



HAL
open science

A facile sonochemical synthesis of shell-stabilized reactive microbubbles using surface-thiolated bovine serum albumin with the Traut's reagent

Xiaochen Ma, Adrien Bussonniere, Qingxia Liu

► To cite this version:

Xiaochen Ma, Adrien Bussonniere, Qingxia Liu. A facile sonochemical synthesis of shell-stabilized reactive microbubbles using surface-thiolated bovine serum albumin with the Traut's reagent. *Ultrasonics Sonochemistry*, 2017, 36, pp.454-465. <10.1016/j.ultsonch.2016.12.033>. <hal-03939945>

HAL Id: hal-03939945

<https://hal.science/hal-03939945v1>

Submitted on 15 Jan 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

A facile sonochemical synthesis of shell-stabilized reactive microbubbles using surface-thiolated bovine serum albumin with the Traut's reagent

Xiaochen Ma, Adrien Bussonniere, and Qingxia Liu

Department of Chemical and Materials Engineering

Donadeo Innovation Centre for Engineering

University of Alberta

9211 - 116 St NW,

Edmonton, Canada T6G 1H9

ABSTRACT

The short lifetime of proteinaceous microbubbles produced using conventional sonication method has hindered their applications in drug delivery and metal removal from wastewater. In this study, we aimed to synthesize stable proteinaceous microbubbles and to demonstrate their reactivity. Our model protein, bovine serum albumin (BSA) was treated with 2-iminothiolane hydrochloride (Traut's reagent) to convert primary amines to thiols before the synthesis of microbubbles. Microbubbles produced with the Traut's reagent-treated BSA (BSA-SH MBs) were initially concentrated at median sizes of 0.5 and 2.5 μm . The 0.5 μm portion quickly vanished, and the 2.5 μm portion gradually shrank to ~ 850 nm within a week and became stabilized afterward for several months under 4 $^{\circ}\text{C}$. Characterizations of BSA-SH MBs by Fourier transform infrared (FTIR) spectroscopy and X-ray photoelectron spectroscopy (XPS) indicated the presence of free unbound thiols and primary amines on their surface, implying the possibility of further surface modification. Based on the zeta potential measurement, the isoelectric point (IEP) of BSA-SH MBs was determined to be 4.5. The attachments of BSA-SH MBs on alumina, silica, and gold surfaces in different pH environments were carried out with a quartz crystal microbalance with dissipation monitoring (QCM-D), demonstrating the reactivities of BSA-SH MBs. At pH 6, the negatively charged BSA-SH MBs were adsorbed onto the alumina surface by electrostatic interaction. Analogously, at pH 4, the adsorption of the positively charged BSA-SH MBs on the silica surface was confirmed. Compared with the

electrostatic interaction, the adsorption of BSA-SH MBs on the gold surface is attributed to the strong gold-thiol bonding effect. This is the first time that a universal approach for stabilizing protein-shelled microbubbles was reported using only one single step of surface treatment of proteins with the Traut's reagent.

KEYWORDS

Microbubbles, BSA, Traut's reagent, stability, longevity, adsorption

INTRODUCTION

In the last decade, microbubbles have been utilized in clinical applications,^[1-11] food industries,^[12, 13] and water treatment^[14, 15]. In clinical practice, as ultrasound contrast agents, microbubbles can improve the quality of the medical imaging^[1-3, 5-11]. Microbubbles have been employed as drug carriers^[1-3, 5, 6] and the loading of therapeutic molecules onto or into the microbubbles can aid the coupling of imaging with therapeutics (theranostics).^[7, 8, 11] Microbubbles have also been used for surface attachments with other functional molecules such as magnetic, semiconductor nanoparticles, and stimulus-responsive polymers.^{4, 9, 10} For example, Lentacker *et al.* developed polycation-coated microbubbles as potential gene delivery carriers;⁴ Park *et al.* demonstrated ultrasound and magnetic resonance imaging applications of microbubbles loaded with metal, metal oxide or semiconductor nanoparticles.¹⁰ Owing to their intrinsic antimicrobial properties, microbubbles have been intensively studied in the food industry as a sterilizing agent to depress the growth of *Bacillus* spores and *Escherichia coli*.^[12, 13] In wastewater treatment, ultrasonic radiation of microbubbles produces a vast amount of free radicals which can efficiently decompose organic compounds.^[14, 15]

Without stabilizers, a short lifetime (~ several hours) limits the use of conventional gas-filled microbubbles.^[16, 17] The pressure difference originating from the curved surface of the bubble, known as the Laplace pressure, spontaneously drives the entrapped gas to diffuse into the liquid phase.^[17, 18] Besides, Ostwald ripening adversely affects the lifetime of microbubbles; specifically, coalescence continues to reduce the surface energy of microbubbles until a complete

phase separation is reached.^[19-21] Surfactants can reduce the surface energy to lengthen the lifetime of microbubbles;^[22, 23] alternatively, biocompatible polymers such as proteins and lipids can be employed to construct a rigid shell, which reduces outward gas diffusion.^[3, 24, 25] Cavalieri *et al.* and Talu *et al.* respectively synthesized lysozyme-shelled and lipid-coated microbubbles with a lifetime of several months.^[24, 25]

The formation of disulfide bond has shown to increase the stability of microbubbles.^[25-28] During the process of acoustic cavitation, superoxide is generated, cross-linking free thiols of cysteine residues.^[25] A higher disulfide content contributes to a thicker shell and thus to a greater stability of microbubbles.^[27] Free thiols can be released internally from proteins with reducing agents such as DL-dithiothreitol (DTT) to enhance inter- and intramolecular disulfide bridging during microbubble formation.^[25, 26, 28] However, such treatment might excessively loosen up the protein structure and gives rise to a lowered hydrophobicity, which reduces the stability of microbubbles.^[27]

