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Fibrin-Targeted Polymerized Shell Microbubbles as Potential Theranostic Agents for Surgical Adhesions

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Abstract

The development of new therapies for surgical adhesions has proven to be difficult as there is no consistently effective way to assess treatment efficacy in clinical trials without performing a second surgery, which can result in additional adhesions. We have developed lipid microbubble formulations that use a short peptide sequence, CREKA, to target fibrin, the molecule that forms nascent adhesions. These targeted polymerized shell microbubbles (PSMs) are designed to allow ultrasound imaging of early adhesions for diagnostic purposes and for evaluating the success of potential treatments in clinical trials while acting as a possible treatment. In this study, we show that CREKA-targeted microbubbles preferentially bind fibrin over fibrinogen and are stable for long periods of time (~48 h), that these bound microbubbles can be visualized by ultrasound, and that neither these lipid-based bubbles nor their diagnostic-ultrasound-induced vibrations damage mesothelial cells in vitro. Moreover, these bubbles show the potential to identify adhesionlike fibrin formations and may hold promise in blocking or breaking up fibrin formations in vivo.

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.8b03692. Representative B mode images used to calculate values in Figure 5b and Supporting Information Table 1; data corresponding to B mode images in Supporting Information Figure 1 (PDF)

The authors declare no competing financial interest.

Graphical Abstract

1. INTRODUCTION

Surgical adhesions are bands of fibrous scar tissue ligating apposing tissues/organs that form in the abdomen following surgery. Adhesions can cause significant morbidity and mortality. A 2010 survey found that more than 7 million abdominal and pelvic surgical procedures of all types were performed in that year,¹ and morbidity studies suggested that some type of adhesion developed after nearly 100% of these procedures. While many adhesions are asymptomatic, at least one-third of patients undergoing invasive surgeries are readmitted an average of two times over a subsequent 2 year period due to complications.^{2,3} Adhesion formation is a major cause of significant postsurgical morbidity, including chronic abdominal or pelvic pain, infertility in women, and potentially fatal intestinal obstructions.⁴⁻⁶ Despite the availability of several FDA-approved adhesion barrier products, the incidence of postsurgical intraperitoneal adhesions has not declined over the last 20 years, and thus the effective treatment of adhesions remains a highly significant clinical problem.⁷

Though a number of potential approaches, both biochemical and physical, have shown the potential for addressing adhesions in animal models, the difficulty of assessing treatment efficacy in human patients (where second surgeries to assess the presence of adhesions are neither practical nor ethical) has made proceeding to clinical trials prohibitively expensive and time-consuming. Because adhesions consist entirely of soft tissue, they are difficult to image using standard clinical imaging techniques, especially in their earliest stages when they are most vulnerable to treatment. While large, mature “acute” adhesions can sometimes be identified by MRI (magnetic resonance imaging), the methodology is not highly reliable; even then, the adhesions themselves may take several months to develop to the point of detectability.⁸ The lack of a means to detect newly forming adhesions also makes early minimally invasive treatment impossible. There is therefore a strong need for an inexpensive, widely available means to detect nascent adhesions with noninvasive diagnostic techniques such as ultrasound or MRI. In addition, a noninvasive technique to break up early-stage adhesions coupled with a reliable detection method (e.g., theranostics approach) would provide an ideal solution to address these adhesion-related complications. As ultrasound imaging is less expensive, more available, and easier to employ than MRI, we have worked to develop a stable, targeted lipid shell microbubble that binds to newly

forming adhesions and acts as an ultrasound contrast reagent (UCR) that can be used to detect and treat early adhesions using only clinical diagnostic ultrasound scanners.

Ultrasound contrast imaging has been established as an advantageous diagnostic modality because it is less costly and more widely available than MRI. Microbubbles of up to 8 μm in diameter have been shown to be a strong contrast agent when injected into the bloodstream.^{9,10} For these accepted intravascular applications, the typically short lifespan of microbubbles (< 10 min) is considered to be an asset.¹¹ In contrast, in the peritoneum, where adhesions form, a contrast reagent must be injected into at most a few localized regions of the abdomen and must be allowed to diffusively permeate throughout the peritoneal cavity to encounter and adhere to its fibrinous adhesion targets.

Peritoneal applications of microbubbles offer some advantages over the more usual intravascular uses of such ultrasound contrast reagents (UCRs). The major potential advantage is no inherent limitation on the bubble size range such as that imposed on typical intravascular UCRs by capillaries in the vasculature. Thus, for purposes of imaging one could use bubbles tens of micrometers in size for enhanced contrast. Furthermore, if one envisions a therapeutic benefit from even modest stable oscillations of such UCRs, then the increased size is also to be desired. On the other hand, one of our goals is to employ only standard clinical ultrasound scanning machines to accomplish imaging and ultimately the breakup of nascent surgical adhesions. The requirement of a volumetric “breathing” response of such bubbles would then place an upper bound on the sizes one could employ with common scan frequencies of 3.5–7 MHz found in clinical scanners. One of our long-term goals is therefore to optimize sizes and shell parameters to balance these competing requirements of imaging and therapy.

