂).

imaging of tissues that are naturally homogenous and difficult to image, such as the breast. If a contrast agent can be developed that binds to the developing tumor vasculature, it can be visualized and then a drug can be delivered to the local area retarding tumor vasculature growth.

Materials and Methods

Materials

Poly (D,L-lactide-co-glycolic acid 50:50, PLGA) (Medisorb 5050 DL 3A, lot 1010-412) was purchased from Alkermes. Poly (vinyl alcohol) (PVA), 88% mole hydrolyzed, with a M_w of 25,000 was from Polysciences, Inc. (1R)-(+)-camphor, GRGDS (Gly-Arg-Gly-Asp-Ser) peptide complex, EDC (1-Ethyl-3-(3-Dimethylamino-Propyl) carbodiimide, sulfo-NHS (N-hydroxysulfosuccinimide), antibiotics (penicillin and streptomycin), and L-glutamine were from Sigma. Dulbecco's modified eagle medium (DMEM), Hank's balanced salt solution, and fetal bovine serum (FBS) were purchased from Fisher. Ammonium carbonate was purchased from J.T.Baker. All other chemicals were reagent grade from Fisher.

Preparation of Microcapsules

Microcapsules were prepared by a patented double emulsion (W/O)/W solvent evaporation process using camphor as a removable core.¹¹ Camphor (0.05 g (0.00011 lbs)) and PLGA (0.5 g (0.0011 lbs)) were dissolved in 10 ml (3.53 x 10⁻⁴ ft³) of methylene chloride. To generate the first (W/O) emulsion, 1.0 ml of 4% ammonium carbonate solution (w/v) was added to the polymer solution and probe sonicated at 115 watts for 30 seconds. The (W/O) emulsion was then poured into a 5% PVA solution and homogenized for 5 minutes at 9,500 rpm. The double emulsion (W/O)/W was then poured into a 2% isopropanol solution and stirred for 1 hour. The capsules were collected by centrifugation, washed three times with hexane, then once with deionized water. The microcapsules were then frozen at -80°C (-112°F) and lyophilized, using a Virtis Benchtop freeze dryer, to remove the camphor and ammonium carbonate core. After freeze drying, the microcapsules appear spherical in shape and have an average size of 1.2 μ m (4.72 x 10⁻⁵ in) - *Figure 2*.

Peptide Conjugation

The platform of our targeted contrast agent is the PLGA, which has a carboxylic acid group that can be used for conjugation. Using carbodiimide chemistry, the carboxylic acid group on the PLGA can be reacted with an amine group, for example an end group on a peptide, and an amide linkage can be formed.²³ This type of reaction can be facilitated using a water-soluble carbodiimide reagent such as ethyl (dimethylaminopropyl) carbodiimide (EDC) and a catalyst, N-hydroxysuccinimide (sulfo-NHS). The peptide conjugation reaction in this study utilizes carboxylic acid carbodiimide chemistry with EDC and sulfo-NHS as the catalysts. The carboxylic acid group (R-COOH) group of the microspheres is conjugated to the amine terminal group of the peptide (R'-NH₂) - *Figure 3*.

