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Theranostic Multilayer Capsules for Ultrasound Imaging and Guided Drug Delivery

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Abstract

Despite the accessibility of ultrasound, the clinical potential of ultrasound-active theranostic agents has not been fully realized because it requires combining sufficient imaging contrast, high encapsulation efficiency, and ultrasound-triggered release in one entity. We report on theranostic polymer microcapsules composed of hydrogenbonded multilayers of tannic acid and poly(*N*-vinylpyrrolidone) that produce high imaging contrast and deliver the anticancer drug doxorubicin upon low-power diagnostic or high-power therapeutic ultrasound irradiation. These capsules exhibit excellent imaging contrast in both brightness and harmonic modes and show prolonged contrast over six months, unlike commercially available microbubbles. We also demonstrate low-dose gradual and high-dose fast release of doxorubicin from the capsules by diagnostic (~ 100 mW/cm²) and therapeutic (> 10 W/cm²) ultrasound irradiation, respectively. We show that the imaging contrast of the capsules can be controlled by varying the number of layers, polymer type (relatively rigid tannic acid versus more flexible poly(methacrylic acid)), and polymer molecular weight. *In vitro* studies demonstrate that 50% doxorubicin release from ultrasound-treated capsules induces 97% cytotoxicity to MCF-7 human cancer cells, while no cytotoxicity is found without the treatment. Considering the strong ultrasound imaging contrast, high encapsulation efficiency, biocompatibility, and tunable drug release, these microcapsules can be used as theranostic agents for ultrasound-guided chemotherapy.

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ASSOCIATED CONTENT

Supporting Information

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Comparison of US contrast intensity from (TA/PVPON-58)g and DOX-(TA/PVPON-58)g capsules and Definity contrast agent; CLSM images of DOX-(TA/PVPON-58)g capsules before and after the fetal bovine serum treatment; US contrast intensity from (TA/PVPON-58)g and DOX-(TA/PVPON-58)g capsules before and after serum treatment; CLSM images of DOX-(TA/PVPON-58)g capsules (2.4 pg DOX/capsule) before and after the therapeutic US treatments; DOX release from DOX-(TA/PVPON-58)g, DOX-(TA/PVPON-58)g, and DOX-(TA/PVPON-1300)g capsules after US treatment at pH = 7.4; dependence of capsule rupture (%) on the capsule architecture; DOX release from DOX-(TA/PVPON-58)g capsules after US treatment at pH = 7.4 before and after the serum exposure (PDF)

The authors declare no competing financial interest.

Graphical abstract

Keywords

ultrasound; drug delivery; multilayer; hydrogen-bonded; tannic acid; doxorubicin

Delivery of therapeutically effective drug concentrations to tumors is often impeded by poor drug solubility, rapid drug clearance, and dose-limiting toxicity.¹ Image-guided drug delivery offers a noninvasive alternative to both surgical resection and systemic drug delivery, allowing higher drug concentrations at tumor sites and reduced side effects.² Ultrasound (US) imaging encompasses low cost, fast real-time visualization, deep penetration in tissues, and noninvasiveness.^{3,4} Importantly, US can trigger drug release *via* inertial cavitation causing mechanical damage to the drug carriers.⁵ Unlike other stimuli, including pH, temperature, and enzymatic degradation, US offers precise control over spatiotemporal drug release and drug transport into solid tumors.^{6–8} The US intensity is easily adjustable from low intensities used for diagnosis ($<720 \text{ mW/cm}^2$) to high-intensity therapeutic irradiation (up to 10^5 W/cm^2) for tumor treatment.⁹

Diagnostic (imaging) and therapeutic (drug delivery) functions can be integrated by theranostic agents for real-time visualization of the drug's intracellular fate.^{10,11} However, the clinical potential of US theranostic agents can only be fully realized by combining sufficient US contrast, high encapsulation efficiency, and US-triggered release in one entity.^{4,12} Although microbubbles (1–8 μm in size) with perfluorocarbons can provide sufficient US contrast, they easily dissolve in blood, have short half-lives once activated,^{8,13} and are challenging for chemical modification and drug loading.^{14,15} Liposomes, nanoparticles, and micelles are more stable and easily modifiable but rarely produce US imaging contrast due to their small size.^{3,16,17} To generate strong US contrast in these nanosized particles, mixing drug-loaded micelles with gasloaded echogenic nanoparticles, co-encapsulating gas and drugs inside nanoparticles, generating gas bubbles around nanocarriers, and physically entrapping echo enhancers have been proposed.^{3,4,18–20} However, along with these complicated fabrication methods, nanocarriers suffer from low drug loading.²¹ Thus, there is an urgent need for a stable theranostic drug carrier with sufficient US contrast and the ability to release large amounts of therapeutics under US treatment.

Polyelectrolyte multilayer microcapsules have emerged as promising US-sensitive drug-delivery carriers due to their high loading capacity and easily adjustable composition and properties.²² These hollow particles are comprised of ultrathin multilayer shells ($<50 \text{ nm}$)

and micron-sized cavities and are assembled through layer-by-layer (LbL) deposition of polymers onto sacrificial templates.^{23,24} Multilayer microcapsules have several advantageous properties such as high drug-loading capacity, the ability to shield therapeutic peptides, protein-based molecules, or potent viral constructs from immunologic clearance, selective permeability allowing sustained/on-demand drug release, as well as easy functionalization for targeting capabilities.^{25,26} Unlike micelles or synthetic vesicles, the chemical and mechanical properties of LbL capsules can be precisely tailored by choosing appropriate polymers, incorporating nanoparticles within the multilayer shell, varying deposition conditions, and controlling the number of layers.^{27–30}

Since US-induced rupture of drug carriers often occurs through mechanical force,³¹ embedding metal and metal oxide nanoparticles in poly(allylamine hydrochloride)/poly-(styrenesulfonate) (PAH)/(PSS) capsules typically improves US sensitivity because of the increased shell density.³² Thus, $\text{Fe}_3\text{O}_4/(\text{PSS}/\text{PAH})_8$ capsules broke into pieces after 60 s sonication at 500 W ($377 \text{ W}/\text{cm}^2$), while particle-free $(\text{PSS}/\text{PAH})_8$ capsules only deformed under the same treatment.³³ The improved US sensitivity of PSS/PAH multilayer capsules was achieved by lowering the capsule stiffness *via* embedding ZnO within the PSS/PAH capsule wall; $\text{ZnO}-(\text{PAH}/\text{PSS})_2$ capsules totally ruptured after 9 s of US at 40 W ($30 \text{ W}/\text{cm}^2$).³⁴ However, those studies involved high-power US in the range of tens to hundreds of watts. Lower power diagnostic US is more desirable in chemotherapy to avoid tissue damage and related side effects.³⁵ In one study, the release of bovine serum albumin from PSS/PAH capsules with embedded Au nanoparticles was triggered by low-intensity, high-frequency US at 3.19 W after 10 min of sonication.³⁶ Computational simulations by Korolovych and co-workers on multilayer capsules revealed that 75% of PSS/PAH capsules modified with ZnO nanoparticles could be successfully destroyed by only 23 mW ($0.33 \text{ W}/\text{cm}^2$) US for 3 s.³⁷ However, the inclusion of nanoparticles complicates capsule synthesis and might challenge capsule biocompatibility and biodegradability. Very recently, *in situ* carbonization of dextran molecules within a $(\text{PSS}/\text{PAH})_4$ shell at 160°C has been shown to result in fluorescent carbon dots, which gave the capsules cytocompatibility, ultrasound responsiveness, reduced permeability, and good fluorescent stability.³⁸