Owing to the Laplace pressure, gas bubbles in the ~ 10 μm size range in aqueous media are inherently unstable to dissolution.¹² Investigators have addressed this instability by adding a shell to the surface to both limit the Laplace pressure and to provide an intrinsic barrier to gas transport across the surface. The shell may be composed of surfactants, lipids, proteins, polymers, or a combination of these materials.^{13,14} Early attempts at microbubble polymer stabilization utilized an albumin or other proteinaceous shells.^{15–17} In addition, for thin-shell microbubbles, investigators have looked at methods for making structures encapsulated by a proprietary double-ester polymer¹⁸ or poly(vinyl alcohol) (PVA).¹⁹

Here we present and test a possible solution to these issues: we describe gas-core polymerized shell microbubbles (PSMs) that display the fibrin binding peptide CREKA and include a UV cross-linkable lipid to enhance bubble stability.

Because of the requirement of stable lipid-based, thin-shelled microbubbles for use in the peritoneal cavity, we have developed a formulation consisting of phospholipids and photopolymerizable lipids that can be used to produce a microbubble shell membrane that is elastic, nonaggregable, and resistant to gas dissolution.²⁰ These structures mimic conventional liposomes or even normal cell membranes but with an important difference: the wall can be cross-linked to any degree desired, preventing the loss of contents over time. This is accomplished by the inclusion of diacetylene-containing lipids that are polymerizable

by UV light.²¹ Polydiacetylene polymers have been studied for 20 years in nanoscopic structures for use in diagnostic and drug applications.^{22–30}

For microbubbles used for adhesion imaging, it is critical to maximize the microbubble stability because the goal is to image the agent after it arrives at a specific anatomical site via diffusion throughout the peritoneum. In previous studies, we found that polydiacetylene-containing microbubbles are stable for at least 90 min,²⁰ but we did not test them for longer (clinically relevant) times, which we examine here. Furthermore, while this type of polymeric material has not yet been tested in humans, the major component in the microbubble assembly is distearoylphosphatidylcholine. As phospholipids are synthesized by mesothelial cells and are responsible for the lubrication of the serosal peritoneal surfaces,³¹ such a phospholipid ultrasound contrast reagent would in many ways be biomimetic.

We chose to use the pentapeptide CREKA because it has been reported to exhibit specificity for cells expressing fibrin.³² This peptide sequence has an N-terminus cysteine residue to enable covalent linkage to a maleimide-terminated lipid. Here, CREKA-PEG2000-DSPE lipids were formulated into polymerized shell microbubbles (PSMs) by cosonication with L- α -phosphatidylcholine hydrogenated soy and h-PEG1-PCDA. The microbubbles were then irradiated with UV light to yield the CREKA-PSM structure containing the colored polydiacetylene polymer. The polydiacetylene polymer not only imparts a degree of physical stability to the structures but also adds strong visible and fluorescence signals that are helpful in quantification and cell binding studies.

2. METHODS AND EXPERIMENTS

2.1. Microfluidic Chip Preparation.

The chips were fabricated with polydimethylsiloxane (PDMS) and glass by applying standard soft lithography techniques.³³ Briefly, the molding silicon wafer was prepared with the SU-8 photoresist by a two-step UV-exposure procedure to obtain channels with a rectangular cross section with heights of 1 and 20 μm (Figure 1a). Briefly, the SU-8 photoresist was spin-coated onto a silicon wafer and UV-exposed using a mask containing 5- μm -wide microchannel arrays (Figure 1a, blue). This process was repeated under careful alignment of the second mask (Figure 1a, gray). The mold was replicated using PDMS (Sylgard 184 elastomer kit, Dow Corning, Germany), which was mixed in 1:10 cross-linker/polymer ratio, gently poured into the mold, and placed under vacuum. After degassing, the mold was cured inside an oven at 65 °C for 4 h, cut into the desired shape, and punched to make inlet and outlet holes. Oxygen plasma treatment was used to irreversibly bond the PDMS layer with a microscope glass slide.

2.2. Polymerized Shell Microbubble (PSM) Synthesis.

2.2.1. Materials.—The components comprising the targeted and nontargeted PSMs are L- α -phosphatidylcholine hydrogenated soy (hydrogenated soy PC), 1,2-distearoyl-*sn*-glycerol-3-phosphoethanolamine-*N*-[methoxy(poly(ethylene glycol))-2000] (m-PEG2000-DSPE; Avanti Polar Lipids, Alabaster, AL, USA), (5-hydroxy-3-oxypentyl)-10,12-pentacosadiynamide (h-PEG1-PCDA), and 1,2-distearoyl-*sn*-glycerol-3-

phosphoethanolamine-*N*-[amino(poly-(ethylene glycol))-2000]-CREKA (CREKA-PEG2000-DSPE) (NanoValent Pharmaceuticals Inc., Bozeman, MT, USA).