The dried microcapsules (100mg (2.2 x 10⁻⁴ lbs)) were

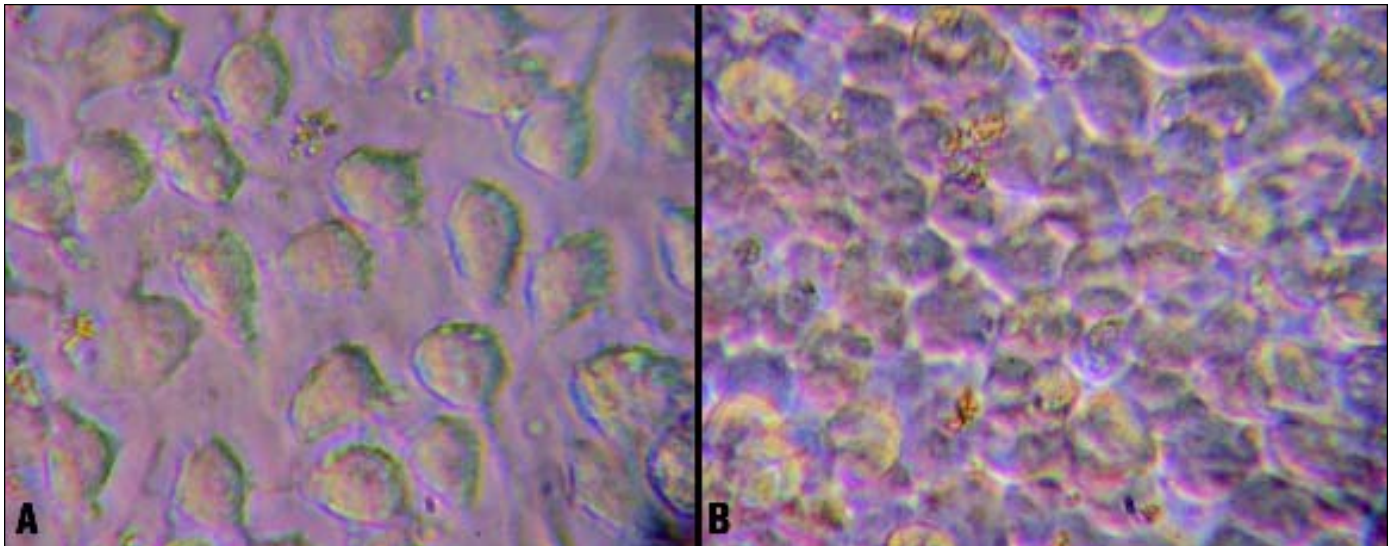


Figure 4. NB2a cells at 0 hours after incubation with PLGA microcapsules, A) unmodified, B) modified. Size bar represents $50\mu\text{m}$ (0.002 in).

combined with 5 mg (1.1×10^{-4} lbs) EDC (1:1 molar ratio of COOH groups in the microcapsules to EDC), 1.4 mg (3.08×10^{-6}) of sulfo-NHS (1:2 molar ratio to EDC), in 10 ml (3.53×10^{-4} ft³) of buffer (0.1M MES, 0.3M NaCl, pH 6.5) and stirred for 15 minutes. RGD peptide (150 μg (3.3×10^{-7} lbs), in a 1:10 molar ratio of COOH groups in the microcapsules to peptide) was then added and stirred for 3 hours.²⁴ The microcapsules were then washed with deionized water, frozen, and lyophilized.

Cell Culture

The NB2a mouse neuroblastoma cells were cultured using growth medium containing 90% DMEM, 10% FBS, 2.5ml (8.83×10^{-5} ft³) of antibiotics (1X) and 2mM L-glutamine. The medium was changed and the cells were split every three days. The experiment was performed on the cells at passage 10. The NB2a cell line was chosen because the cell line was being used in our lab for another project and we were very familiar with the cell culture techniques. Furthermore, since

the attachment of the RGD peptide was being examined, the NB2a cell line was acceptable because the RGD peptide sequences bind a variety of receptors, some of which are on the NB2a cell line.

Static Attachment & Microscope Imaging

Cells, ~95,000, were plated in each well of a 12-well cell culture plate with growth medium, total volume 3ml (1.06×10^{-4} ft³). After three days, the cells became confluent. The cells were then washed with 3ml (1.06×10^{-4} ft³) of Hank's balanced salt solution and replaced with a 3ml (1.06×10^{-4} ft³) modified medium containing microcapsules, either PLGA with or without RGD peptide, suspended in the growth medium at a concentration of 0.5 mg/ml (0.031 lbs/ft³). The cells were then incubated for 0, 1, 6, and 24 hours. After each specified time point, the medium was removed and the cells were washed again with 3ml (1.06×10^{-4} ft³) of Hank's balanced salt solution. The cells were then viewed under a Wesco Verta 7000 series microscope. Digital pictures were taken using an