In this paper, we reported, for the first time, a facile synthesis of long-lived microbubbles with surface-treated proteins. Traut's reagent, which reacts with primary amines (Figure 1a), was applied to our model protein BSA to render thiol groups externally.^[29] Upon the ultrasonic radiation, the thiolated BSA (BSA-SH) forms the shell of microbubbles with air entrapped inside (BSA-SH MBs). Within a week, BSA-SH MBs gradually shrank to a critical size of ~850 nm while forming a thicker shell. As a result of bubble shrinkage, the increased Laplace pressure was balanced by the thickened shell, reaching an equilibrium and stabilizing for several months. The characterizations of microbubbles by Fourier transform infrared (FTIR) spectroscopy and X-ray photoelectron spectroscopy (XPS) indicated the presence of primary amines and thiols after the microbubble synthesis. Quartz crystal microbalance with dissipation monitoring (QCM-D) illustrated their reactivities on silica, alumina, and gold surfaces, shedding light on the possibilities of utilizing BSA-SH MBs in other applications such as drug delivery and metal removal from wastewater.

MATERIALS AND METHODS

Lyophilized BSA ($\geq 96\%$, A2153), Traut's reagent ($\geq 98\%$, I6256), ethylenediaminetetraacetic acid (EDTA) ($\geq 98.5\%$, ED-100G), silica nanoparticles (10-20 nm, 637238) and alumina nanoparticles (< 50 nm, 544833) were purchased from Sigma-Aldrich. Phosphate buffered saline (PBS, 28372) and spin desalting columns (PI-89891) were acquired from ThermoFisher Scientific. All reagents were used as received without further treatment. Milli-Q water with a resistivity of $18.2 \text{ M}\Omega\cdot\text{cm}$ was used for preparing solutions. Stock solutions of hydrochloric acid (HCl, 0.1 M) and sodium hydroxide (NaOH, 0.1M) were used to adjust the pH of solutions.

Synthesis of microbubbles using surface-treated BSA

EDTA (5 mM) was added to PBS to prevent oxidation of free sulfhydryls,^[30] and the EDTA-PBS mixture was adjusted to pH 8 with 0.1 M NaOH to assist effective thiolation of BSA with Traut's reagent.^[29, 31] BSA (50 mg in 0.75 ml EDTA-PBS) and Traut's reagent solutions (0, 0.5, 2, and 5 mg in 0.25 ml EDTA-PBS) were prepared, and BSA and Traut's reagent were allowed to react by mixing to form 1 ml solutions of 0, 5, 20, and 50 times molar excess of Traut's reagent. The mixtures were incubated for 1 hour at 23°C with a gentle swirling of 60 rpm (New Brunswick Innova 42/42R) before decanting into spin desalting columns to stop the reaction and remove the excess Traut's reagent. The mixtures were placed in a water bath (Fischer Scientific Isotemp Bath 4100 R20) at 45°C for 10 minutes (Figure 1c) to moderately loose up the structure of the thiolated BSA (BSA-SH). A conformational change of BSA does not occur below 40°C , and undesired aggregations start to take place at approximately 50°C .^[32-34] Ultrasonic radiation of 20 kHz was applied to the mixtures with a sonic dismembrator (Fisher ScientificTM Model 705). A temperature probe was used to monitor the solution temperature throughout the ultrasonic radiation (Figure 1b). An ultrasound impulse of 23% amplitude, equivalent to 173 W, was applied for 45 sec. The combination of amplitude and duration was selected based on empirical evidence. After the microbubble synthesis, two separate phases were formed. In brief, the foam phase containing the large, visible bubbles was at the top, and the aqueous phase containing the desired microbubbles was at the bottom. The synthesized

microbubbles were separated from the large, visible bubbles using a pipet. The separated aqueous phase was then centrifuged at 300 relative centrifugal force (RCF) for 10 min to collect the microbubbles,^[35] and then the top layer of the supernatant was resuspended in PBS. Milli-Q water was used to resuspend the supernatant for the QCM-D measurements. The centrifugal collection was repeated for three times, and the collected microbubbles were later stored at 4 °C.

Scanning electron microscope (SEM) imaging

To study morphology and the shell thickness of BSA-SH MBs, SEM images were collected using a field emission scanning electron microscope (FESEM: Carl-Zeiss Sigma) operated at an acceleration voltage of 5 kV. A small aliquot of the as-synthesized solution of BSA-SH MBs was dropped on a silicon wafer and left to evaporate overnight. To measure the shell thickness of the synthesized MBs under different conditions, a cross section of the shell was needed, and it was obtained by gently scraping the air-dried BSA-SH MBs with a piece of fine sandpaper.^[36] Following the breakage of microbubbles and the exposure of their cross sections, SEM images of them were obtained and the thickness of their shells was measured using ImageJ.^[37] In brief, a number of lines were drawn using ImageJ to label the thickness of the shell, from the outside to the inside boundary of the shell. The length of these lines were automatically calculated by ImageJ and averaged out to the thickness of the shell. For a better conductivity, a carbon thin film was sputter-coated on the sample surface before the imaging collection.

Size distribution, zeta potential, surface tension measurements

ImageJ software^[37] and a ZetaPALS particle size analyzer (Brookhaven Instrument, USA) were both adopted for size distribution measurements. Images of microbubbles at different stages were collected with a digital microscope (KEYENCE, VHX-700F) and histograms were accordingly generated via ImageJ. The size distributions were additionally measured using a ZetaPALS dynamic light scattering detector (Brookhaven Instruments, USA); 300 measured values were used to generate a size distribution histogram. The zeta potentials of silica nanoparticles, alumina nanoparticles, and BSA-SH MBs at different pH values were also

measured by the ZetaPALS module. Before measurements, the suspensions containing these agents were respectively diluted using PBS and adjusted to various pH values with HCl and NaOH. The zeta potential at each pH value was averaged over ten measurements. The surface tension of the surface-treated BSA aqueous solution was measured using a Theta Optical Tensiometer T200 (Biolin Scientific, Stockholm, Sweden). A quartz cell was used to contain the BSA-SH solution, and an air bubble was generated from a gastight syringe with a needle. The air bubble was recorded and analyzed using the Theta software for the measurement of the surface tension.