2.2.2. Preparation of Targeted and Nontargeted PSMs.—CREKA-PEG2000-DSPE, hydrogenated soy PC, and h-PEG1-PCDA were dissolved in chloroform in a molar ratio of 15:60:25 (respectively). A thin film of lipids was formed after evaporating the solvent and was rehydrated with a mixture of glycerol/1,2 propanediol/water in a ratio of 10:10:80 (respectively) to achieve a final concentration of 5.2 $\mu\text{mol/mL}$ (total lipid). The solution was tip-sonicated with a Branson Digital Sonifier (20 kHz) at 10% amplitude for 10 min. The lipid solution was added to the solution inlet, and nitrogen gas was added to the air inlet of the microfluidic device. The gas pressure was increased to 18 psi with a solution flow rate of 0.25 mL/min. The microbubble solution was collected and polymerized with a Spectrolinker XL-1000 UV cross-linker at 253 nm and $5000 \times 100 \mu\text{J}/\text{cm}^2$ to form polymerized shell microbubbles (PSM). Nontargeted microbubbles were produced in the same manner except that CREKA-PEG2000-DSPE was substituted with m-PEG-2000-DSPE.

2.3. Microbubble Characterization.

The microbubble size was measured with bright-field imaging using a Zeiss Axiovert S100 inverted microscope. The microbubble solution (1.5 μL , $\sim 10^7$ PSM/mL) was placed on a CellVision glass slide to a depth of 10 μm . Images were recorded at 20 $\times$ magnification with 200 ms of exposure time and were analyzed using the ImageJ particle sizer function to determine the diameter of the microbubbles. The concentration of each batch of microbubbles was determined using a Coulter Z2 analyzer. The microbubble stability was determined in phosphate-buffered saline (PBS) and in LP-9 medium (a 1:1 solution of medium 199 and Ham F12 with 10% fetal bovine serum (FBS), 10 ng/mL epidermal growth factor (EGF), and 400 ng/mL hydrocortisone) to a final concentration of 10^5 PSM/mL. The microbubble solutions were stored at 37 $^{\circ}\text{C}$. Size and concentration measurements were determined as described above at 2, 24, and 48 h.

2.4. Fibrin Targeting.

The binding of targeted and nontargeted microbubbles to fibrin and fibrinogen was assessed to confirm the binding specificity of the targeted microbubbles. Fibrinogen (2 mL) at a concentration of 10 mg/mL (bovine plasma, Sigma-Aldrich) was used to prepare the fibrinogen- and fibrin-coated wells in six-well polystyrene plates. For fibrin generation, 50 μL of thrombin (bovine plasma, Sigma-Aldrich) at 100 units/mL was added to fibrinogen-containing wells.³⁴ The plates were agitated at room temperature for 1 h, and all wells were washed with PBS buffer. Aliquots of microbubbles (50 μL , $\sim 10^7$ PSM/mL) were added to fibrin- and fibrinogen-coated wells and incubated for 30 min at room temperature. The bubble solution was then removed from the wells, and each well was washed twice with 1 mL of PBS. Because the PCDA polymers undergo a color change after cross-linking and fluoresce at an excitation wavelength of 547 nm, the binding of targeted and nontargeted polydiacetylene-containing microbubbles was determined by measuring the sample fluorescence (ex/em 547/580 nm) using a SoftMax Pro plate reader. Specific microbubble

binding to each well was then calculated by difference, with sample readings being subtracted from the control reference reading generated by the original plate.

2.5. LP-9 Cell Culture and Microbubble Cytotoxicity Assay.

Human LP-9 mesothelial cells (Coriell Cell Repositories, Camden NJ), were cultured on Corning BioCoat collagen-coated plasticware in growth medium containing a 1:1 mixture of Medium 199 and Ham's F12 media, 10% FBS, 10 ng/mL EGF, and 400 ng/mL hydrocortisone. Freshly made targeted and nontargeted PSMs were diluted in LP-9 growth medium to concentrations of 10^5 microbubbles/mL and 10^7 microbubbles/mL and were placed on live cells. As controls, additional cells were placed in a solution of 10:10:80 glycerol/1,2 propanediol/water (the solution in which fresh PSMs are dissolved) with media, but there were no microbubbles. Cells were returned to a 37 °C, 5% CO₂ incubator. Cells were removed from the incubator after 2, 24, or 48 h. All media and PSMs were removed, and cells were washed with sterile phosphate-buffered saline. Cells were then exposed to a solution containing 1 μ M ethidium homodimer-1 and 1 μ M calcein, AM (both from Invitrogen LIVE/DEAD Viability/Cytotoxicity Kit) in sterile PBS for 30 min at room temperature. Fluorescent images of cells were acquired using an Olympus Ix81 microscope, an Olympus mercury arc lamp illumination system, and a COOLSNAP HQ2 camera. The ratio of live to dead cells was quantified from three representative images of each condition with the experiment performed in triplicate. Each combination of PSM type (targeted vs nontargeted), PSM concentration (0, 10^5 , and 10^7 microbubbles/mL), and time point (2, 24, and 48 h) was quantified. The resulting fluorescent images were analyzed using Metamorph, ImageJ, Matlab, and Excel.