Previously, we have shown that biocompatible microcapsules of hydrogen-bonded tannic acid and poly(*N*-vinylpyrrolidone) (TA/PVPON) have antioxidant, immunomodulatory, and cytoprotective properties^{39–41} and can be used for long-term drug storage.⁴² In this work, we demonstrate that nanoparticle-free TA/PVPON microcarriers possess a high US-imaging contrast and can deliver encapsulated therapeutics under both low-intensity diagnostic (power intensities of $0.1 \text{ W}/\text{cm}^2$) and high-intensity therapeutic ($>10 \text{ W}/\text{cm}^2$) US irradiation. We illustrate that the US-imaging contrast intensity of the multilayer capsules can be controlled by varying the number of layers in the capsule shell, polymer type (rigid TA versus the more flexible poly(methacrylic acid), PMAA), and polymer molecular weight. Using a medical US approach, we show that DOX-loaded (TA/PVPON) capsules have innate imaging contrast with higher intensity than commercially available contrast agents upon continuous US irradiation at 10 MHz. The US application time and acoustic power as well as solution pH effects on tuning US-triggered DOX release are also demonstrated. Given the prolonged and strong US imaging contrast, high encapsulation efficiency, biocompatibility, and finely tunable drug release under both diagnostic and therapeutic US,

these microcapsules can be used as theranostic agents for efficient tracking and delivery of therapeutics in tumor tissues and provide important design insights for future development of theranostic microcarriers for imaging-guided US-triggered cancer therapy.

RESULTS AND DISCUSSION

Response of (TA/PVPON) Capsules to Diagnostic (Imaging) Ultrasound

The doxorubicin-loaded (TA/PVPON)_n capsules were obtained *via* hydrogen-bonded LbL assembly of TA and PVPON using 5- μ m sacrificial silica cores with the subscript *n* denoting the number of TA/PVPON bilayers, as developed previously (Figure 1a).⁴² Since capsule rigidity is dependent on the capsule diameter (*d*) and the shell thickness (*t*),⁴³ we first investigated the effect of the *t/d* ratio of DOX-free TA/PVPON capsules on the capsules' US sensitivity in terms of mechanical deformation. The *t/d* ratio of the shell was controlled by varying (a) the molecular weight of PVPON from 58 to 1300 kDa (labeled as PVPON-58 and PVPON-1300, respectively), (b) the number of deposited bilayers, and (c) the sacrificial template size (Table 1). Complete dissolution of the sacrificial templates was confirmed using AFM and SEM (Figure 1c–e). The US sensitivity of these capsules was explored by checking the integrity and permeability of the capsule shell after US treatment. It is worth mentioning that the US power intensity of ~100 mW/cm² and the corresponding mechanical index (MI) of 1.3 used here are both below the FDA maximum intensity and MI of 720 mW/cm² and 1.9, respectively, for clinical imaging and diagnostic purposes.⁴⁴ We will therefore refer to the ultrasound conditions used in these experiments as diagnostic US.

Capsule integrity and permeability after US was evaluated using confocal microscopy *via* a FITC-dextran fluorescent probe and compared to that of untreated capsules (results for treated capsules in Table 1). Confocal microscopy image analysis revealed that (TA/PVPON-1300)₄ capsules (*t/d* = 1.8×10^{-2}) remained spherical with no significant deformation after the US treatment (Figure 2a, b). Only $12 \pm 3\%$ of the original (TA/PVPON-1300)₄ capsules became permeable to the fluorescent probe (*M_w* = 2000 kDa) indicating the formation of pores in the capsule shell after exposure to diagnostic US (Figure 2b). The size of the induced pores should be comparable to the hydrodynamic diameter of the FITC-dextran probe, *D_h* ~ 54 nm.⁴⁶ In drastic contrast, (TA/PVPON-58)₄ capsules (*t/d* = 1.4×10^{-2}) expanded from $3.5 \pm 0.2 \mu\text{m}$ to $4.9 \pm 0.7 \mu\text{m}$ and ruptured ($28 \pm 2\%$) after the US treatment (Figure 2c, d). Apparently, the greater mechanical stability of (TA/PVPON-1300)₄ capsules (due to the thicker shell⁴⁷) compared to that of (TA/PVPON-58)₄ capsules resulted in lower US sensitivity of the former and disintegration (or rupture) of the latter (Figure 2d).

A similar effect of enhanced mechanical integrity with an increasing *t/d* ratio is observed when the size of the capsules is changed. Specifically, 2- μ m (TA/PVPON-58)₁₅ capsules made on solid silica cores with a shell thickness (*t* = $47 \pm 1 \text{ nm}$) comparable to 3.5 μ m (TA/PVPON-58)₄ capsules (*t* = $52 \pm 2 \text{ nm}$, from porous CaCO₃ cores) were prepared and treated with US. As summarized in Table 1, decreasing the size of (TA/PVPON-58) capsules from 3.5 to 2 μ m while maintaining similar shell thickness (first and second table entries) prevented the capsules from bursting during the treatment. The same trend was observed in further CLSM studies where the number of layers was altered to change the *t/d* ratio: when *n* was increased from 4 to 15, (TA/PVPON-58)_n capsules remained impermeable to the 2000

kDa FITC-dextran after US irradiation due to higher rigidity of the shells (Figure 2e, f). However, these (TA/PVPON-58)₁₅ capsules became more permeable to a small molecular weight dye (Alexa Fluor 488 succinimidyl ester; $M_w = 643$ Da) after the US treatment as compared to untreated capsules, with a sizable increase in permeability from $30 \pm 5\%$ to $57 \pm 5\%$ (Figure 2g, h) indicating that perforation of capsules with a higher t/d ratio and mechanical stability is still possible through smaller pores using diagnostic US.