FTIR and XPS measurements

BSA was used as purchased. Lyophilized BSA-SH, BSA-SH MBs, and BSA-SH MBs reacted with alumina nanoparticles were collected using a freeze dryer (Labconco FreeZone 4.5) and subjected to FTIR spectroscopy and XPS. FTIR spectra were recorded with 32 scans at a resolution of 1 cm^{-1} using a Nicolet iS50 FT-IR spectrometer equipped with an iS50 automated beamsplitter exchanger (ABX) at wavenumber 700 to 4000 cm^{-1} . For a non-destructive analysis, the attenuated total reflectance (ATR) module was adopted instead of the diffuse reflectance infrared Fourier transmission spectroscopy (DRIFTS) or potassium bromide (KBr) method which involves sample grinding and destruction. High-resolution X-ray photoelectron spectra of N1s and S2p were obtained using an XPS (Kratos AXIS 165) with monochromatic Al $K\alpha$ X-rays (1486.6 eV). The X-ray source was applied at 210 W, and the pressure was kept at $2.5\text{E-}8$ torr. A pass energy of 20 eV at an increment of 0.1 eV was used to collect the high-resolution XPS output.

QCM-D measurements

QCM-D measurements were performed using a D300 QCM-D (Q-Sense Västra Frölunda, Sweden) with a QAFC 302 axial flow measurement chamber. The silica, alumina, and gold sensors (QSX303), supplied by Q-Sense AB, were quartz crystals coated with SiO_2 , Al_2O_3 , and Au, respectively. Before the injection of solutions, all sensors were placed in the chamber and excited to their fundamental resonant frequency at $\sim 4.9\text{ MHz}$ using an AC voltage. The

chamber was thermally controlled at 23 °C, and the flow rate was adjusted to 25 $\mu\text{L}/\text{min}$ using a flow dispenser (ISM935C, ISMATEC). Background solutions consisting of Milli-Q water pre-adjusted to the same pH as the solution of BSA-SH MBs were injected into the chamber for 10 min. Following the background solution, the solution of BSA-SH MBs flowed into the chamber for 30 min, and then the chamber was rinsed with the original background solution for 10 min.

RESULTS AND DISCUSSION

Spatial and temporal size distributions of BSA-SH MBs

As the size of microbubbles plays a critical role in governing their applications, the spatial and temporal size distributions were respectively studied. Because the Traut's reagent dosage is directly linked to the conversion efficiency of primary amines to thiols, its effect on the size distribution of the microbubbles formed was determined. In the absence of the Traut's reagent, microbubbles were observed to be present in a size range of 0.4 - 4.0 μm (Figure 2a) by image analysis and in a size range of 0.4 - 1.8 μm (Figure S1a) by dynamic light scattering. Upon introducing the Traut's reagent, a bimodal distribution of microbubbles separately centered at 0.5 and 2.5 μm , respectively, were observed (Figure 2b, c, S1b, c). On the contrary, such a trend was not present when the molar excess of Traut's reagent was increased to 50 times (Figure 2d, S1d). To determine an optimal ratio of the Traut's reagent to BSA, the stability of microbubbles was tested. In detail, microbubbles originally sized at $\sim 0.5 \mu\text{m}$ were merely capable of maintaining the bubble structure for a few hours, consistent with the reported short lifetime of conventional BSA microbubbles.^[26, 38, 39] On the other hand, microbubbles initially with an average size of 2.5 μm were longer-lived, and the bubbles gradually shrank to $\sim 850 \text{ nm}$ in the days following the synthesis. At 20 times BSA molar excess of the Traut's reagent, such a long-lived fraction of the synthesized microbubbles was observed to be the greatest (Figure 2c, S1c), and this condition was therefore considered to be optimal for the synthesis of BSA-SH MBs and the subsequent characterizations (Figure 3, 7, 8, 9, 10, 11). The higher dosage of the Traut's reagent, the more surface coverage of thiols after the reaction, which results in more disulfide bonds for strengthening the shell. In other words, the formation of the long-lived microbubbles was a result of the optimal surface coverage of thiols. It was therefore

hypothesized that a molar excess of the Traut's reagent of 5 times BSA or lower did not provide sufficient surface coverage of thiol groups available for synthesizing stable microbubbles. On the other hand, an overly high surface coverage of thiol groups rendered by high dosages such as 50 times molar excess of Traut's reagent to BSA created excessive steric hindrance to stable microbubble formation. This argument was experimentally supported through the measurement of the shell thickness of the microbubbles synthesized with surface-treated BSA with different molar excesses of the Traut's reagent (Figure 6a, S4a-d). In detail, the untreated BSA generated a shell of 45 ± 10 nm upon ultrasonic radiation while the treated BSA formed microbubbles with a thicker shell. The 20 times molar excess of the Traut's reagent gave rise to the greatest thickness of the subsequently generated microbubbles, 155 ± 20 nm, further explaining their long-term stability. However, an excessively low or high molar excess of the Traut's reagent such as 5 or 50 times failed to enhance the lifetime of the synthesized microbubbles as much as the optimal 20 times molar excess, shown as moderately thickened shell thicknesses of 75 ± 20 nm and 85 ± 10 nm, respectively.

