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Multifunctional Polymer Microbubbles for Advanced Sentinel Lymph Node Imaging and Mapping

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14. ABSTRACT

The goal of this Postdoctoral Award was twofold. First, the proposed research sought to design microbubble ultrasound contrast agents with enhanced dye loading capacity for imaging and surgical labeling of sentinel lymph nodes. Second, the training plan sought to improve the PI's scientific development through a combination of junior scientist mentoring, grant-writing, and external presentations. Owing to poor yields and dispersities through the original proposed fabrication plan, a new synthesis plan led to some progress in creating uniform dye loading, although problems with yield remain. The PI also made progress in identifying polymer and chemical structures that break easily under ultrasound. On the training side, the PI has been quite successful in obtaining additional external funding through NIH, presenting at numerous conferences, and mentoring several students. In addition, he applied for multiple faculty positions, obtained three offers, and will begin a tenure-track faculty position at the University of Colorado Boulder Department of Chemical and Biological Engineering in Summer 2012.

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Introduction

The purpose of this training grant was twofold. First, the goal of the research portion was to create new types of contrast agents for imaging and identifying the sentinel lymph node for breast cancer patients. Current sentinel lymph node identification techniques have significant background signal at the injection site and require opening the patient to determine their labeling efficacy. Microbubbles are excellent contrast agents for ultrasound imaging of lymphatic architecture, but they are too unstable to reliably label the sentinel lymph node for removal. The proposed research sought to create microbubbles with large amounts of dye loaded into their shells that could be released specifically into the sentinel lymph node at the clinician's discretion. Thus to develop this contrast agent, new microbubble shell structures were fabricated with a combination of dye-loaded polymer for fluorescence detection and phospholipid for stability, along with a mechanism for releasing the dye within the node.

Second, the training portion of the grant was designed to both provide the PI with both experience in mentorship and grantsmanship while encouraging the PI to improve his standing within the scientific community. The text devoted to this section will discuss mentoring experience, funding opportunities pursued, external and internal talks presented. In addition, the PI's successful pursuit of a tenure-track faculty position will be discussed.

Body

The original proposal was divided into a research plan and a training plan, and each section will be discussed separately.

Research Plan:

The overall goal of the research project was to create microbubbles that can be used to (1) image the tumor lymphatics through noninvasive ultrasound and then (2) label the sentinel lymph node through release of dye trapped in the bubble's stabilizing layer. The first step in this process was to create stable microbubbles in high yield with high loadings of releasable fluorescent dye. In the proposed research plan, microbubbles would be stabilized through a mixture of dye-labeled poly(acrylic acid) (PAA) and phospholipid. To synthesize such a polymer, approximately 25% of the acid groups on PAA were converted to thiols through amidation with cysteamine, and then about 5% of these were functionalized with a maleimide-fluorescein derivative (Fig. 1a). This synthesis was straightforward and reproducible. This polymer was mixed with 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), followed by probe sonicating the mixture under a headspace of perfluorobutane to induce microbubble formation.[1] The microbubbles were in general large, with an average diameter of 4-5 μm. However, two problems arose with this approach. First, microbubble yields were at least a full order of magnitude less than other formulation methods. Given that bubbles are inherently only temporarily stable due to the positive Laplace Pressure on the gas, this synthetic route already starts at a significant disadvantage. Second, polymer coverage was found to be inconsistent and nonuniform on the bubble surface. For example, Figures 1b and 1c show large microbubbles that have only patches of the green fluorescent polymer. Given these two difficulties, it was decided that an alternate dye-loading procedure should be pursued.

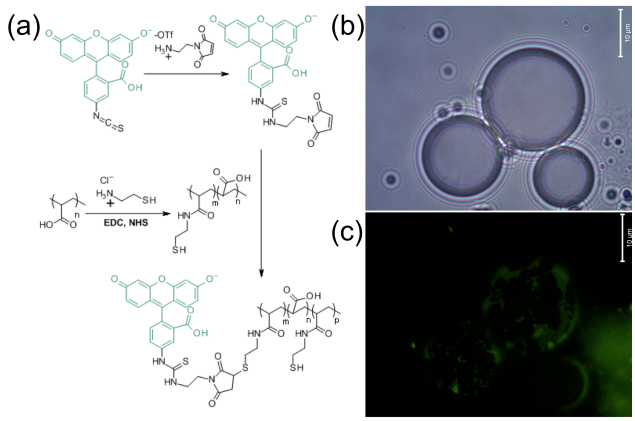

Figure 1: One-pot polymer-lipid microbubbles. (a) Synthesis of thiolated poly(acrylic acid) with fluorescein attached. (b) Bright field image of large bubbles stabilized by polymer and phospholipid interactions. (c) Green fluorescence image shows incomplete and inconsistent polymer covering on microbubble surface.