Our results suggest that the transition from rupture to perforation of (TA/PVPON-58) capsules by diagnostic US lies within the t/d ratio of $1.4 \times 10^{-2} < t/d < 2.3 \times 10^{-2}$. This finding agrees with that of previously reported PFO-PLLA capsules where the US sensitivity increased as the t/d ratio decreased.⁴⁸ Similarly, the cavitation doses for 1,2-dilignoceroyl-*sn*-glycero-3-phosphocholine-coated microbubbles were higher than that of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine at the same US pressure due to increased shell stiffness *via* the increased alkyl chain length.⁴⁹ Our results show that the hydrogen-bonded TA/PVPON capsules demonstrate US sensitivity without nanoparticle modification of the capsule shell due to their lower mechanical rigidity. The (TA/PVPON) capsules in solution are much softer than their ionically paired PSS/PAH counterparts modified with nanoparticles for US sensitivity. For instance, the Young's modulus of (TA/PVPON-58) is 0.89 MPa at pH = 7.4,⁴⁵ while it is 580 MPa for PSS/PAH capsules and therefore was necessary to include up to four layers of ZnO nanoparticles to lower the Young's modulus to 30 MPa in studies using those capsules.³⁴ Importantly, along with the t/d ratio, the mechanical rigidity of TA/PVPON capsules can be controlled by the PVPON molecular weight. As demonstrated previously, by decreasing the molecular weight of PVPON from 1300 to 360 kDa and to 55 kDa, the Young's modulus of the (TA/PVPON) shell in the wet state could be decreased from 4.3 to 1.6 and to 0.89 MPa, respectively.⁴⁵ Thus, the nanoparticle-free hydrogen-bonded (TA/PVPON) system affords improved biocompatibility and controllable US-triggered drug release *via* regulation of the capsule shell composition and thickness.

Echogenic Properties of TA/PVPON Capsules

As discussed previously, achieving US imaging contrast with stable, efficient carriers is a challenging task. However, echogenicity in drug carriers can be amplified when there is a phase difference between the carrier interior (e.g., hydrophobic; gas) and the environmental media (e.g., hydrophilic; water).⁵⁰ Since hydrophobic cargo can induce a hydrophobic environment inside the capsule, we encapsulated doxorubicin (DOX) within (TA/PVPON-58) capsules using our previously developed procedure.⁴²

In theranostic applications, the size of the particle is one of the most important parameters controlling its functionality. For instance, microbubbles larger than 4 μm had strongly enhanced imaging contrast and prolonged circulation compared to those 1–2 μm in size.⁵¹ We therefore constructed 5- μm capsules as this size would allow the capsules to pass pulmonary capillaries while still producing maximum US contrast.⁵² The 5- μm TA/PVPON capsules are large enough to exhibit a high scattering cross-section and, therefore, high echogenicity.⁵³ The 8-bilayer (TA/PVPON-58) capsules with a t/d ratio of 1.8×10^{-2} were loaded with 0.4 pg of DOX/capsule (see the Methods). The echogenic properties of DOX–

(TA/PVPON-58)₈ and DOX-free (TA/PVPON-58)₈ capsules were investigated by observing the US contrast from a suspension of capsules in water using both brightness-modulated (B-mode) and harmonic imaging modes on a SONIX RP US system as employed in a previous study.⁵⁴ While B-mode imaging is a popular method of general sonography, harmonic mode provides better resolution for objects that exhibit a nonlinear response to US, including almost all US contrast agents.^{55,56}

We discovered that DOX-free (TA/PVPON-58) capsules produce US imaging contrast in both imaging modes (Figures 3 and 4). It has been shown that high imaging contrast is observed in rigid US contrast agents and is explained by the strong sound scattering ability of those agents.⁵⁷ In our studies, the contrast intensity increased by ~36% with growing shell thickness (from 4 to 8 bilayers) in B-mode. Similarly, increasing the shell rigidity by using a higher molecular weight PVPON (1300 kDa instead of 58 kDa) in the capsules resulted in a ~30% higher contrast intensity in both modes for (TA/PVPON)₈ capsules (Figure 3a, b). To further illustrate this point and the importance of TA as a shell component, we prepared hydrogen-bonded capsules with poly(methacrylic acid) (PMAA) in place of TA. As a linear polymer, PMAA is less rigid than the bulky TA molecule, and indeed, the contrast intensity in B-mode was ~50% higher in the case of TA/PVPON capsules compared to those constructed with PMAA (Figure 3a). These results suggest that the imaging contrast of the capsules can be controlled by changing the shell rigidity *via* varying the number of deposited layers within the shell, the polymer type (rigid TA versus more flexible PMAA), and the polymer molecular weight. A similar observation was reported by Church *et al.*, wherein the sound scattering ability of gas microbubbles increased upon increasing the shell rigidity which resulted in better US contrast at 2–5 MHz.⁵⁷

Our results showing higher US contrast intensity with increasing capsule stiffness agree with previous reports on other types of US contrast agents. For instance, increasing the stiffness of lipid-coated microbubbles by increasing the alkyl chain length from C16 to C22 led to a better US video contrast, better US contrast persistence, and longer circulation time.⁵⁸ Uhlendorf *et al.* reported increasing linear ultrasonic response upon increasing microbubble shell thickness,⁵⁹ which is similar to our observations (Figure 3a).

Remarkably, the US-imaging contrast of (TA/PVPON) capsules was significantly enhanced after DOX encapsulation. The CLSM image in Figure 4a shows that the DOX–(TA/PVPON-58)₈ capsule interiors are homogeneously filled with red fluorescence due to DOX. The US images of DOX–(TA/PVPON-58)₈ capsules in B-mode (Figure 4b) and harmonic mode (Figure 4c) illustrate a distinctive imaging contrast enhancement. The bright elongated spots are due to capsule movement. The DOX–(TA/PVPON-58)₈ capsules showed much higher contrast compared to DOX-free (TA/PVPON-58)₈ capsules under similar capsule concentrations in both modes (Figure S1a). The better contrast from DOX–(TA/PVPON-58)₈ capsules likely stems from the more hydrophobic capsule interior from loaded DOX. Specifically, the US intensity from DOX–(TA/PVPON-58)₈ was 8 times higher in B-mode and 2 times higher using harmonic imaging at the highest concentration (1.6×10^6 particles/mL) when compared to DOX-free (TA/PVPON-58)₈ capsules (Figure S1a). In addition, the analysis of subjective pixel intensity in both harmonic and B-mode images demonstrates an approximately linear dependence between the concentration of DOX–(TA/

PVPON-58)₈ capsules and the intensity of contrast (Figure 4d). A similar dependence was observed in DOX-free (TA/PVPON-58)₈ capsules (Figure S1a). As the DOX loading amount was increased from 0.4 to 2.4 pg/capsule, the US contrast intensity increased (Figure S1c), confirming the effect of increased hydrophobicity of the capsule interior upon DOX encapsulation on the increased US imaging contrast of the capsules.