At the aforementioned optimal condition, temporal size distributions of BSA-SH MBs were accordingly measured using image analysis and dynamic light scattering. A roughly bimodal size distribution with two peaks at 2.5 and 0.5 μm appeared following the synthesis (Figure 2c, 4a). As mentioned above, the unstable portion sized at 0.5 μm , pointed by the red arrows (Figure 4a), were mostly vanished within a few hours and completely disappeared in 1 day after the synthesis (Figure 4b). The long-lived portion initially centered at 2.5 μm , on the other hand, gradually shrank in the days after the synthesis and eventually stabilized at the critical size of 850 nm (Figure 4b-d, 5b-d, S2b-d). Immediately after the synthesis, the concentration of BSA-SH MBs was measured to be $8.5 \pm 1.4 \times 10^7$ bubbles/ml, and it was dropped to $3.8 \pm 1.0 \times 10^7$ bubbles/ml within 3 days (Figure S3). The collapse of the sub-micron portion was the major driving force lowering the overall concentration, but the stabilized long-lived portion was well maintained afterwards (Figure S3). The short lifetime of the 0.5 μm portion was attributed to its incomplete thiolation during the surface treatment. As a result, fewer thiols of the starting material gave rise to fewer cross-linkers of disulfide bonds in the synthesized microbubbles that easily collapsed under the Laplace pressure. In contrast, the long-lived portion of 2.5 μm with enough cross-linkers was able to sustain its spherical structure over

time. The shrinkage of BSA-SH MBs was experimentally observed to be associated with the thickening of their shells; more specifically, the initial shell thickness of 155 ± 20 nm gradually increased to 280 ± 30 nm within a week, and then eventually stabilized at 290 ± 25 nm (Figure 6b, S4e). The volume change of the shell materials (ΔV_{shell}) could be roughly estimated by assuming BSA-SH MBs were perfectly spherical without losing shell materials during their shrinkage. Immediately after the synthesis, BSA-SH MBs, with an average radius of $1.25 \mu\text{m}$ and an average shell thickness of 155 nm, the shell volume (V_0) could be estimated using $V_0 \approx (1.25 \mu\text{m}^3 - (1.25-0.155) \mu\text{m}^3) 4\pi/3 \approx 2.68 \mu\text{m}^3$. Analogously, the shell volume of BSA-SH MBs after one week (V_1) was $V_1 \approx (0.425 \mu\text{m}^3 - (0.425-0.29) \mu\text{m}^3) 4\pi/3 \approx 0.311 \mu\text{m}^3$. It could be readily seen that their shell volume was greatly compressed along with the shrinkage of BSA-SH MBs, indicating the adoption of a more close-packed shell over time. As the dominant driving force of the bubble shrinkage, the Laplace pressure can be calculated using the Young-Laplace equation given as $\Delta P = 2\gamma/R$ where γ and R are the surface tension and spherical radius, respectively.^[40] The measured surface tension of the thiolated BSA, BSA-SH, was determined to be 50.0 mN/m (Figure S5) and it was slightly lower than that of the untreated BSA, $51-53$ mN/m.^[41, 42] With a starting diameter of $2.5 \mu\text{m}$, the Laplace pressure inside the newly synthesized microbubbles was 0.79 atm, which was considered to be the most probable driving force of the microbubble shrinkage. The condition of the microbubble stabilization was reported to be $E_b > 2\sigma R/3d^2$, where E_b , σ , R , and d represent the bulk modulus of the shell, the interfacial tension between the air and solution, the radius of microbubbles, and the thickness of the shell.^[36, 43] After the microbubble shrinkage, the bulk modulus of the shell was calculated to be at least 3.79×10^5 N/m² to stabilize microbubbles with a final diameter of 850 nm and a thickened shell of ~ 290 nm. Driven by the Laplace pressure, microbubbles continued to shrink until reaching their critical size where the Laplace pressure was eventually overcome by the thickened, more close-packed shell.^[44-46]

FTIR and XPS measurements of BSA-SH MBs

FTIR spectra of BSA, BSA-SH, and BSA-SH MBs were respectively recorded to track the changes in bonding throughout the synthesis of BSA-SH MBs (Figure 7). For a non-destructive quantitative analysis, the peak heights of amide I (C=O stretching) and amide II (C-N

stretching and N-H bending) respectively at 1650 and 1532 cm^{-1} were manually fixed, and then the relative magnitude of other bonds was compared accordingly. Compared to the undetectable presence of free thiols in untreated BSA (Figure 7a), an appreciable increase in thiol content in BSA-SH (Figure 7b) was shown in the peak intensity at 2510 cm^{-1} , confirming the successful thiolation of BSA. The slight shift of the experimental value from the literature value, typically in the region of 2550–2600 cm^{-1} ,^[47] may arise from the abundant intra- and intermolecular hydrogen bondings.^[48] Although the formation of BSA-SH MBs greatly consumed free thiols,^[24-28] there were still free thiols remaining on the surface shown with a reduced peak intensity at 2510 cm^{-1} (Figure 7c). The peaks of the C-N stretching and the CH_2 rocking vibrations of the primary amine at 1235 and 1390 cm^{-1} , respectively throughout the synthesis (Figure 7a-c) suggested the presence of the primary amine.^[49, 50] Other major peaks at 1075, 3040, and 3381 were attributed to the C-O stretch of primary alcohol, the O-H stretch of carboxylic acid, and the O-H stretch of alcohols, respectively.^[49-51] Based on the FTIR data, free thiols were successfully rendered onto BSA after the surface treatment, and partially consumed during the microbubble formation; on the other hand, the primary amines were present throughout the synthesis.