2.6. Ultrasound Cytotoxicity Assay.

LP-9 human mesothelial cells were cultured on collagen-coated plates as described above. Cells were exposed to 10^5 PSMs (targeted or nontargeted) per milliliter of growth medium and compared against controls with no PSMs added. Cells were allowed to acclimate to the presence of the PSMs for 30 min in a 37 °C, 5% CO₂ incubator. All ultrasound exposures employed a Terason t2000 (Terason, Burlington, MA) using a 7L3 probe driven in B-mode with a center frequency of 5 MHz, a pulse repetition frequency (PRF) of 10 kHz, and a pulse duration of 600 ns (roughly three cycles). All exposure parameters were independently measured with a calibrated Onda HNR-0500 needle hydrophone (Onda Corporation, Sunnyvale, CA). Cells under all conditions were treated with ultrasound generated by a Terason Ultrasound System equipped with a 7L3 probe for 1 min. Cells cultured in the absence of bubbles and the absence of ultrasound treatment served as controls. Following ultrasound exposure, all cells were washed with sterile PBS and stained with the Invitrogen LIVE/DEAD Viability/Cytotoxicity Kit as described above. The samples were imaged and analyzed as described above. All experiments were repeated in triplicate with a different PSM preparation used each time. The cellular viability was calculated from analyses of three images from each plate in each experiment.

2.7. Ultrasound Contrast Imaging of Fibrin Binding with Microbubbles.

Experiments were performed in a setup consisting of 1% agarose gels formed in 100 cm Petri dishes placed atop 1% agarose gels in 150 cm Petri dishes. Ultrasound transmission gel

was used as a coupler between the top Petri dish and the bottom agarose gel to minimize ultrasound scattering. A region of fibrin was formed on the top agarose gel for some conditions by adding 500 μL of 20 mg/mL fibrinogen, followed by 25 μL of 100 U/mL thrombin. The fibrin solidified as a thin film that covered approximately 25% of the surface of the gel after 30 min of incubation at 37 °C. For control conditions, no fibrin was added. Plates were washed with PBS before adding a 5 mL solution of fibrin-targeted or nontargeted PSMs (at 10^5 microbubbles/mL) to the agarose plates with and without the fibrin coating. Plates were incubated with PSMs for 30 min at room temperature, and unbound PSMs were washed off with a single PBS rinse. Plates were refilled with fresh PBS, and images were obtained using a Terason t2000 (Terason, Burlington, MA) with a 7L3 probe. The gel interface containing fibrin and PSMs was located at a fixed distance of 1 cm. The peak positive pressure at 1 cm was 0.4 MPa. Images were analyzed in ImageJ with the z-axis brightness profile, and the experiment was performed in triplicate.

3. RESULTS AND DISCUSSION

3.1. Polymerized Shell Microbubble (PSM) Size Is Stable over 48 h.

The microfluidic chip generated 2.5×10^6 microbubbles/min on average, and the microbubbles were cross-linked (yielding PSMs) immediately after production. The rapid rate of PSM production enables sufficient microbubbles for use in preclinical studies, which typically use $\sim 10^8$ microbubbles/mL. Furthermore, the microfluidic chip generates microbubbles with tighter (more monodisperse) size distributions, which have been shown to improve US contrast intensity in vivo.³⁵ Nontargeted and fibrin-targeted PSMs were incubated in both PBS and media at 37 °C. The size of each PSM sample was measured at 2, 24, and 48 h. Figure 2 shows the mean diameter of the PSMs over the time course. The standard deviation of all of the PSM populations increases over time, specifically at 24 and 48 h; however, the mean diameter of the PSMs does not vary with statistical significance. Under all conditions, the PSM populations demonstrate a consistent mean diameter.

While it can certainly be inferred from our results that the PSMs likely remain stable for even longer than 48 h, in vivo pilot studies by our group suggest that 48 h is the minimum amount of time for unbound PSMs to be cleared in the peritoneum, leaving only bound PSMs for accurate imaging and treatment. As it is important to image and treat adhesions during the brief time frame in which they are still in their nascent, fibrinous state and most vulnerable, we view 48 h as the time point that is most relevant to the potential clinical applications we are currently pursuing with these PSMs.

3.2. PSMs Do Not Affect the Cellular Viability.

To ensure that the injected lipid formulation used in our PSMs would not be toxic to the mesothelial cells, we subjected cultured LP-9 cells, a human mesothelial cell line, to several concentrations of PSMs and assessed the cellular viability 2, 24, and 48 h after PSM exposure. The highest concentration tested, 10^7 PSM/mL, was chosen to represent the highest feasible concentration achievable by our current PSM production methods. The lower concentration tested, 10^5 PSMs/mL, was chosen on the basis of our preliminary tests suggesting this to be an optimal concentration for in vivo imaging. Three time points were

chosen to be representative of the time frame where new adhesions forming after surgery are most vulnerable. At all time points and conditions tested, the cell viability remained high and remained similar to that of untreated cells (Figure 3). In all treatments and at all time points, the cellular viability was greater than 90%. These in vitro results are a promising indicator of safety. Further in vivo tests will be necessary to confirm the safety of the PSMs.