The DOX-free (TA/PVPON-58)₈ capsules demonstrated US contrast comparable to that of the Definity commercial contrast agent in both modes (Figure S1). Significantly, this result was achieved using a MI of only 1.1, while perfluoropentane-filled iron-doped silica shells recently reported by Kummel *et al.* demonstrated contrast similar to that of Definity using US with a higher MI of 1.9.⁶⁰ The MI used in our study is lower and therefore safer for tissues. Moreover, DOX encapsulation increased the contrast intensity of the DOX-(TA/PVPON-58)₈ capsules 6-fold compared to that of the commercial contrast agent in B-mode under the same imaging conditions (Figure S2).

In stark contrast to previously reported nanosized contrast agents which showed US contrast upon specific activation (such as releasing CO₂ from CaCO₃ at low pH⁴ or releasing perfluorohexane bubbles from PLGA microparticles triggered by laser irradiation,^{3,61}) only in B-mode,⁶² the TA/PVPON capsules in this work exhibit excellent US contrast in harmonic imaging mode as well, which allows for better resolved US images. In addition, no extra triggers are required for activation of the DOX-(TA/PVPON-58)₈ capsules, which should allow monitoring of the DOX-(TA/PVPON-58)₈ capsule delivery process in real time. Importantly, the echogenicity of DOX-(TA/PVPON-58)₈ capsules is highly stable as no significant change in the US contrast intensity from the capsules was observed in either harmonic or B-mode after storing the DOX-(TA/PVPON-58)₈ capsules in solution for 6 months (Figure 4e), unlike commercially available microbubbles and synthetic gas-generating nanoparticles whose US contrast intensity diminishes over time after activation.^{4,62} For instance, Definity has a half-life of only 1.3 min after activation.⁶³ Thus, the DOX-(TA/PVPON-58)₈ capsules represent an example of stable activation-free US contrast agents with intensity comparable to or higher than a relevant commercially available contrast agent.

Under physiological conditions, a protein corona might form around the capsules affecting their US sensitivity. Therefore, we studied the effect of serum treatment on the US imaging contrast of the DOX-(TA/PVPON)₈ capsules. The surface charge of the capsules and their size did not change significantly after the serum treatment, with corresponding zeta potential values of -37 ± 3 and -33 ± 2 mV and sizes of 3.6 ± 0.3 and 3.7 ± 0.2 μm , respectively, before and after the treatment (Figure S3). Both DOX-free and DOX-(TA/PVPON-58) capsules had good US contrast in both modes after the serum exposure (Figure S4). These results indicate that the DOX-free and DOX-(TA/PVPON-58)₈ capsules are highly stable in serum and that the capsules possess stable echogenicity under physiological conditions.

Use of Diagnostic Ultrasound for Sustained DOX Release from TA/PVPON Capsules

Delivery of DOX from DOX-(TA/PVPON-58)₈ capsules was first investigated upon exposure to diagnostic US (2.25 MHz, 115 mW/cm²) for 5 min at pH = 7.4 at 25 °C. Figure 5a shows that $7.6 \pm 0.7\%$ of loaded DOX was released from the capsules after the 5 min

treatment in contrast to only $1.2 \pm 0.1\%$ of DOX released from the control (untreated) capsules after 24 h (Figure 5a). The US-treated DOX-(TA/PVPON-58)₈ capsules preserved their spherical shape. After the second US exposure (15 min), an additional $9 \pm 1\%$ of DOX was released from the capsules with no further DOX release when the US was ceased (Figure 5a). These results indicate that the drug release from the capsules occurs only when the diagnostic US is applied and is negligible without the treatment.

To mimic the intracellular pH (pH = 6.5–5 in endosomes and pH < 5 in primary/secondary lysosomes),⁶⁴ we exposed the capsules to US in buffers of pH 5 and 3. It is important to note that TA/PVPON capsules are stable under acidic conditions and do not dissolve.⁴² The cumulative drug release from DOX-(TA/PVPON-58)₈ capsules after the US treatment was $16 \pm 2\%$ and $33 \pm 3\%$ at pH = 5 and pH = 3 (25 °C), respectively (Figures 5b,c). Much lower DOX release was observed for the untreated capsules at pH = 5 ($4 \pm 1\%$) and pH = 3 ($9 \pm 3\%$), respectively (Figure 5b,c). Previously, we have shown that exposure of capsules to pH = 7.4 or to pH = 5 resulted in negligible release of DOX after 24 h with 0.96 ± 0.01 and $1.60 \pm 0.02\%$ of the released drug, respectively,⁴² which agrees with the current control results. The release of DOX only during the US exposure implies that the drug release occurs through US-induced pore formation in the capsule shell which likely self-heals when the treatment is ceased.³⁶ The larger amount of DOX released from the capsules under diagnostic US at pH = 3 (Figure 5c) can be explained by enhanced hydrophilicity and solubility of DOX under acidic conditions as well as by the enhanced US cavitation in acidic media.^{65,66} The pH-dependent DOX release from (TA/PVPON-58)₈ capsules under diagnostic US suggests a potential use of this approach for controllable US-triggered low-dose chemotherapy which could be achieved by multiple burst treatments of TA/PVPON capsules.

Use of Therapeutic US for High-Dose DOX Release from TA/PVPON Capsules

To demonstrate the potential of our capsules for therapeutic treatment, we explored higher US power intensities such as those used in ablative therapy.⁶⁷ We used 20 kHz US at power intensities of 14–257 W/cm² for up to 80 s, which lies within the range of conditions classified as therapeutic US (100–100000 W/cm²).⁶⁸

We investigated the minimum power intensity capable of inducing at least 10 µg/mL DOX release from one-time US exposure at a capsule concentration of 1.38×10^7 capsules/mL (a ratio of 100 capsules per cell in a standard MTT assay) with 2.4 pg DOX/capsule. A DOX dose of ~10 µg/mL can kill 90% of human breast cancer MCF-7 cells after 48-h incubation.⁶⁹ The amount of DOX released from the capsules was measured 30 min after applying 20 s of therapeutic US under various power intensities. Figure 6a shows that the cumulative release after 48 h was 22 ± 4 , 39 ± 3 , 51 ± 1 , 60 ± 2 , and $68 \pm 3\%$ for power intensities of 14, 28, 57, 99, and 255 W/cm², respectively. We also found that 50% DOX release is comparable to ~10 µg/mL of DOX (Table S1). This data indicates the minimum US power intensity to induce ~10 µg/mL DOX is ~57 W/cm². Significantly, the drug release after 255 W/cm² US irradiation reached ~17 µg/mL, indicating that DOX-(TA/PVPON)₈ capsules can be used to boost the therapeutic effectiveness of high intensity US in thermal ablation for tumor tissues.