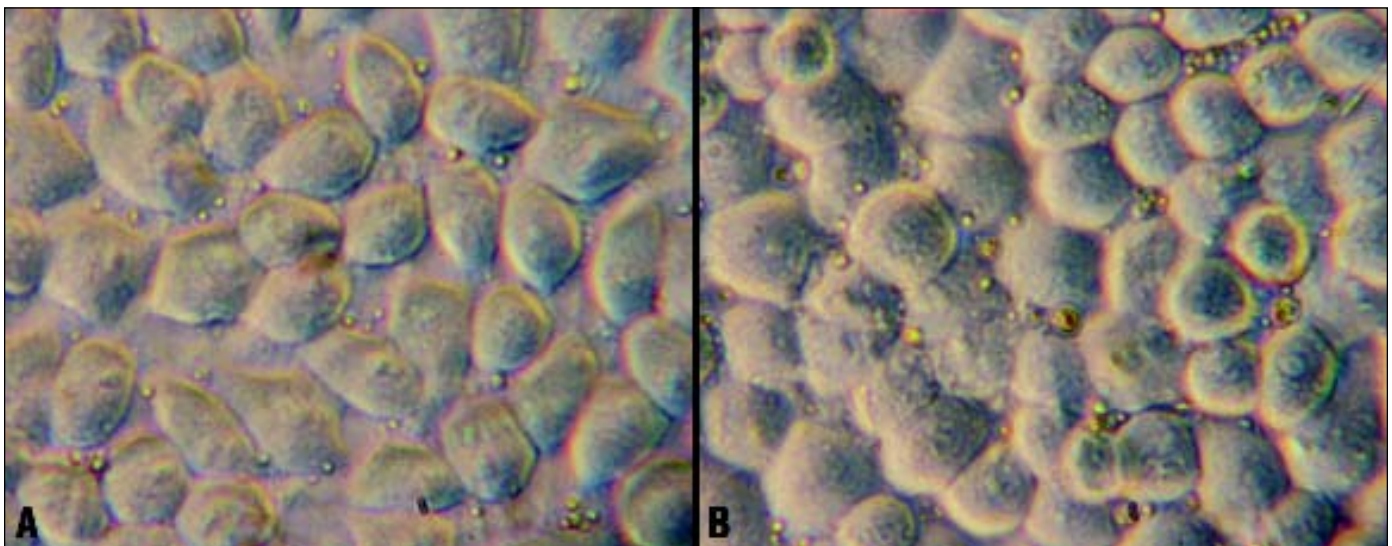


Figure 5. NB2a cells at 1 hour after incubation with PLGA microcapsules, A) unmodified, B) modified. Size bar represents $50\mu\text{m}$ (0.002 in).

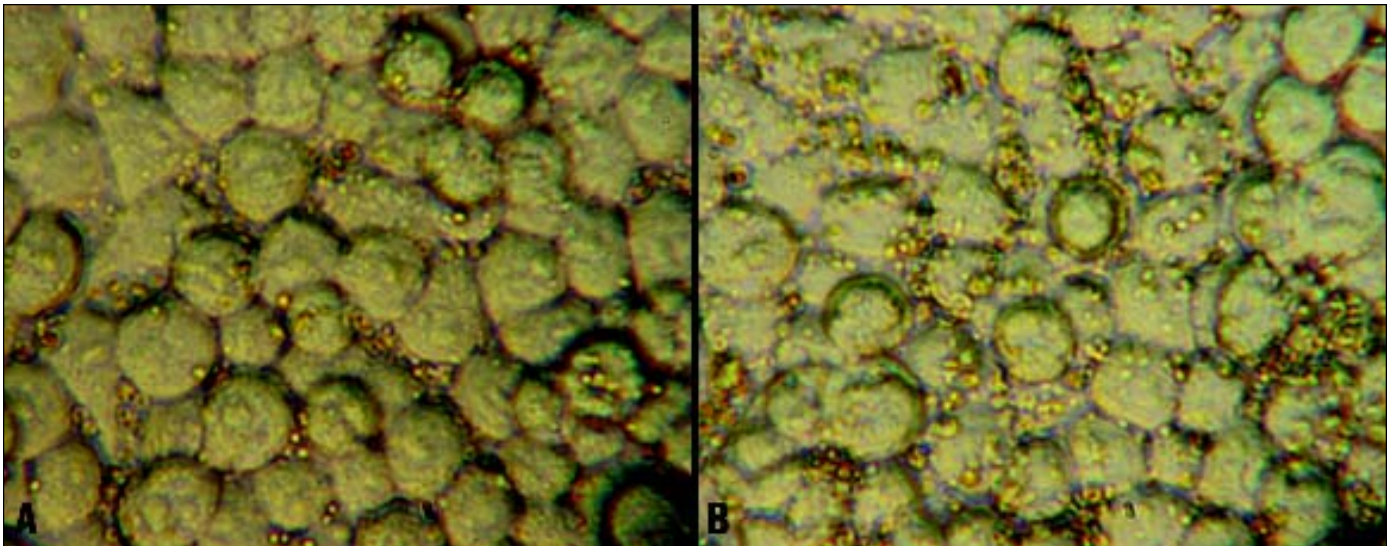


Figure 6. NB2a cells at 6 hours after incubation with PLGA microspheres, A) unmodified, B) modified. Size bar represents $50\mu\text{m}$ (0.002 in).

Olympus DP11 digital camera interfaced with the microscope at a magnification of 1000x.

Results

At 0 hours, where the microspheres were pipetted on and immediately taken off, both the PLGA and RGD conjugated PLGA microspheres show little to no attachment - *Figure 4*. The PLGA and RGD bound PLGA microspheres showed limited attachment after one hour as seen by *Figure 5*. The microspheres conjugated with RGD peptide attach to a greater degree than microspheres without peptide conjugated to its surface, after 6 hours of incubation - *Figure 6*. The microspheres are contrast agent filled with a gas, air in this case, and are naturally buoyant due to their gas content. Even though they are buoyant in the medium, many still attach to the cells, suggesting the peptide coating is mediating an interaction between the cells and the microspheres that results in attachment. Furthermore, the attraction of attachment was not only greater than the buoyancy, but also withstood washing with buffered saline solution after the given time point, suggesting definitive attachment.