The next attempted fabrication procedure was to deposit the dye-loaded polymer in a layer-by-layer fashion onto preformed lipid-stabilized microbubbles. The adhesive properties of poly(methacrylic acid) (PMA) are pH-sensitive. At neutral pH, the polymer is negatively charged and has little affinity for hydrogen bond acceptors. Below its pKa of 6.8, however, the protonated PMA can hydrogen bond to a number of H-bond acceptors, including both phospholipids[2] and poly(vinyl pyrrolidone) (PVP)[3] (Fig. 2a). The synthetic strategy was to first create microbubbles stabilized by phospholipid, then incubate the bubbles in successive solutions of PMA-fluorescein and PVP at acidic pH to deposit the layers (Fig. 2b). Following this, the polymer shell would be crosslinked through disulfide formation, which would allow the layers to be stable at neutral pH. Ideally, then, the external force caused by the rapid size oscillations of the microbubble would be sufficient to break these covalent bonds mechanically[4] and allow dye adhesion to the lymph node macrophages. The negative pressure on a gas bubble during the ultrasound pulse has been shown to break even thick polymer shells,[5] so a thin polymer shell should be susceptible to this mechanical stress.

In practice, bubbles stabilized by lipid alone could be produced at excellent yields, approximately 10^8/mL, which is similar to commercial formulations. Next, polymer was found to deposit quite efficiently onto the lipid shell. However, during successive depositions and washes, the centrifuge washing steps necessary to remove excess polymer from the surrounding buffer caused significant bubble loss, by up to an order of magnitude for each polymer deposition step. Bubble loss was found to be very dependent on operator error, as removing subnatant from a tube of suspended bubbles could be very tricky to do efficiently and consistently. Thus, depositing three pairs of layers, a typical structure for LBL deposition, required six washing steps, and the resultant bubble yield was inadequate. In addition, the bubble destruction during the purification process led to significant amounts of polymer being deposited onto the bubble surface non-uniformly, as the collapsed bubbles would contain large amounts of polymer that would bridge between the two (Figure 2c-d).

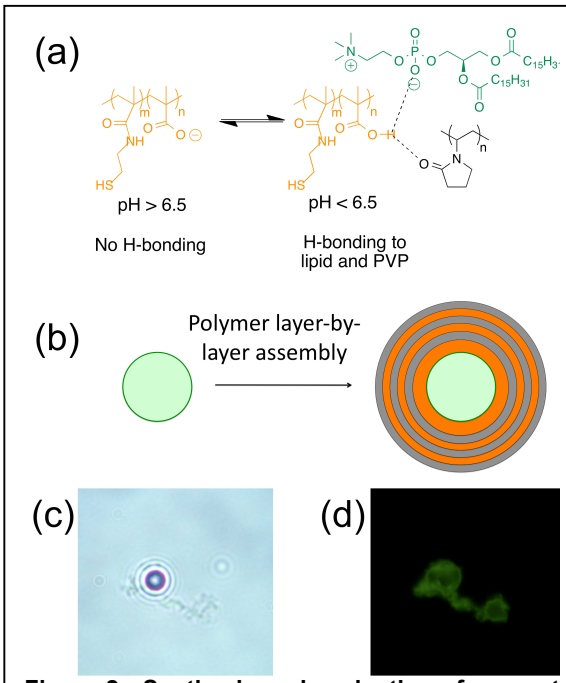

Figure 2: Synthesis and evaluation of one-pot polymer-lipid microbubbles. (a) Polymethacrylic acid will bond to both phospholipid and poly(vinyl pyrrolidone) at acidic pH, allowing layer-by-layer deposition. (b) Alternating layers of PMA and PVP were deposited onto phospholipid-shelled microbubbles. (c) Bright field image of sample microbubble (~4 μm diameter) with visible polymer tail. (d) Green fluorescence image shows significant undesired PMA attached to microbubble surface.

As a side note, the cleaving of polymer crosslinking strands through microbubble oscillations had not been shown previously, and studies were performed to evaluate the viability of this approach. First, the layer-by-layer assembly was performed on lipid-coated silica microparticles, which were made by simply sonicating commercial silica microspheres with a lipid suspension.[6] PMA and PVP were then deposited as before, and the green fluorescence is seen easily (Figure 3). The polymer-coated spheres were then subjected to a focused ultrasound beam at 2.25 MHz and 1000 1.5 MPa sine pulses at 2 Hz for 30 minutes. After this, the green fluorescence disappears from the silica surface. While this does not model a microbubble oscillation, the silica surface can act as a nucleation site for bubble growth, and thus ultrasound energy is concentrated there, albeit not to the same degree.