Our release data also agree with the mechanical deformation results in Figure 6b wherein increasing the power intensity from 14 to 255 W/cm² caused an increase in the number of broken capsules from 25 ± 11 to 73 ± 6%, respectively (Figure 6b). Confocal microscopy analysis revealed that the DOX-(TA/PVPON-58)₈ capsules treated with US at 14 W/cm² for 20 s displayed some degree of rupture and damage while the 255 W/cm² treatment samples were completely broken (Figure S5a–c). Less capsule rupture and a lower drug release (~5 µg/mL) were observed after capsules were treated at 14 W/cm² for 20 s. It is likely that no cavitation occurred under these conditions and that the drug-release mechanism may rely on thermal energy from the US.⁷⁰ When the power intensity is above 60 W/cm², cavitation (mechanical energy) is the dominant factor in rupturing and triggering the drug release.⁷⁰ It is noteworthy that at all power intensities, drugs released from microcapsules can benefit from the sonoporation effect in which US energy enhances the permeability of cellular membranes.⁷¹

The effect of US application time on the DOX release from the capsules at 14 W/cm² is shown in Figure 6c. By increasing the time from 20 to 40, and to 80 s, the cumulative DOX release can be increased from 22 ± 3 to 35 ± 2 and to 41 ± 2%, indicating that treatments for longer than 80 s are necessary to achieve 50% DOX release per US treatment at 14 W/cm² (Table S1, Supporting Information). In contrast to the 20-s treatment at 14 W/cm², the longer 80-s US treatment resulted in the majority of capsules being fully ruptured (Figure S5d). Our results demonstrate that US at 14 W/cm² for 20 s (280 J/cm² total energy delivered over the treatment time) was enough to rupture and deform (TA/PVPON-58)₈ capsules. In contrast, much higher intensity (280 W/cm²) was required to break ionically paired nanoparticle-free PSS/PAH multilayer capsules.^{34,36,72} In our work, only 1120 J/cm² was applied total to not only disrupt capsules but also release the drug, while 14841 J/cm² was needed to release 35% of the encapsulated protein from (PSS/PAH)₄ capsules.³⁶

We explored the effect of the increased shell thickness of DOX-(TA/PVPON) capsules on the cumulative DOX release under therapeutic US. The capsule shell thickness was varied by (a) increasing (TA/PVPON-58) bilayers from 4 to 8 and (b) using PVPON with a larger molecular weight of 1300 kDa. The calculated *t/d* ratio of the capsules loaded with 2.4 pg of DOX/capsule was 9×10^{-3} , 18×10^{-3} , and 21.5×10^{-3} for DOX-(TA/PVPON-58)₄, DOX-(TA/PVPON-58)₈, and DOX-(TA/PVPON-1300)₈, respectively. No DOX was released from the DOX-(TA/PVPON-1300)₈ capsules during the 24 h following the US treatment, while less stiff DOX-(TA/PVPON-58)₄ and DOX-(TA/PVPON-58)₈ capsules demonstrated sustained and burst drug release, respectively, with the corresponding DOX amounts of 27 ± 2 and 30 ± 7% (Figure S6a). After 72 h, the cumulative release from the DOX-(TA/PVPON-1300)₈ and DOX-(TA/PVPON-58)₈ capsules did not change, but it reached 71 ± 10% for DOX-(TA/PVPON-58)₄ capsules (Figure S6a). The data agrees with the corresponding amount of ruptured capsules calculated using confocal microscopy where increasing the *t/d* ratio from 9×10^{-3} to 18×10^{-3} and to 21.5×10^{-3} for DOX-(TA/PVPON-58)₄, DOX-(TA/PVPON-58)₈ and DOX-(TA/PVPON-1300)₈ capsules resulted in 93 ± 2%, 42 ± 15%, and 4 ± 1% rupture of capsules (Figure S6b). In contrast, the DOX release from nontreated capsules was insignificant (Figure S6a). These data are in good correlation with those obtained for 4 and 15 bilayer capsules under diagnostic conditions that revealed increased US sensitivity with decreasing *t/d* ratio (Table 1). Importantly, after

serum exposure, DOX-(TA/PVPON)₈ capsules could release DOX upon US treatment with only ~5% decrease in cumulative drug release (Figure S7).

Anticancer Activity of DOX-(TA/PVPON) Multilayer Capsules Induced by Therapeutic US

To explore the antitumor activity of the capsules, the DOX-(TA/PVPON-58)₈ capsules (2.4 pg DOX/capsule) treated with therapeutic US and untreated capsules were added to a 24-well plate containing 10⁵ MCF-7 cells in each well (the capsule-to-cell ratio = 100:1) along with cell culture media (RPMI, 10% FBS) in an MTT assay. The DOX-free (TA/PVPON-58)₈ capsules and the 10 µg/mL DOX solution were used as negative and positive controls for cytotoxicity, respectively. Figure 7 demonstrates that DOX-free (TA/PVPON-58)₈ and untreated DOX-(TA/PVPON-58)₈ capsules were noncytotoxic after 48-h incubation with the cells resulting in 99 ± 4% and 114 ± 10% viability of the cells, respectively. The increased cell viability observed in the case of the untreated DOX-(TA/PVPON-58)₈ capsules can be ascribed to the growth of the MCF cells induced by trace amounts of DOX released from the capsules (0.96 ± 0.01%) under these conditions.^{73,74} In drastic contrast, 48-h incubation of the cells with capsules treated with therapeutic US resulted in a cell viability of only 14 ± 4%, which is similar to that of the positive control (DOX solution), which killed 94 ± 1% of the MCF-7 cells. The viability of the cells incubated with DOX-(TA/PVPON-58)₈ capsules after they were treated with the diagnostic US did not decrease due to an increase of capsule stiffness by a higher drug loading (2.4 pg DOX/capsule) compared to the diagnostic US sensitive capsules (0.4 pg DOX/capsule).