High resolution XPS of S2p (Figure 8) and N1s (Figure 9) was conducted to track the changes in chemical bonding throughout the synthesis of BSA-SH MBs. For S2p spectra, the peaks were fitted using S2p doublets consisting of S2p_{3/2} and S2p_{1/2} with a 2:1 area ratio and a splitting of 1.2 eV according to the spin-orbit coupling.^[52, 53] Before the thiolation, only disulfide bond was observed with the S2p_{3/2} and S2p_{1/2} peaks at 163.5 and 164.7, respectively (Figure 8a).^[52, 54, 55] After the surface treatment of BSA to BSA-SH, along with the pre-existing disulfide bond, free thiols appeared with the S2p_{3/2} and S2p_{1/2} peaks at 162.7 and 163.9, respectively (Figure 8b). In disulfide bond, sulfur was equipped with a negative charge of 1; however, in thiols, sulfur was reduced to a negative charge of 2, endowing a better ability to release the electron upon the X-ray excitation. The downward shifts of the S2p_{3/2} and S2p_{1/2} peaks were attributed to the higher electron-releasing tendency of the sulfur in thiols in comparison with that in the disulfide bond. After the synthesis of BSA-SH MBs, the mutual existence of disulfide bond and thiol were indicated by their S2p_{3/2} and S2p_{1/2} peaks at 163.5 and 164.7, and 162.7 and 163.9, respectively (Figure 8c). In addition to their co-existence, their relative amounts could be estimated by comparing the relative peak area of either their S2p_{3/2} or S2p_{1/2} peak. In specific, the

content of free thiols was increased from indiscernible to 48.2 % (Figure 8a, b) after the surface thiolation, and it was dropped to 26.3 % after the bubble synthesis (Figure 8b, c). In agreement with the FTIR analysis (Figure 7), the S2p spectra illustrated the successful thiolation that renders free thiols onto BSA and the remaining presence of free thiols albeit being partially consumed by the synthesis of BSA-SH MBs.

The binding energy peaks of the N1s spectra at 400.3, 399.6, and 398.9 eV correspond to H-bonded nitrogen, secondary amines, and primary amines, respectively (Figure 9).^[56-58] The partial consumption of primary amines was confirmed through a decrease in peak area at 398.9 eV from 20.92 to 12.04 % after the thiolation with Traut's reagent (Figure 9a, b). The subsequent sonochemical formation of the microbubbles had a negligible effect on the content of primary amine from 12.04 to 12.74% (Figure 9b, c). At the optimal dosage of the Traut's reagent, 20 times molar excess, a partial conversion of primary amine to thiol occurred during the surface treatment of native BSA, and their co-existence after the synthesis of BSA-SH MBs was consistently indicated from the FTIR spectra (Figure 7) and XPS binding energies of S2p (Figure 8) and N1s (Figure 9).

Zeta potential and QCM-D adsorption measurements

The zeta potentials of BSA-SH MBs were measured in a broad range of pH 3 to 9 (Figure 10). As the pH increases, the zeta potentials of BSA-SH MBs decreases, exhibiting the same trend of the native BSA.^[59] The isoelectric point (IEP), referred to the pH where a molecule carries a zero net charge, was determined to be 4.5 for the synthesized BSA-SH MBs from the zeta potential measurement (Figure 10). The IEP of native BSA is theoretically reduced from 5.6 to 4.1 if a complete conversion occurs from primary amine-containing lysine with a pKa of 9.7 to thiol-containing cysteine with a pKa of 5.1.^[60, 61] The difference between the measured and theoretical values might be attributed to an incomplete conversion of the primary amines to thiols as shown by FTIR (Figure 7) and XPS (Figure 8, 9) analyses. Also, zeta potential measurements of two common metal oxide nanoparticles (alumina and silica) were also performed to demonstrate the opposite signs of zeta potentials to BSA-SH MBs and thus the possible electrostatic interactions over certain pH regions. Consistent with the literature values,^[62-66]

alumina and silica nanoparticles were measured to be positively and negatively charged, respectively from pH 3 to 9 (Figure 10). It could be readily seen that, below its IEP of 4.5, BSA-SH MBs favor an electrostatic interaction with the negatively charged silica while, above its IEP, BSA-SH MBs are expected to interact with the positively charged alumina.

To probe the reactivity of BSA-SH MBs, QCM-D adsorption tests were performed; specifically, BSA-SH MBs were introduced onto alumina (Figure 11a) and silica (Figure 11c) surfaces at certain pH regions by electrostatic interactions and onto gold surfaces (Figure 11e) by the gold-thiol bonding effect. The Sauerbrey equation states that the mass adsorbed on a piezoelectric crystal and its resonant frequency are inversely related; in other words, a decline of the resonance frequency indicates the occurrence of adsorption.^[67] Extensive adsorptions of BSA-SH MBs on an alumina sensor at pH 6 (Figure 11b) and on a silica sensor at pH 4 (Figure 11d) were confirmed through significant drops in frequency after the injection at 10 min. Immediately following the drop, both sensors underwent a zig-zag fluctuation of frequencies before they were rinsed off with the background solution at 30 min, revealing a dynamic interaction between microbubbles and sensor. Free microbubbles constantly compete with and replace a small portion of the loosely-bound microbubbles on the sensor, reaching a dynamic equilibrium. The slight increment in frequency at 30 min on both sensors depicts the injection of background solution which removed loosely-bound microbubbles. The pH values were selected to create opposite charges between BSA-SH MBs and the sensors. At pH 6, BSA-SH MBs with an IEP of 4.5 carry a negative charge while the alumina sensor is positively charged; the oppositely charged surfaces favor adsorption. Analogously, the strong interaction at pH 4 between positively charged BSA-SH MBs and a negatively charged silica sensor is shown as a sharp decrease in frequency (Figure 11d). At pH 10, both the BSA-SH MBs and the alumina sensor are negatively charged, leading to a minor, unspecific adsorption (Figure S6a). Similarly, no appreciable adsorption was observed between BSA-SH MBs and the silica sensor at pH 4, a value at which both surfaces are positively charged (Figure S6b). Regarding the viscoelastic properties of the adsorbed BSA-SH MBs, a large-scale adsorption (Figure 11b, d), in comparison with a minor adsorption (Figure S6a, b), gave rise to a larger increment of dissipation which was positively related to their viscoelasticity. To test the reactivity of the surface thiols, BSA-SH MBs were also introduced to react with the gold sensor at pH 6 where both the microbubbles and