3.3. PSMs Treated with Standard Clinical Ultrasound Do Not Damage Mesothelial Cells in Culture.

We have shown that PSMs alone do not appear to have any toxic effect on mesothelial cells, but this alone is not sufficient because we have proposed using the PSMs as an ultrasound contrast agent or as an ultrasound-triggered treatment to break up fibrinous adhesions. We therefore further demonstrate that ultrasound-treated PSMs do not appear to kill mesothelial cells. Our PSMs are intended to be used with typical clinical ultrasound, which is generally considered to be one of the safest methods of clinical imaging. Regarding the breakup of nascent adhesions, we have suggested that concentrated PSMs could break up fibrin or other soft tissue. To this end, we tested the effect of ultrasound in the presence of PSMs on the viability of cultured mesothelial cells. This is important to investigate because inflammation associated with adhesion formation is triggered by surgery-associated cell/tissue trauma. Thus, the rationale of these studies was to confirm that our treatment methods would not damage cells, resulting in the formation of further adhesions. We compared untreated cells to cells treated with ultrasound in the presence of fibrin-targeted on nontargeted PSMs (no fibrin is externally added for all conditions) or no PSMs. Our concern here is not whether bound PSMs agitated by ultrasound will damage the fibrin to which they are bound, which would actually be desirable in vivo, but instead whether their vibrations are damaging to the living cells of the mesothelial lining to which they *should not* bind directly. Under all conditions, the cell viability remains high, >90%, and is similar to that of the untreated controls (Figure 4). While further in vivo results are necessary over a longer time course to definitively show that ultrasound treatment in the presence of PSMs is not harmful to cells in the mesothelium and does not result in further inflammation and adhesion formation, these results, combined with the results of the PSM exposure study, strongly suggest that our PSMs would be safe near the mesothelium.

3.4. Specific Binding of Fibrin-Targeted PSMs Confirmed by Fluorescence and Ultrasound Imaging.

The PSM fluorescence intensity signal (Figure 5a) shows that fibrin-targeted PSMs bind to fibrin compared to nontargeted PSMs. Furthermore, neither the fibrin-targeted nor nontargeted PSMs showed binding to fibrinogen or untreated polystyrene.

If PSMs are to be widely used as theranostic agents for surgical adhesions, then they must be responsive to commonly available diagnostic ultrasound. By design, PSMs should not require either high pressures or high temporal average intensities to achieve our desired goals, and thus their employment should not require specialized acoustic transducers. A unique feature of our PSMs is the partially polymerized shell. The shell allows a bubble dynamic response demonstrably suitable for imaging (Figure 5b, Figure S1, and Table S1) while also maintaining greater than 48 h of passive stability (Figure 2) as well as contrast

stability for an ultrasound exposure duration of 1 min. While these results imply a certain regime of bubble dynamics, the dynamic response of these PSMs and the shell's rheometric properties remain to be determined in future work. Our observations above would imply that these bubbles undergo stable volumetric pulsations of low enough amplitude to rule out both parametric shape oscillations and more violent inertial collapses. Because it is demonstrated (Figure 2) that passive diffusive dissolution is virtually eliminated, it seems likely that growth by rectified diffusion would also be suppressed for ultrasound exposure consistent with clinical radiological scan conditions.

A common misconception is that bubbles must be resonant and thus restrictively size-matched to a given ultrasound frequency to be effective as either contrast or therapy vehicles. A related misconception is the implicit assumption that bubbles are resonant at all, that is, that they demonstrate a peak in their response as a function of frequency near their natural frequency. A critical radius R_{crit} smaller than which bubbles are nonresonant is estimated by eq 1

$$R_{\text{crit}} \cong \sqrt{\frac{\mu_{\text{eff}}^2}{\rho_1 P_{\text{eff}}}} \quad (1)$$

where the effective viscosity is $\mu_{\text{eff}} = \mu_l + \beta\mu_{\text{shell}}$, the effective static pressure is $P_{\text{eff}} = P_0 + \beta G_{\text{shell}}$, μ_l is the viscosity of the host liquid, μ_{shell} is the surface dilatational viscosity of the shell, P_0 is the ambient pressure plus the Laplace pressure, and G_{shell} is the shell shear elastic modulus.³⁶ We will assume that the ratio of shell volume to bubble volume is $\beta \approx 0.1$. A clean-surface unshelled air bubble in water ($\mu_l = 0.001$ Pa s, $P_0 = 0.1$ MPa) fails to be resonant for sizes smaller than $R_{\text{crit}} \approx 10$ nm; in 45% Hct blood, the corresponding critical size is $R_{\text{crit}} \approx 500$ nm. However, the addition of an elastic shell with, for example, $G_{\text{shell}} = 10$ MPa and $\mu_{\text{shell}} = 0.5$ Pa s increases the estimated critical radius R_{crit} to almost $5 \mu\text{m}$.³⁷ This implies that our PSMs are either not resonant or are only weakly so. This is potentially beneficial with regard to our goal of requiring only commonly available diagnostic ultrasound scanners: a nonresonant bubble has a flat response over a large frequency range below and near its natural frequency, which renders its response robust and insensitive to variations in the center frequency of the clinical scanner. For our example of $2 \mu\text{m}$ bubbles with assumed $G_{\text{shell}} = 10$ MPa, the natural frequency is about 5 MHz. These conditions are amenable to the majority of existing clinical scanners.

Our intent is to use our PSMs to detect and treat fibrinous adhesions. Thus, we assessed the ability of CREKA-conjugated PSMs to bind to fibrin and found that fibrin treated with fibrin-targeted PSMs is detectable using standard B-mode ultrasound. Specifically, by comparing B-mode images of surfaces treated with targeted or nontargeted PSMs in the presence or absence of fibrin, fibrin is detectable in an ultrasound image. Figure 5b shows the normalized grayscale brightness taken from representative images of the four conditions. Targeted PSMs bound to fibrin have more than twice the brightness of the control conditions (Figure 5b). In live (real-time) ultrasound, we have observed that with a probe moving across the fibrin-coated region the contrast between the fibrinous regions of the targeted

PSM-treated plate and the nonfibrin regions is even more striking than a still image can properly capture. These results are clinically relevant and demonstrate the feasibility of PSMs for potential adhesion detection: fibrin-targeted PSMs are detectable under ultrasound, stable over a clinically relevant period of time, and bind to fibrin in vitro.