CONCLUSION

We have demonstrated theranostic microcarriers: polymer multilayer capsules composed of hydrogen-bonded TA/PVPON which show a strong imaging contrast when diagnostic US is applied. We have shown that the US imaging contrast of nanoparticle-free TA/PVPON capsules can be controlled by (1) increasing capsule shell thickness and (2) controlling polymer rigidity and (3) polymer molecular weight. Increasing the capsule stiffness resulted in increased US imaging contrast. In addition, the US contrast intensity increases as the DOX loading amount per capsule is increased. A better US contrast from DOX-(TA/PVPON-58) capsules can be generated in both brightness and harmonic modes which are relevant for general and contrast agent related sonography. Importantly, DOX-(TA/PVPON-58)₈ capsules demonstrated prolonged and stable US contrast intensities over six months compared to a commercially available microbubble US contrast agent. We also illustrated that the US sensitivity of TA/PVPON capsules to diagnostic US for deformation can be tuned *via* varying the ratio of the capsule shell thickness to its size. The low-dose gradual release of anticancer drug DOX from the (TA/PVPON) multilayer capsules was demonstrated upon application of diagnostic US. We show that in maximized drug loading the DOX-(TA/PVPON-58)₈ capsules are opened by therapeutic US to release DOX, which can be useful for controlled release of high drug doses upon a single US application. We found the US application time and acoustic power as well as solution pH are crucial for tuning US-triggered DOX release. In addition, we also reported that TA/PVPON capsules can express US contrast and release the drug after exposure to serum. The capsule stability in serum should ensure their viability in intravenous administration for US-guided

chemotherapy. Finally, 50% DOX release from the DOX-(TA/PVPON-58)₈ capsules was achieved by application of therapeutic US and induced 97% cytotoxicity to MCF-7 cancer cells. No cytotoxicity from either DOX-free or DOX-loaded (TA/PVPON-58)₈ capsules was observed in the absence of the therapeutic US. The current study illustrates the strong potential of the TA/PVPON capsules as a theranostic agent for efficient cancer therapy using a medical US approach. Given the ease of surface functionalization of the capsules *via* copolymerization of the PVPON used as a shell component,^{39,40} providing the TA/PVPON capsules with targeting capabilities is a valid route of extending the therapeutic efficacy of this system. In addition, the ability of PVPON to prevent protein adsorption on the surfaces of PVPON-coated polymeric particles *in vitro* and *in vivo* due to its very hydrophilic nature (similarly to PEG)^{75,76} allows the potential to obviate clearance by the reticuloendothelial system, imparting a “stealth” capability for *in vivo* use. Keeping in mind this ability and a recent report of spherical TA/PVPON capsules 2 μm in size passing across 0.8- μm fenestrations of a membrane,⁷⁷ we envision an entirely different approach to treating solid human tumors of numerous pathologic subtypes including common metastatic malignancies such as breast melanoma, colon, prostate, and lung, utilizing the TA/PVPON capsules as a delivery platform. These capsules can protect encapsulated therapeutics from degradation or clearance prior to reaching the target and have a native US contrast as a means of visualizing the drug release. They can release their encapsulated drug cargo in specific locations *via* externally applied ultrasound exposure. Future *in vivo* studies will be important to explore the capsules’ blood circulation half-lives as well as their biodistribution.

METHODS

Materials

Polyethylenimine (PEI, average M_w = 25 kDa), tannic acid (1700 Da), poly(*N*-vinylpyrrolidone) (average M_w = 1300 kDa, PVPON-1300; and 58 kDa, PVPON-58), 46 wt % hydrofluoric acid, fluorescein isothiocyanate (FITC)-dextran, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich. Alexa Fluor 488 succinimidyl ester was from Invitrogen. Porous silica (5- and 3- μm average sizes) and solid silica ($2.0 \pm 0.1 \mu\text{m}$) microspheres were obtained from Restek and Polysciences, Inc., respectively. Doxorubicin hydrochloride (DOX) was purchased from LC Laboratories. Ultrapure deionized water (18.2 M Ω cm) was used for solution preparation (Evoqua). NaH₂PO₄ and Na₂HPO₄ buffers of ACS grade (Sigma-Aldrich) were used. Definity (Perflutren Lipid Microspheres) were obtained from Lantheus Medical Imaging, Inc. Poly(methacrylic acid) (PMAA; average M_w = 100 kDa), calcium chloride (CaCl₂), and sodium carbonate (Na₂CO₃) were purchased from Fisher-Scientific. Fetal bovine serum (FBS, 10%) was purchased from Mediatech Inc.

Synthesis of TA/PVPON Hollow Capsules

The silica and CaCO₃ particles (1.5 mL of 10% dispersion) were first coated with PEI deposited from a 1 mg/mL aqueous solution for 10 min in a 1.5 mL Eppendorf centrifuge tube. To fabricate CaCO₃ particles, 5 mL of 0.3 M Na₂CO₃ solution was rapidly poured into 5 mL of 0.3 M CaCl₂ solution, and the mixture was agitated for 30 s using a magnetic stirrer. The precipitated particles were filtered off, washed with DI water followed by acetone

rinsing, and air-dried at room temperature. The resulting CaCO_3 cores were of 3–4 μm in size. (TA/PVPON) capsules were prepared by coating TA and PVPON layers sequentially in pH = 6 buffer. TA/PVPON bilayers were allowed to adsorb onto the particle surfaces from 0.5 mg/mL polymer solutions in 0.01 M phosphate buffer at pH = 6 for 10 min each. After each deposited layer, the polymer excess was removed using centrifugation at 3000 rpm for 1 min, and the particles were rinsed twice with rinsing solution (0.01 M phosphate buffer at pH = 6) using centrifugation. Alternating polymer deposition was continued until a desired number of (TA/PVPON) $_n$ bilayers (n) were achieved. Dissolution of silica templates and CaCO_3 cores were conducted in 8% hydrofluoric acid and EDTA (0.3 M, pH = 7), respectively, and followed by dialysis in DI water for 4 days (Float-A-Lyzer, MWCO = 8–10 kDa, Spectrum Laboratories) to yield hollow (TA/PVPON) capsules.

For US contrast intensity measurements, PMAA/PVPON hydrogen-bonded capsules were synthesized by depositing alternating PMAA (0.5 mg/mL) and PVPON (0.5 mg/mL) layers on PEI-coated porous silica particles at pH = 3, while TA/PVPON-1300 capsules were made by depositing alternating TA (0.5 mg/mL) and PVPON (0.5 mg/mL) layers on PEI-coated porous silica particles at pH = 6. The porous silica particles were dissolved using 8 wt % HF to yield hollow capsules, and the capsules were purified by dialysis as described above.

Loading DOX in TA/PVPON Capsules

First, DOX hydrochloride was converted to the free amine as described previously.⁴² The DOX was then dissolved in CHCl_3 to result in either 2 mg/mL (for diagnostic US-triggered release) or 10 mg/mL (for therapeutic US-triggered release) solution and added to porous silica cores (40 mg) in a 1.5-mL Eppendorf tube using a 0.2- μm pore syringe filter. After sealing and shaking overnight using a Fisher Scientific shaker, the centrifuge tube was then opened and kept in a vacuum oven at 40 °C for 12 h. The PEI was adsorbed onto the dried DOX-loaded cores from 1 mg/mL aqueous solution for 10 min, and then the cores were separated from the polymer solution using centrifugation. After a triple rinse with 0.01 M phosphate buffer at pH = 6, the cores were coated with 8-bilayer TA/PVPON-58 multilayer from the corresponding 0.5 mg/mL polymer solutions (0.01 M phosphate buffer, pH = 6) using 10 min for polymer layer adsorption. Phosphate buffer (0.01 M) at pH = 6 was used for two rinses after each deposited polymer layer. The silica cores were dissolved in 1% hydrofluoric acid to yield the DOX–(TA/PVPON-58) $_8$ capsules with encapsulated DOX. Concentration of capsules in solutions was determined using a hemocytometer (Fisher Scientific).