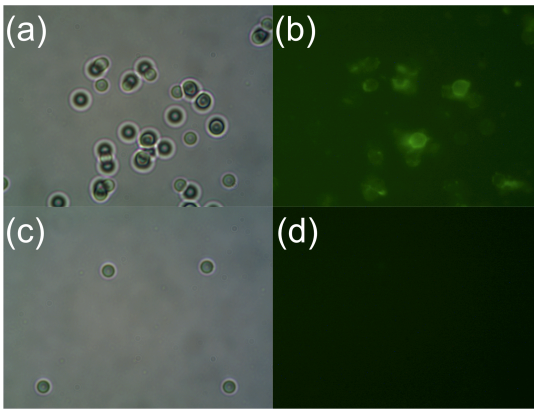

Figure 3: Bright field and green fluorescence microscopic images of polymer-lipid silica microspheres before (a, b) and after (c, d) 30 minutes of ultrasound. After ultrasound insonation, the green fluorescence is removed from the silica sphere surface.

Training Plan:

The overall goal of the training plan was to further my readiness for a faculty position through the mentorship of junior scientists, application for and acquisition of external funding, and improving my external name recognition.

Over the course of the grant I was very active in mentoring junior students. I was fortunate to work with an advisor who believed strongly in allowing senior mentored researchers to work closely and mentor junior students. During the project term I mentored a subgroup of one Ph. D student, one M.S. student, and three undergraduates, all of whom worked on projects related to the intersection of colloids, interfaces, and polymer science, which is the future research direction that I would like to pursue as a faculty member. I have taught the students how to perform synthesis, microbubble formulation, ultrasound evaluation studies, microscopy, spectroscopy, and other related laboratory techniques. Training was given both through a formal weekly meeting and through routine informal discussions on almost a daily basis.

To help support these students and their research, I actively applied for external research grants, through ghostwriting for my advisor and informal advising to a number of research proposals. First and foremost, this includes an R21, which was funded and helps to support the Ph. D student. I have also submitted two other R21s and an NSF; none of these were funded.

In the proposed research period I was was very active in presenting at internal and external conferences. Highlights include invited presentations at the American Chemical Society National Meeting (March 29, 2011), a UCSD-Corning workshop (February 17, 2011), the Biophotonics Industry Forum in San Francisco (January 20, 2012), and the Nanoscience and Emerging Technology in Cancer Research Workshop in San Diego (December 10, 2011). I also attended and presented a poster at the NCI Alliance for Nanotechnology in Cancer Annual Meeting in Boston in November 2011, and I have received an invitation to present at the next meeting in Houston. I also presented an invited talk at the ACS National Meeting in March 2012 and gave another oral presentation at the Materials Research Society Spring Meeting in April 2012.

Finally, while it was not an explicit part of my training plan, I devoted a significant amount of time to preparing, applying, and obtaining faculty positions. During the fall and winter of 2011-2012, I applied to approximately 70 schools. Of these, I obtained interviews at UCSD Chemistry, University of Maryland Bioengineering, University of Texas Austin Biomedical Engineering, University of Colorado Boulder Chemical and Biological Engineering, and Lehigh University Bioengineering. Of these, I obtained offers at Boulder and Lehigh, as well as an offer from UCSD Nanoengineering (my home department), and I have accepted the offer at Boulder. My postdoctoral advisors have cleared the importation of my work described in the DOD grant, as these were my original ideas and not those of my mentors.

Key Research Accomplishments

- Manufactured polymer-lipid microbubbles with enhanced dye loading.
- Evaluated distribution and stability of loaded microbubbles.
- Created microbubbles with polymer deposited stepwise onto lipid-shelled microbubbles.
- Evaluated distribution and stability of layer-by-layer polymer-loaded microbubbles.
- Performed pilot studies to evaluate polymers containing frangible crosslinking units.

Reportable Outcomes

- Applied for ~70 tenure-track faculty positions.
- Gave five onsite interviews.
- Obtained two written job offers and one verbal.
- Committed to University of Colorado, Boulder, Department of Chemical and Biological Engineering.

- Obtained funded R21 research grant (Percentile = 4.0).
- Applied to three other grants, none funded.
- Presented invited research talk at American Chemical Society National Meeting (Anaheim).
- Presented invited research talk at UCSD-Corning Workshop (San Diego).
- Presented invited talk at UC Biophotonics Industry Forum (San Francisco).
- Presented invited research talk at Nanoscience and Emerging Technology in Cancer Research Workshop (San Diego).
- Presented invited poster at NCI Alliance for Nanotechnology in Cancer Annual Meeting.
- Presented invited research talk at American Chemical Society National Meeting (San Diego).
- Presented research talk at Materials Research Society Annual Spring Meeting (San Francisco).
- Invited to present at NCI Alliance for Nanotechnology in Cancer Annual PI Meeting (Houston).
- Managed a subgroup of 5 students, including one Ph. D and one M.S. student.

Conclusion

In summary, progress in the proposed research plan was slower than expected due to the need to reconfigure experimental protocols to adjust for poor results in the intended research direction, as well as early termination of the grant upon promotion to tenure-track faculty. However, some of the directions pursued in this research project have led to new ideas in surface modification of colloids and particles, which will be pursued in my independent position.

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Appendices

None.

Supporting Data

None.