4. SUMMARY AND CONCLUSIONS

Our primary goals in designing a microbubble to target surgical adhesions for ultrasound imaging and possible treatment were twofold: to design a microbubble that is stable for at least 48 h and attach to these microbubbles an easily synthesized fibrin-binding motif. Our results show that we have achieved both of these objectives. The microfluidic technique for producing the polymerizable shell microbubbles is fast and efficient, and the process of cross-linking them to boost their stability requires only UV light exposure. These PSMs do not show significant changes in diameter over a 48 h period, suggesting that they remain stable for at least that long. We have shown that CREKA-bound PSMs bind preferentially to fibrin in vitro, even over its precursor protein fibrinogen. The bubbles do not appear to be harmful to mesothelial cells in vitro, even when treated with ultrasound. This is significant, not only for any future clinical uses for these microbubbles but also for in vivo animal model studies that are likely the next step in studying the effectiveness of PSMs in detecting and treating surgical adhesions. Finally, and perhaps most conclusive of all, we have shown that we are able to detect the presence of fibrin in an in vitro setting using clinical ultrasound and our PSMs.

While microbubbles are known to be effective ultrasound contrast agents, traditional preparation techniques limit the longevity of the microbubbles (often intentionally, for microbubbles injected into the circulation), thus limiting the current uses of microbubble contrast agents. Our studies show that more stable microbubbles can be synthesized quite easily, greatly expanding the possible targets and situations where microbubble contrast agents can be used. The CREKA fibrin-targeting peptide, a simple pentapeptide, can be easily synthesized and attached to lipids that can be incorporated into the microbubble shell. We have not yet tested other binding molecules that could be used to bind PSMs to other targets but PEG tethers are commonly used for displaying molecular targeting molecules, and it is therefore reasonable to assume that other targeting moieties could be used to make PSMs to target a wide variety of biological surfaces.

Our immediate concerns moving forward, having shown the potential effectiveness of PSMs in vitro, is to test them in vivo. A few critical preliminary pilot studies appear to further support our in vitro observations, though further studies are necessary to confirm this.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- (1). Centers for Disease Control. Number of All-Listed Procedures for Discharges from Short-Stay Hospitals, by Procedure Category and Age: United States; 2010.
- (2). Schnüriger B; Barnmparas G; Branco BC; Lustenberger T; Inaba K; Demetriades D Prevention of Postoperative Peritoneal Adhesions: A Review of the Literature. *Am. J. Surg* 2011, 201 (1), 111–121. [PubMed: 20817145]
- (3). Arung W; Meurisse M; Detry O Pathophysiology and Prevention of Postoperative Peritoneal Adhesions. *World J. Gastroenterol. WJG* 2011, 17 (41), 4545–4553. [PubMed: 22147959]
- (4). Lyell DJ; Caughey AB; Hu E; Daniels K Peritoneal Closure at Primary Cesarean Delivery and Adhesions. *Obstet. Gynecol* 2005, 106 (2), 275–280. [PubMed: 16055575]
- (5). Duron J-J Postoperative Intraperitoneal Adhesion Pathophysiology. *Colorectal Dis. Off. J. Assoc. Coloproctology G. B. Irel* 2007, 9 (Suppl 2), 14–24.
- (6). Scott JW; Olufajo OA; Brat GA; Rose JA; Zogg CK; Haider AH; Salim A; Havens JM Use of National Burden to Define Operative Emergency General Surgery. *JAMA Surg* 2016, 151 (6), e160480–e160480. [PubMed: 27120712]
- (7). Ward BC; Panitch A Abdominal Adhesions: Current and Novel Therapies. *J. Surg. Res* 2011, 165 (1), 91–111. [PubMed: 20036389]
- (8). Lienemann A; Sprenger D; Steitz HO; Korell M; Reiser M Detection and Mapping of Intraabdominal Adhesions by Using Functional Cine MR Imaging: Preliminary Results. *Radiology* 2000, 217 (2), 421–425. [PubMed: 11058638]
- (9). Fuechsel F; Cosgrove D Ultrasound Contrast Agents: Basic Principles and Clinical Applications, 2nd ed.; Martin Dunitz, 2001; pp 327–334.
- (10). Faez T; Emmer M; Kooiman K; Versluis M; van der Steen AFW; de Jong N. 20 Years of Ultrasound Contrast Agent Modeling. *IEEE Trans. Ultrason. Ferroelectr. Freq. Control* 2013, 60 (1), 7–20. [PubMed: 23287909]
- (11). Fisher NG; Christiansen JP; Leong-Poi H; Jayaweera AR; Lindner JR; Kaul S Myocardial and Microcirculatory Kinetics of BR14, a Novel Third-Generation Intravenous Ultrasound Contrast Agent. *J. Am. Coll. Cardiol* 2002, 39 (3), 530–537. [PubMed: 11823094]
- (12). Epstein PS; Plesset MS On the Stability of Gas Bubbles in Liquid-Gas Solutions. *J. Chem. Phys* 1950, 18 (11), 1505.
- (13). Sirsi S; Borden M Microbubble Compositions, Properties and Biomedical Applications. *Bubble Sci., Eng., Technol* 2009, 1 (1–2), 3–17. [PubMed: 20574549]
- (14). Paefgen V; Doleschel D; Kiessling F Evolution of Contrast Agents for Ultrasound Imaging and Ultrasound-Mediated Drug Delivery. *Front. Pharmacol* 2015, 6, doi: DOI: 10.3389/fphar.2015.00197
- (15). D'Arrigo JS Aromatic Proteinaceous Surfactants Stabilize Long-lived Gas Microbubbles from Natural Sources. *J. Chem. Phys* 1981, 75 (2), 962–968.
- (16). Keller MW; Feinstein SB; Watson DD Successful Left Ventricular Opacification Following Peripheral Venous Injection of Sonicated Contrast Agent an Experimental Evaluation. *Am. Heart J* 1987 114 (3), 570.
- (17). Grinstaff MW; Suslick KS Air-Filled Proteinaceous Microbubbles: Synthesis of an Echo-Contrast Agent. *Proc. Natl. Acad. Sci. U. S. A* 1991, 88 (17), 7708–7710. [PubMed: 1652761]
- (18). Cui W; Bei J; Wang S; Zhi G; Zhao Y; Zhou X; Zhang H; Xu Y Preparation and Evaluation of Poly(L-Lactide-Co-Glycolide)(PLGA) Microbubbles as a Contrast Agent for Myocardial Contrast Echocardiography. *J. Biomed. Mater. Res., Part B* 2005, 73B (1), 171–178.
- (19). Cavalieri F; El Hamassi A; Chiessi E; Paradossi G Stable Polymeric Microballoons as Multifunctional Device for Biomedical Uses: Synthesis and Characterization. *Langmuir* 2005, 21 (19), 8758–8764. [PubMed: 16142958]