Ultrasound Imaging of DOX–(TA/PVPON) $_8$ and (TA/PVPON) $_8$ Capsules

Aqueous suspensions of DOX–(TA/PVPON-58) $_8$ and (TA/PVPON) $_8$ capsules (with either PVPON-58 or PVPON-1300) were added into an ultrasonically invisible container (balloon) and allowed to distribute evenly within the balloon. The capsule concentrations of 0, 3.78×10^5 , 7.56×10^5 , 1.13×10^6 , and 1.51×10^6 capsules/mL with 90 mL total volume in the balloon were prepared. The imaging was run at physiological pH for these concentrations. The US contrast intensity was measured using a SONIX RP scanner with a L14-5 transducer (Ultrasonix Medical Corp, Richmond, BC) and a pulse-inversion harmonic imaging preset (transmission at 5 MHz, receive at 10 MHz). Images were collected in both harmonic and B-

mode then analyzed using custom Matlab software (Mathworks Inc., Natick, MA) which measured average pixel intensity in a specific user-defined region-of-interest (ROI). For TA/PVPON and PMAA/PVPON hydrogen-bonded capsules, the capsule concentration was 2×10^5 capsules/mL with 60 mL total balloon volume. The imaging was performed at pH = 3 (0.01 M phosphate buffer).

Ultrasound Imaging of Definity Contrast Agents

Definity contrast agents were activated *via* agitation using a Vialmix (Lantheus Medical Imaging) agent activator for 45 s and then suspended in aqueous solution within an ultrasonically invisible container (balloon) and allowed to distribute evenly.⁷⁸ The contrast agent concentrations were 0, 3.78×10^5 , 7.56×10^5 , 1.13×10^6 , and 1.51×10^6 contrast agents/mL with 90 mL total volume in the balloon. Contrast agents were imaged in the same manner as capsules as described above.

Fetal Bovine Serum Treatment of DOX-Free (TA/PVPON)₈ and DOX-(TA/PVPON)₈ Capsules

The 3.5- μ m DOX-free (TA/PVPON)₈ and DOX-(TA/PVPON)₈ capsules were treated with 10% FBS for 24 h followed by several rinses with the buffer before any measurements. Serum-treated and untreated capsules were imaged using the US and the procedure described above. The capsule concentration for each imaging experiment was 1.36×10^6 capsules/mL.

US-Triggered DOX Release

For diagnostic US treatment,^{44,79–82} the custom setup included a single element (1.90 cm in diameter) immersion transducer (Olympus, Waltham, MA) in series with a signal generator (AFG3022B, Tektronix, Beaverton, OR) and a power amplifier (A075, Electronics and Innovation, Rochester, NY). The following acoustic parameters were used: 2.25 MHz US frequency, 2.0 MPa pressure amplitude, 1000 cycle pulse length, 1.0 s pulse repetition period, and 3, 30, 180, 300, and 900 s duration of exposure. US pressure measurements were performed in a water bath using a hydrophone (Model HGL-0400, ONDA, Sunnyvale, CA) and a preamplifier setup in series with a digital oscilloscope (TDS2014B, Tektronix, Beaverton, OR) for voltage monitoring and recording. The solution immersed transducers were manipulated by a precision stepper motor (Velmex, Inc., Bloomfield, NY) to locate the spatial peak pressure maximum. The latter was determined by converting voltage to pressure using hydrophone calibration data. For high-intensity therapeutic US, a 20 kHz ultrasonic probe (Fisher Scientific Model 120 Sonic Dismembrator) (0.3 cm diameter) with a tunable output of acoustic power was used. The US power was obtained from the digital display of operating parameters. Specifically, the ultrasonic probe was immersed in a 1.5 mL Eppendorf tube containing capsule solution ($\sim 1.38 \times 10^7$ capsules, 0.01 M NaH₂PO₄ buffer at pH = 7.4). The capsules were treated under different ultrasonic modes, times and power. The percentage of ruptured capsules N_R , % after the US treatment was calculated using the following equation: $N_R = 1 - N_A/N_B$, where N_A is the number of nonruptured capsules after US treatment and N_B is that before the treatment. The nonruptured capsules were counted using a hemocytometer (Fisher Scientific). The serum-exposed capsules were treated with therapeutic US at 57 W/cm² for 20 s.

Release of DOX from TA/PVPON Capsules

The release of DOX from TA/PVPON capsules was quantified using UV-vis spectroscopy.⁴² The capsule DOX loading was assessed after the DOX-loaded capsules were completely ruptured by the exposure of the capsule solution (0.01 M phosphate buffer, pH = 7.4) to high power US using an ultrasonic probe (20 kHz, 7 W, 100 W/cm², 5 min). The DOX amount released into the solution was measured using UV-vis spectroscopy and quantified using the DOX calibration curve ($\lambda_{\text{max}} = 480 \text{ nm}$). To obtain cumulative DOX release profiles over a selected time after low intensity US treatment, the capsule suspensions were centrifuged, and the absorbance of the supernatants was measured in the range from 800 to 200 nm.

Confocal Laser Scanning Microscopy (CLSM)

CLSM images were obtained using Nikon A1R+ confocal microscope equipped with a 63 $\times$ oil immersion objective. For imaging, a drop of capsule dispersion was added to a buffer solution in an 8-well Lab-Tek chamber (Electron Microscopy Sciences), and the capsules were imaged after 5 h allowed for capsules to settle. For capsule permeability, a drop of capsule solution was added to the chamber, half-filled with FITC-dextran aqueous solutions or Alexa Fluor 488 fluorescent dye (Invitrogen) (1 mg/mL), and permeability toward the probe was analyzed after 15 min of the capsule exposure.

Atomic Force Microscopy (AFM)

AFM height images of TA/PVPON capsules were collected on dry samples using Multimode 8 (Bruker) in the Soft Tapping mode in air. AFM probes were purchased from Bruker (resonance frequency $\sim 300 \text{ kHz}$, tip radii 10 nm). For the preparation of capsules for AFM imaging, a drop of the capsule suspension was placed on a cleaned silicon wafer and air-dried inside a Petri dish prior to AFM imaging. The capsule single wall thickness was determined as half of the height of the collapsed flat regions of dried capsules.

