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Letter

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Multimodal Detection of a Small Molecule Target Using Stimuli-Responsive Liposome Triggered by Aptamer−Enzyme Conjugate

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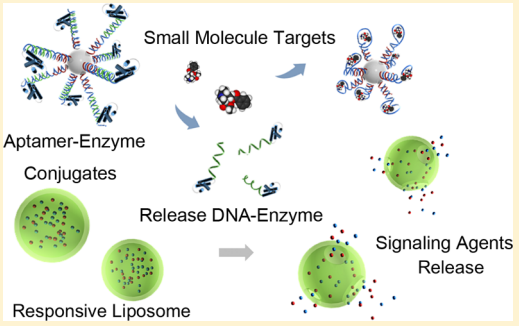
Supporting Information

ABSTRACT: Nanomaterials which can respond to external stimuli have attracted considerable attention due to their potential applications in sensing and biomedicine. One of the most promising classes of such materials is the stimuli-responsive liposome that can release its contents in response to a specific target. Despite recent progress, development of liposomes responsive to small molecular targets remains a challenge, due to the difficulty in designing the transduction process to link between target binding and triggered release, even though small molecular metabolites play important roles in many biological processes. Herein, we demonstrate a combination of an aptamer (apt) for target recognition and enzyme phosphatidylcholine 2-acetylhydrolase (PLA_2) for rupture of liposome. As a proof-of-concept, cocaine molecules were used to trigger the release of the enzyme. The exposure to DNA−PLA_2 conjugates induced the rupture of liposome containing uranin and gadopentetic acid (Gd-DTPA), allowing multimodal fluorescent and MRI detection of cocaine. The strategy demonstrated in this work can be generally applied to other imaging modalities by loading different imaging agents, as well as other targets by using different functional DNAs.



An active research topic in the field of bionanotechnology is the development of multimodal detection agents. Toward this goal, there has been significant interest in nanomaterials with the ability to load multiple payloads in an inert and protected state during transportation or circulation,[1] in which different detection agents are combined in one vehicle to allow simultaneous detection in a variety of complementary modes.[2] These systems have further been refined into stimuli-responsive nanomaterials, which allow a greater degree of control over the timing, position, and selectivity of the payloads release profile.[3]

The general principle behind the stimuli-responsive nanomaterials lies in the fact that a specific physical, chemical, and biological stimulus can trigger the conformational or compositional changes of the nanomaterials, inducing payload release.[4] Because of these desirable features, a variety of stimuli-responsive nanomaterials have been reported, including gold nanomaterials,[5] quantum dots,[6] dendrimers,[7] and nanoemulsions.[8] Each of these platforms possesses unique fabrication components and can promote the release of loaded imaging agents due to external stimuli.

Among the varieties of stimuli-responsive nanomaterials, liposomes have shown significant promise due to their superior biocompatibility, high loading efficiency, and versatility in terms of size and surface modification.[9] As a result, liposome products have been approved by FDA for clinical use for two decades.[10] Recent research has focused on transforming passive liposomes into stimuli-responsive liposomes triggered by a wide range of physical stimuli, including magnetic or electric fields,[11] temperature,[12] light,[13] and acoustic waves.[14] Liposomes responsive to biochemical stimuli, such as pH, redox, enzymes, and host−guest recognitions,[15−17] have also been explored but to a much less extent. Despite these progress, one important class of biological molecules is particularly underrepresented as trigger elements: the small molecular metabolites. A generalized stimuli-responsive liposome system that allows responsiveness to specific small molecular targets remains a significant challenge. The barrier lies in the design of the signal transduction process between the small molecular target recognition and liposome triggered release.