- (20). Park Y; Luce AC; Whitaker RD; Amin B; Cabodi M; Nap RJ; Szleifer I; Cleveland RO; Nagy JO; Wong JY Tunable Diacetylene Polymerized Shell Microbubbles as Ultrasound Contrast Agents. *Langmuir* 2012, 28 (8), 3766–3772. [PubMed: 22260537]
- (21). Hupfer B; Ringsdorf H; Schupp H Liposomes from Polymerizable Phospholipids. *Chem. Phys. Lipids* 1983, 33 (4), 355–374. [PubMed: 6680051]
- (22). Storrs RW; Tropper FD; Li HY; Song CK; Sipkins DA; Kuniyoshi JK; Bednarski MD; Strauss HW; Li KCP Paramagnetic Polymerized Liposomes as New Recirculating MR Contrast Agents. *J. Magn. Reson. Imaging* 1995, 5 (6), 719–724. [PubMed: 8748492]
- (23). Sipkins DA; Cheresch DA; Kazemi MR; Nevin LM; Bednarski MD; Li KC Detection of Tumor Angiogenesis in Vivo by AlphaVbeta3-Targeted Magnetic Resonance Imaging. *Nat. Med* 1998, 4 (5), 623–626. [PubMed: 9585240]
- (24). Li KCP; Guccione S; Bednarski MD Combined Vascular Targeted Imaging and Therapy: A Paradigm for Personalized Treatment. *J. Cell. Biochem* 2002, 87 (S39), 65–71. [PubMed: 12210723]
- (25). Hood JD Tumor Regression by Targeted Gene Delivery to the Neovasculature. *Science* 2002, 296 (5577), 2404–2407. [PubMed: 12089446]
- (26). Yavlovich A; Singh A; Tarasov S; Capala J; Blumenthal R; Puri A Design of Liposomes Containing Photopolymerizable Phospholipids for Triggered Release of Contents. *J. Therm. Anal. Calorim* 2009, 98 (1), 97–104. [PubMed: 20160877]
- (27). Mackiewicz N; Gravel E; Garofalakis A; Ogier J; John J; Dupont DM; Gombert K; Tavitian B; Doris E; Ducongé F Tumor-Targeted Polydiacetylene Micelles for In Vivo Imaging and Drug Delivery. *Small* 2011, 7 (19), 2786–2792. [PubMed: 21837631]
- (28). Qin G; Li Z; Xia R; Li F; O'Neill BE; Goodwin JT; Khant HA; Chiu W; Li KC Partially Polymerized Liposomes: Stable against Leakage yet Capable of Instantaneous Release for Remote Controlled Drug Delivery. *Nanotechnology* 2011, 22 (15), 155605. [PubMed: 21389566]
- (29). Federman N; Chan J; Nagy JO; Landaw EM; McCabe K; Wu AM; Triche T; Kang H; Liu B; Marks JD; et al. Enhanced Growth Inhibition of Osteosarcoma by Cytotoxic Polymerized Liposomal Nanoparticles Targeting the Alcam Cell Surface Receptor; DOI: 10.1155/2012/126906, accessed Oct 31, 2018.
- (30). Girgis MD; Federman N; Rochefort MM; McCabe KE; Wu AM; Nagy JO; Denny C; Tomlinson JS An Engineered Anti-CA19–9 Cys-Diabody for Positron Emission Tomography Imaging of Pancreatic Cancer and Targeting of Polymerized Liposomal Nanoparticles. *J. Surg. Res* 2013, 185 (1), 45–55. [PubMed: 23827791]
- (31). Müller SA; Treutner KH; Anurov M; Titkova S; Oettinger AP; Schumpelick V Experimental Evaluation of Phospholipids and Icodextrin in Re-Formation of Peritoneal Adhesions. *Br. J. Surg* 2003, 90 (12), 1604–1607. [PubMed: 14648743]
- (32). Zhou Z; Wu X; Kresak A; Griswold M; Lu Z-R Peptide Targeted Tripod Macrocyclic Gd(III) Chelates for Cancer Molecular MRI. *Biomaterials* 2013, 34 (31), 7683–7693. [PubMed: 23863450]
- (33). Duffy DC; McDonald JC; Schueller OJA; Whitesides GM Rapid Prototyping of Microfluidic Systems in Poly-(Dimethylsiloxane). *Anal. Chem* 1998, 70 (23), 4974–4984. [PubMed: 21644679]
- (34). Park YC; Zhang C; Kim S; Mohamedi G; Beigie C; Nagy JO; Holt RG; Cleveland RO; Jeon NL; Wong JY Microvessels-on-a-Chip to Assess Targeted Ultrasound-Assisted Drug Delivery. *ACS Appl. Mater. Interfaces* 2016, 8 (46), 31541–31549. [PubMed: 27781429]
- (35). Streeter JE; Gessner R; Miles I; Dayton PA Improving Sensitivity in Ultrasound Molecular Imaging by Tailoring Contrast Agent Size Distribution: In Vivo Studies. *Mol. Imaging* 2010, 9 (2), DOI: 10.2310/7290.2010.00005.
- (36). Church CC The Effects of an Elastic Solid Surface Layer on the Radial Pulsations of Gas Bubbles. *J. Acoust. Soc. Am* 1995, 97 (3), 1510–1521.
- (37). Hoff L; Sontum PC; Hovem JM Oscillations of Polymeric Microbubbles: Effect of the Encapsulating Shell. *J. Acoust. Soc. Am* 2000, 107 (4), 2272–2280. [PubMed: 10790053]