the gold sensor (IEP = 5.2)^[68] are negatively charged excluding the possibility of electrostatic interactions. A significant degree of adsorption of BSA-SH MBs, as well as a dramatically increased viscoelasticity were observed on a gold sensor (Figure 11f). Compared to the charge neutralization in the previous scenarios (Figure 11a-d), the strong bonding effect between gold and thiol might explain the greater magnitude (Figure 11e, f) of the interaction between BSA-SH MBs and gold surface.^[52, 69-71] Also, no zig-zag fluctuation of frequencies was observed for the adsorption of BSA-SH MBs on the gold sensor, further indicating the strong gold-thiol bond without any appreciable competing effect from the free microbubbles. Based on the QCM-D tests, the reactivities of carboxylic and amine groups of BSA-SH MBs were indirectly testified through electrostatic attachments onto the alumina and silica sensors; in addition, the reactivity of the surface thiol groups was directly proved by reacting with the gold sensor. With these reactive functional groups on their surfaces, BSA-SH MBs could be used to load drug molecules through either bond formation or electrostatic interactions. Alternatively, BSA-SH MBs could be directly used to remove metal nanoparticles from wastewater through column flotation. As shown from the QCM-D tests, these synthesized microbubbles have great affinity to metal surfaces; therefore, they can collect metal or metal ions as they float from the bottom to the top of a wastewater column. The novel, facile synthesis of stable proteinaceous microbubbles provides opportunities of expanding their utilizations in either drug delivery or wastewater treatment. Moreover, these microbubbles exhibit an acoustic attenuation coefficient in the order of magnitude of conventional microbubbles^[72, 73] (0.53 dB/cm at 3.3 MHz, measurements detailed in the SI) suggesting possible applications in acoustic imaging.

CONCLUSIONS

A facile synthesis of proteinaceous microbubbles with an increased stability was demonstrated using the surface-thiolated BSA with the Traut's reagent. At the optimal molar excess of the Traut's reagent, 20 times, BSA-SH MBs showed an initial size distribution that peaked at 2.5 and 0.5 μm , respectively. The 0.5 μm portion vanished within three days, and the 2.5 μm portion gradually shrank to and eventually stabilized at 850 nm for several months. FTIR spectra and XPS analyses showed the thiol, primary amine, and carboxylic groups on the surface

of BSA-SH MBs, and these functional groups were proved to be reactive via the QCM-D adsorptions tests. Based on these reactive functional groups, applications of BSA-SH MB in drug delivery and water purification could be further exploited through either electrostatic interactions or bond formations.

ACKNOWLEDGMENTS

Financial support from the Natural Science and Engineering Research Council of Canada (NSERC) and the Canadian Centre for Clean Coal/Carbon and Mineral Processing Technologies (C5MPT) are greatly appreciated.

REFERENCES

1. Blomley, M. and D. Cosgrove, *Microbubble echo-enhancers: a new direction for ultrasound?* The Lancet, 1997. **349**(9069): p. 1855-1856.
2. Cosgrove, D., *Echo enhancers and ultrasound imaging.* European journal of radiology, 1997. **26**(1): p. 64-76.
3. Calliada, F., et al., *Ultrasound contrast agents: basic principles.* European journal of radiology, 1998. **27**: p. S157-S160.
4. Lentacker, I., et al., *Ultrasound-responsive polymer-coated microbubbles that bind and protect DNA.* Langmuir, 2006. **22**(17): p. 7273-7278.
5. Ferrara, K., R. Pollard, and M. Borden, *Ultrasound microbubble contrast agents: fundamentals and application to gene and drug delivery.* Biomedical Engineering, 2007. **9**.
6. Cosgrove, D. and C. Harvey, *Clinical uses of microbubbles in diagnosis and treatment.* Medical & biological engineering & computing, 2009. **47**(8): p. 813-826.
7. Ferrara, K.W., M.A. Borden, and H. Zhang, *Lipid-shelled vehicles: engineering for ultrasound molecular imaging and drug delivery.* Accounts of chemical research, 2009. **42**(7): p. 881-892.
8. Qin, S., C.F. Caskey, and K.W. Ferrara, *Ultrasound contrast microbubbles in imaging and therapy: physical principles and engineering.* Physics in medicine and biology, 2009. **54**(6): p. R27.
9. Yang, F., et al., *Superparamagnetic iron oxide nanoparticle-embedded encapsulated microbubbles as dual contrast agents of magnetic resonance and ultrasound imaging.* Biomaterials, 2009. **30**(23): p. 3882-3890.
10. Park, J.I., et al., *Microbubbles loaded with nanoparticles: a route to multiple imaging modalities.* Acs Nano, 2010. **4**(11): p. 6579-6586.
11. Kiessling, F., et al., *Ultrasound microbubbles for molecular diagnosis, therapy, and theranostics.* Journal of nuclear medicine, 2012. **53**(3): p. 345-348.