Another important aspect of the attachment to examine is the location of attachment of the microspheres to the cells. Definite attachment was determined by the amount of microcapsules attached to the top surface of the cells. Since there are gaps at the junctions of many cells, it could be possible that the microcapsules adhered to the proteins secreted in the gaps by the cells, and thus were not considered in determining the amount of microcapsules that attached to cell at each time point. At time 0, *Figure 4*, the attachment of both types of microspheres on top of cells is not present. After only one hour of incubation, *Figure 5*, the peptide-modified microcapsules have only 3 attached versus 0 for the unmodified microcapsules. However, after 6 hours, *Figure 6*, the attachment of the peptide-modified microcapsules is much greater than the unmodified microcapsules. Approximately 50 peptide-modified microcapsules attach on top of cells as compared to approximately 16 for the unmodified

microcapsules. The results of this initial proof of concept study show the feasibility of using a polymer contrast agent to target cells through receptors expressed during certain pathologies, such as cancer.

Conclusions

These qualitative results suggest that RGD modified microspheres can be used to target the receptors overexpressed in cancer cells. This opens up the possibilities for targeted therapeutic imaging and drug delivery using microspheres as the vehicle.

Since the initial proof of concept study, several more studies have been conducted that have strengthened the initial hypothesis. The time of incubation for attachment has been dropped to as little as five minutes, representing a more realistic physiological situation. The binding ability of the microspheres has been tested on a human breast cancer cell line, MDA-MB-231 (courtesy of Dr. Janet Price, MDA Cancer Center). The selectivity of the adhesion was tested by first blocking the targeted receptors with the same peptide that was conjugated on the surface of the microspheres. The blocked sample set showed limited adhesion with respect to PLGA and RGD bound PLGA microspheres, indicating that the adhesion is mediated through receptors that bind RGD peptide sequences. The dynamics of cell adhesion have also been tested under flow conditions using a parallel plate flow system and the results are in line with the static attachment studies.

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About the Authors



Justin Lathia is in his last year of a five-year BS/MS dual degree program at Drexel University in Philadelphia, PA. He is majoring in biomedical engineering with concentrations in biomaterials, tissue engineering, and ultrasound imaging. He has completed two six-month co-op programs with Merck & Co., Inc. in the area of biological vaccines.

Lathia is working on his thesis, which deals with the surface

modification of ultrasound contrast agents for cancer targeting, in the Microencapsulation and Drug Delivery Lab in the School of Biomedical Engineering, Science, and Health Systems. In 2002, he was named the school's most outstanding undergraduate student. Lathia was the winner of the ISPE International Undergraduate Student Poster Competition in 2001 and represented the Delaware Valley Chapter as a finalist in the graduate division in the 2002 International Student Poster Competition.



Dalia El-Sherif received her BA in biology from Temple University in 1998. In her last two years of undergraduate work, she began working with Dr. Margaret Wheatley's Microencapsulation and Drug Delivery Laboratory in the School of Biomedical Engineering, Science and Health Systems at Drexel University. After receiving her undergraduate

degree, she enrolled in the PhD program at Drexel and continued to work with Dr. Wheatley on her research. During the course of her PhD studies, she explored the business end of the biotechnology and pharmaceutical fields. She is currently a PhD candidate at Drexel. She also works for PA Consulting Group, a leading management, systems, and technology consulting firm focused on helping companies innovate and succeed by speeding up time to market, extending product lifespans, keeping abreast of technological changes and providing market insight.



Nikhil Dhoot attended Manjunatha Vidyalaya in Dombivli, India and graduated sixth in his class for the Senior Secondary Certificate examination in 1990. He attended Junior College at V. G. Vaze College in Bombay, India and obtained his Higher Secondary Certificate in 1992. He graduated from the Karnataka Regional Engineering

College, Surathkal, India with a Bachelor of Engineering in chemical engineering in 1996. He ranked second in his class and was selected for the best student award by the Department of Chemical Engineering for the academic year 1994-95. Nikhil joined the Department of Chemical Engineering at Drexel University in the fall of 1996. He was a recipient of the Teaching Assistant Excellence award for the academic year 1996-97 and received the American Institute of Chemists award for an outstanding graduate student in Chemical Engineering during the academic year 1997-98. His work has been presented at several national and international conferences. He is currently working at Emisphere Technologies Inc., in Parrytown, NY.



Margaret A. Wheatley is a Professor in the School of Biomedical Engineering at Drexel University. Her expertise is in the area of controlled drug delivery and contrast agents for diagnostic ultrasound. She holds a PhD in chemical engineering from University of Toronto, an MS in biochemistry and a BS in chemistry, both from Oxford University, UK.

She did her post doctorate with Dr. Langer at MIT.

Drexel University, Microencapsulation and Drug Delivery Laboratory, School of Biomedical Engineering, Science and Health Systems, 3141 Chestnut St., Philadelphia, PA 19104. 