Figure 1.

(a) Schematic drawing of the microfluidic stepemulsification geometry used to produce microbubbles. The chip had channels with different heights of 1 μm (blue) and 20 μm (gray). (b) Optical image of the microchannel array at the end of the microfluidic chip containing 5- μm -wide channels for bubble production (scale bar 100 μm). (c) Image of the microfluidic chip (scale bar 1 cm).

Figure 2.

The polymerized shell microbubble (PSM) stability and diameter do not change significantly over time. The mean diameters of nontargeted (NT-PSM) and fibrin-targeted (T-PSM) polymerized shell microbubbles (PSMs) are shown over time. The PSMs were incubated in PBS or media at 37 °C. The data are expressed as the mean $\pm$ SD.

Figure 3.

LP-9 cellular viability after PSM exposure. LP-9 cells were exposed to 10^5 and 10^7 fibrin-targeted and nontargeted PSMs (T-PSM and NT-PSM, respectively) for 2, 24, and 48 h prior to assessing the cellular viability. Viability is expressed as the total number of dead cells divided by the total number of cells ($n = 3$). Fluorescent images were obtained for each time point and condition and were analyzed and quantified using ImageJ. Data is expressed as the mean $\pm$ SEM.

Figure 4.

LP-9 cellular viability after treatment with PSM and ultrasound. LP-9 cells were exposed to either 10^5 targeted or nontargeted PSM (T-PSM and NT-PSM, respectively) in the absence and presence of ultrasound treatment. Cellular viability was assessed in triplicate experiments using the LIVE/DEAD assay. Data is expressed as the mean $\pm$ SEM.

Figure 5.

Assessment of the specificity of fibrin binding of targeted PSMs. (a) Targeted microbubbles bind specifically to fibrin. Agarose solution (1%) was added and allowed to solidify in 100 cm Petri dishes. Fibrinogen, fibrin, or no addition (control) was added to the agarose layers. Fibrin-targeted or nontargeted PSMs (10^5 , T-PSM and NT-PSM, respectively) were added to each plate and incubated for 30 min at room temperature. Unbound PSMs were rinsed, and the fluorescence (ex/em 547/580) for each condition was measured using an OptiMax plate reader. Data is expressed as the mean $\pm$ SEM. (b) Targeted microbubbles (T-PSM) bound to fibrin are visible in B-mode ultrasound. Agarose gels (1%) were prepared in 100 cm Petri dishes with fibrin blotted (with “no fibrin” controls). Plates were exposed to nontargeted or fibrin-targeted PSMs (T-PSM, NT-PSM) for 30 min and then washed. B-mode ultrasound images were taken, and ImageJ was used to calculate grayscale values for fibrin and nonfibrin regions or similarly located regions on the “no fibrin” (– fibrin) plates. These values were averaged, and the fibrin (or corresponding) region’s average was divided by the “no fibrin” region. Error bars are uncertainties in the ratio.