12. Ishikawa, H., et al., *Inactivation of Bacillus spores by the supercritical carbon dioxide micro-bubble method*. *Bioscience, biotechnology, and biochemistry*, 1997. **61**(6): p. 1022-1023.
13. Kobayashi, F., et al., *Inactivation of Escherichia coli by CO₂ microbubbles at a lower pressure and near room temperature*. *Transactions of the ASABE*, 2009. **52**(5): p. 1621-1626.
14. Suslick, K.S., *Sonochemistry*. *science*, 1990. **247**(4949): p. 1439-1445.
15. Xu, Q., et al., *A comparative study of microbubble generation by mechanical agitation and sonication*. *Innovative Food Science & Emerging Technologies*, 2008. **9**(4): p. 489-494.
16. Epstein, c.e.P. and M.S. Plesset, *On the Stability of Gas Bubbles in Liquid-Gas Solutions*. *The Journal of Chemical Physics*, 1950. **18**(11): p. 1505-1509.
17. Kloek, W., T. van Vliet, and M. Meinders, *Effect of bulk and interfacial rheological properties on bubble dissolution*. *Journal of Colloid and Interface Science*, 2001. **237**(2): p. 158-166.
18. Kabalnov, A., et al., *Dissolution of multicomponent microbubbles in the bloodstream: 1. Theory*. *Ultrasound in medicine & biology*, 1998. **24**(5): p. 739-749.
19. Lemlich, R., *Prediction of changes in bubble size distribution due to interbubble gas diffusion in foam*. *Industrial & Engineering Chemistry Fundamentals*, 1978. **17**(2): p. 89-93.
20. Binks, B.P., *Modern aspects of emulsion science*. 1998: Royal Society of Chemistry.
21. Taylor, P., *Ostwald ripening in emulsions*. *Advances in colloid and interface science*, 1998. **75**(2): p. 107-163.
22. Chatterjee, D. and K. Sarkar, *A Newtonian rheological model for the interface of microbubble contrast agents*. *Ultrasound in Medicine and Biology*, 2003. **29**(12): p. 1749-1757.
23. Barak, M. and Y. Katz, *Microbubbles - Pathophysiology and clinical implications*. *Chest*, 2005. **128**(4): p. 2918-2932.
24. Talu, E., et al., *Long-term stability by lipid coating monodisperse microbubbles formed by a flow-focusing device*. *Langmuir*, 2006. **22**(23): p. 9487-9490.
25. Cavalieri, F., et al., *Ultrasonic synthesis of stable, functional lysozyme microbubbles*. *Langmuir*, 2008. **24**(18): p. 10078-10083.
26. Grinstaff, M.W. and K.S. Suslick, *Air-filled proteinaceous microbubbles: synthesis of an echo-contrast agent*. *Proceedings of the National Academy of Sciences*, 1991. **88**(17): p. 7708-7710.
27. Cavalieri, F., et al., *One-pot ultrasonic synthesis of multifunctional microbubbles and microcapsules using synthetic thiolated macromolecules*. *Chemical Communications*, 2011. **47**(14): p. 4096-4098.
28. Vong, F., et al., *A comparison of the physical properties of ultrasonically synthesized lysozyme-and BSA-shelled microbubbles*. *Ultrasonics sonochemistry*, 2014. **21**(1): p. 23-28.
29. Traut, R.R., et al., *Methyl 4-mercaptobutyrimidate as a cleavable crosslinking reagent and its application to the Escherichia coli 30S ribosome*. *Biochemistry*, 1973. **12**(17): p. 3266-3273.
30. Tarbell, D., *The mechanism of oxidation of thiols to disulfides*. *Organic sulfur compounds*, 1961. **1**: p. 97-102.

31. Jue, R., et al., *Addition of sulfhydryl groups of Escherichia coli ribosomes by protein modification with 2-iminothiolane (methyl 4-mercaptobutyrimidate)*. *Biochemistry*, 1978. **17**(25): p. 5399-5406.
32. Clark, A., et al., *ELECTRON MICROSCOPY OF NETWORK STRUCTURES IN THERMALLY-INDUCED GLOBULAR PROTEIN GELS*. *International Journal of Peptide and Protein Research*, 1981. **17**(3): p. 380-392.
33. Takeda, K., et al., *Conformational change of bovine serum albumin by heat treatment*. *Journal of protein chemistry*, 1989. **8**(5): p. 653-659.
34. Yamasaki, M., H. Yano, and K. Aoki, *Differential scanning calorimetric studies on bovine serum albumin: II. Effects of neutral salts and urea*. *International journal of biological macromolecules*, 1991. **13**(6): p. 322-328.
35. Feshitan, J.A., et al., *Microbubble size isolation by differential centrifugation*. *Journal of colloid and interface science*, 2009. **329**(2): p. 316-324.
36. Rovers, T.A., et al., *Temperature is key to yield and stability of BSA stabilized microbubbles*. *Food Hydrocolloids*, 2016. **52**: p. 106-115.
37. Abràmoff, M.D., P.J. Magalhães, and S.J. Ram, *Image processing with ImageJ*. *Biophotonics international*, 2004. **11**(7): p. 36-42.
38. Gedanken, A., *Preparation and properties of proteinaceous microspheres made sonochemically*. *Chemistry—A European Journal*, 2008. **14**(13): p. 3840-3853.
39. Sirsi, S., et al., *Lung surfactant microbubbles*. *Soft Matter*, 2009. **5**(23): p. 4835-4842.
40. Graf, K. and M. Kappl, *Physics and chemistry of interfaces*. 2006: John Wiley & Sons.
41. Tripp, B.C., J.J. Magda, and J.D. Andrade, *Adsorption of globular proteins at the air/water interface as measured via dynamic surface tension: concentration dependence, mass-transfer considerations, and adsorption kinetics*. *Journal of colloid and interface science*, 1995. **173**(1): p. 16-27.
42. McClellan, S.J. and E.I. Franses, *Effect of concentration and denaturation on adsorption and surface tension of bovine serum albumin*. *Colloids and Surfaces B: Biointerfaces*, 2003. **28**(1): p. 63-75.
43. Meinders, M.B. and T. van Vliet, *The role of interfacial rheological properties on Ostwald ripening in emulsions*. *Advances in colloid and interface science*, 2004. **108**: p. 119-126.
44. Du, Z., et al., *Outstanding stability of particle-stabilized bubbles*. *Langmuir*, 2003. **19**(8): p. 3106-3108.
45. Subramaniam, A.B., et al., *Colloid science: Non-spherical bubbles*. *Nature*, 2005. **438**(7070): p. 930-930.
46. Azmin, M., et al., *Dissolution of coated microbubbles: the effect of nanoparticles and surfactant concentration*. *Materials Science and Engineering: C*, 2012. **32**(8): p. 2654-2658.
47. Tingaut, P., R. Hauert, and T. Zimmermann, *Highly efficient and straightforward functionalization of cellulose films with thiol-ene click chemistry*. *Journal of Materials Chemistry*, 2011. **21**(40): p. 16066-16076.
48. Desiraju, G.R. and T. Steiner, *The weak hydrogen bond: in structural chemistry and biology*. Vol. 9. 2001: Oxford University Press on Demand.
49. Stewart, J.E., *Vibrational spectra of primary and secondary aliphatic amines*. *The Journal of Chemical Physics*, 1959. **30**(5): p. 1259-1265.