Scanning Electron Microscopy (SEM)

SEM analysis was performed using a FEI Company QuantaTM 650 FEG SEM operated at 10 kV. Samples were prepared by depositing a drop of a particle or capsule suspension on a clean silicon wafer and allowing it to dry at room temperature. Before imaging, dried specimens were sputter-coated with 5 nm silver coating using a Denton sputter-coater.

Cytotoxicity of DOX-(TA/PVPON) Capsules (MTT Assay)

The human breast adenocarcinoma MCF-7 (ATCC) cells were grown under the 37 °C (air/CO₂ ratio of 95/5%) in T-75 culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics/antimycotics. When reaching more than 80% confluence, the cells were detached by using 0.05% Trypsin and resuspended in a new flask. The procedure was repeated until a total number of cells reached 3×10^6 cells (cell counting was performed using Trypan Blue method). The cells were seeded in 24-well plates (1×10^5 cells per well) and incubated overnight. The DOX-(TA/PVPON-58)₈ capsules containing 2.4 μg of DOX/capsule and exposed to therapeutic US (20 kHz, 1 W, 14 W/cm², 15 min) and diagnostic US (2.25 MHz,

2.0 MPa, 115 mW/cm², 15 min), control untreated DOX–(TA/PVPON-58)₈ capsules, and DOX-free (TA/PVPON-58)₈ capsules were added to the cells in the capsule-to-cell ratio of 100:1 (1×10^7 capsules/well, 1 mL of liquid per well) and incubated for 48 h. After that, liquid medium was aspirated, the cells were washed once with PBS, and the MTT cytotoxicity assay (ATCC) was performed using a Thermo Scientific NanoDrop spectrophotometer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Schematic illustration of hydrogen-bonded TA/PVPON multilayer capsules loaded with doxorubicin (DOX) (inset shows capsule cross-section and PVPON and TA molecular structures). (b) Schematic representation of US-triggered capsule perforation/destruction followed by DOX release from the capsule. AFM images of (c) (TA/PVPON-58)₄ and (d) (TA/PVPON-1300)₄ capsules. (e) SEM image of (TA/PVPON-58)₁₅ capsules.

Figure 2.

CLSM images of (TA/PVPON)₄ capsules with PVPON-1300 (a, b) and PVPON-58 (c, d) before (a, c) and after (b, d) diagnostic US (180 s, 2.25 MHz, 115 mW/cm² power intensity) followed by incubation with 2000 kDa of FITC-dextran. CLSM images of (TA/PVPON-58)₁₅ capsules after incubation with 2000 kDa of FITC-dextran before (e) and after (f) the diagnostic US. CLSM images of (TA/PVPON-58)₁₅ capsules before (g) and after (h) the diagnostic US followed by incubation with Alexa Fluor 488. The arrows point to the perforated capsules filled with the dye. The scale bar is 5 μm in all images.

Figure 3.

US contrast intensity from 4- and 8-bilayer (PMAA/PVPON-58) and (TA/PVPON-58) capsules and 8-bilayer (TA/PVPON-1300) capsules under (a) B-mode (10 MHz transmit and receive frequency) and (b) harmonic mode (5 and 10 MHz transmit and receive frequency, respectively). The capsule concentration was 2×10^5 capsules/mL. Data shown are representative of three independent experiments performed with triplicates for each sample. *** $p < 0.001$, ** $p < 0.05$.

Figure 4.

(a) CLSM images of drug-loaded DOX-(TA/PVPON-58)₈ capsules at pH = 7.4. US images of DOX-(TA/PVPON-58)₈ capsules obtained under (b) B-mode and (c) harmonic mode. (d) US contrast intensity from DOX-(TA/PVPON-58)₈ capsules (5 MHz transmit frequency). Dashed lines are linear fits of the experimental data points. (e) US contrast intensity from DOX-(TA/PVPON-58)₈ capsules (3.78×10^5 capsules/mL) before and after 6-month storage (pH = 7.4, 0.01 M phosphate buffer, 25 °C) (n.s. = not significant).

Figure 5.

Release of DOX from DOX-(TA/PVPON-58)₈ capsules (labeled as DOX-capsules) *via* treatment with diagnostic US for 5 min (2.25 MHz, 115 mW/cm²) at (a) pH = 7.4, (b) pH = 5, and (c) pH = 3. Arrows indicate the point at which the US was applied (ON). Statistical analysis: *t* test, ****p* < 0.001.

Figure 6.

(a) DOX release from DOX-(TA/PVPON-58)₈ capsules (2.4 pg DOX/capsule loading) at various US power intensities using therapeutic conditions (20 s, 20 kHz) at pH = 7.4. The arrow indicates the point at which the US was applied (ON). (b) Dependence of the capsule rupture (%) on US power intensity (W/cm²) after 20 s (pH = 7.4). (c) DOX release from DOX-(TA/PVPON-58)₈ capsules (2.4 pg DOX/capsule loading) after the therapeutic US (20 kHz) at 14 W/cm² (pH = 7.4). (d) Dependence of capsule rupture (%) on the time of US treatment at 14 W/cm² (pH = 7.4). *****p* < 0.0001; ****p* < 0.001; ***p* < 0.01.

Figure 7.

Cell viability of MCF-7 cancer cells after 48-h incubation with free DOX, control (TA/PVPON-58)₈ capsules, DOX-(TA/PVPON-58)₈ capsules, therapeutic US (T-US)-treated DOX-(TA/PVPON-58)₈ capsules (20 kHz, 257 W/cm²); and diagnostic US (D-US)-treated DOX-(TA/PVPON-58)₈ capsules (2.25 MHz, 115 mW/cm²). All experiments were carried out in triplicate, and the standard deviation is denoted using error bars. **** $p < 0.0001$ for comparison of cell viability between DOX-(TA/PVPON-58)₈ capsules and therapeutic US (T-US)-treated DOX-(TA/PVPON-58)₈ capsules.

Table 1

US Sensitivity of (TA/PVPON)_n Capsules^a

samples	sacrificial template	shell thickness (nm)	size (μm)	<i>t/d</i> ratio (10 ⁻²)	shell changes	ruptured capsules (%)
(TA/PVPON-58) ₄	porous CaCO ₃	52 ± 2	3.5 ± 0.2	1.42–1.46 ^b	rupture	28 ± 2
(TA/PVPON-58) ₁₅	solid silica	47 ± 1	2.0 ± 0.1	2.28–2.32 ^c	perforation	0
(TA/PVPON-1300) ₄	porous CaCO ₃	62 ± 5	3.5 ± 0.2	1.73–1.81 ^b	perforation	0

^aUS exposure: 3 min, 2.25 MHz, power intensity 115 mW/cm².^bCapsule shell thickness was measured by AFM.^cCapsule shell thickness was calculated as per a previous study.⁴⁵ Capsule size was determined by CLSM at pH = 7.4.