50. Barth, A., *The infrared absorption of amino acid side chains*. Progress in biophysics and molecular biology, 2000. **74**(3): p. 141-173.
51. Socrates, G., *Infrared and Raman characteristic group frequencies: tables and charts*. 2004: John Wiley & Sons.
52. Castner, D.G., K. Hinds, and D.W. Grainger, *X-ray photoelectron spectroscopy sulfur 2p study of organic thiol and disulfide binding interactions with gold surfaces*. Langmuir, 1996. **12**(21): p. 5083-5086.
53. Ishida, T., et al., *High resolution X-ray photoelectron spectroscopy measurements of octadecanethiol self-assembled monolayers on Au (111)*. Langmuir, 1998. **14**(8): p. 2092-2096.
54. Bain, C.D., H.A. Biebuyck, and G.M. Whitesides, *Comparison of self-assembled monolayers on gold: coadsorption of thiols and disulfides*. Langmuir, 1989. **5**(3): p. 723-727.
55. Nazari, A.M., P.W. Cox, and K.E. Waters, *Copper ion removal from dilute solutions using ultrasonically synthesised BSA-and EWP-coated air bubbles*. Separation and Purification Technology, 2014. **132**: p. 218-225.
56. Zhang, F. and M. Srinivasan, *Self-assembled molecular films of aminosilanes and their immobilization capacities*. Langmuir, 2004. **20**(6): p. 2309-2314.
57. Eby, D.M., et al., *Probing the molecular structure of antimicrobial peptide-mediated silica condensation using X-ray photoelectron spectroscopy*. Journal of Materials Chemistry, 2012. **22**(19): p. 9875-9883.
58. Yu, B., et al., *Functionalized graphene oxide/phosphoramidate oligomer hybrids flame retardant prepared via in situ polymerization for improving the fire safety of polypropylene*. RSC Advances, 2014. **4**(60): p. 31782-31794.
59. Rezwani, K., et al., *Bovine serum albumin adsorption onto colloidal Al₂O₃ particles: A new model based on Zeta potential and UV-Vis measurements*. Langmuir, 2004. **20**(23): p. 10055-10061.
60. Vogli, R. and D. Oreopoulos, *Amino acid sequence in bovine serum albumin*. Clin. Chim. Acta, 1973. **48**: p. 353.
61. Huang, B.X., H.-Y. Kim, and C. Dass, *Probing three-dimensional structure of bovine serum albumin by chemical cross-linking and mass spectrometry*. Journal of the American Society for Mass Spectrometry, 2004. **15**(8): p. 1237-1247.
62. Parks, G.A., *The isoelectric points of solid oxides, solid hydroxides, and aqueous hydroxo complex systems*. Chemical Reviews, 1965. **65**(2): p. 177-198.
63. Sprycha, R., *Electrical double layer at alumina/electrolyte interface: I. Surface charge and zeta potential*. Journal of colloid and interface science, 1989. **127**(1): p. 1-11.
64. Kosmulski, M., *Chemical properties of material surfaces*. Vol. 102. 2001: CRC press.
65. Júnior, J.A.A. and J.B. Baldo, *The behavior of zeta potential of silica suspensions*. New Journal of Glass and Ceramics, 2014. **4**(02): p. 29.
66. Rajan, Y.C., B.S. Inbaraj, and B.H. Chen, *Synthesis and characterization of poly (γ -glutamic acid)-based alumina nanoparticles with their protein adsorption efficiency and cytotoxicity towards human prostate cancer cells*. RSC Advances, 2015. **5**(20): p. 15126-15139.
67. Sauerbrey, G., *Verwendung von Schwingquarzen zur Wägung dünner Schichten und zur Mikrowägung*. Zeitschrift für physik, 1959. **155**(2): p. 206-222.

68. Phan, H.T., et al., *Investigation of bovine serum albumin (BSA) attachment onto self-assembled monolayers (SAMs) using combinatorial quartz crystal microbalance with dissipation (QCM-D) and spectroscopic ellipsometry (SE)*. PloS one, 2015. **10**(10): p. e0141282.
69. Grönbeck, H., A. Curioni, and W. Andreoni, *Thiols and disulfides on the Au (111) surface: The headgroup-gold interaction*. Journal of the American Chemical Society, 2000. **122**(16): p. 3839-3842.
70. Li, T., et al., *Characterization of floc size, strength and structure under various coagulation mechanisms*. Powder technology, 2006. **168**(2): p. 104-110.
71. Jadzinsky, P.D., et al., *Structure of a thiol monolayer-protected gold nanoparticle at 1.1 Å resolution*. Science, 2007. **318**(5849): p. 430-433.
72. De Jong, N., et al., *Ultrasonic characterization of ultrasound contrast agents*. Medical & biological engineering & computing, 2009. **47**(8): p. 861-873.
73. Raymond, J.L., et al., *Broadband attenuation measurements of phospholipid-shelled ultrasound contrast agents*. Ultrasound in medicine & biology, 2014. **40**(2): p. 410-421.