To meet the challenge, the use of DNA aptamers, which can be selected from a large DNA library to bind targets with high selectivity and specificity,[18] has emerged as a promising solution to bridge the gap in a signal transduction process for a wide range of small molecular targets.[19] Taking advantage of this ability, aptamers have been combined with different signaling agents to develop many types of sensors for small molecules,[19] including fluorescence,[20] colorimetry,[21] electrochemistry,[22] and magnetic resonance.[23] Building upon these successes, we report

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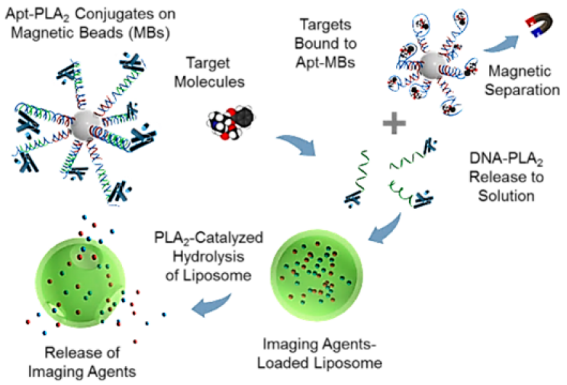
herein a stimuli-responsive liposome system, which allows multimodal detection of small molecule targets, such as cocaine, in a sensitive and selective manner by using an aptamer–enzyme triggered response pathway. The presence of cocaine molecules triggers the release of DNA–enzyme conjugates, which break the lipid bilayer of liposomes, causing release of the payloads. Fluorescence and MRI detection of cocaine were demonstrated using liposomes loaded with corresponding signaling agents, indicating a broad scope of this approach for different detection modalities.

RESULTS AND DISCUSSION

In order to develop a stimuli-responsive liposome that is responsive to small molecular targets, we need to build a link between recognition of the target molecules and rupture of the liposome to release the payloads. We chose a class of heat-stable, calcium-dependent enzymes phosphatidylcholine 2-acetylhydrolase (PLA_2) to provide such a link. In aqueous solution, PLA_2 catalyzes the hydrolysis of the 2-acyl bond of 3-*n*-phosphoglycerides, a major component of biological membranes, generating lysolipid and fatty acid as the major products.[24] Therefore, a direct relationship can be established between the concentrations of PLA_2 and the amount of ruptured liposomes. More importantly, because of the efficient enzymatic turnover, PLA_2 with nanomolar concentration can lead to the rupture of liposomes of much higher under ambient conditions, making it an ideal catalyst to release the signaling agents and amplify the "turn-on" signals.

The next challenge is to establish the relationship between target and PLA_2 concentrations. Inspired by the design of aptamer sensors, we propose a target-triggered enzyme releasing process using a DNA–PLA_2 conjugate to establish the relationship (Figure 1). The PLA_2 enzyme can be



signaling agents and amplify the 'turn-on' signals.

Figure 1. Scheme of stimuli-responsive liposomes responsive to small molecular targets for the multimodal detection.

immobilized onto magnetic beads (MBs) through DNA hybridization between an aptamer attached to MBs and its complementary DNA conjugated to the PLA_2, forming MB-supported conjugates. The binding of the target molecules by the aptamer weakens the hybridization between the aptamer and DNA–PLA_2 conjugate, resulting in release of the conjugate. The PLA_2 enzyme that remains on the MB will be removed from the solution through magnetic separation. The released conjugates can catalyze the hydrolysis of liposomes with signaling molecules inside, resulting in the release of signaling agents into the solution. Under optimized conditions, the concentration of released signaling agents is proportional to the concentration of released enzymes and therefore is proportional to the target molecules. The combination of aptamer, PLA_2, and liposomes allows the transduction between targets and signal output. Since different signaling agents can be encapsulated in the liposome and different aptamers can be obtained through systematic evolution of ligands by exponential enrichment (SELEX),[25,26] this strategy provides a general platform for detecting small molecule targets with multiple signal outputs.

The conjugation of thiolated linker DNA to PLA_2 was carried out by using a heterobifuntional linker sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), which can link the surface amines on the PLA_2 with DNA (Figure 2a). To demonstrate the successful conjugation

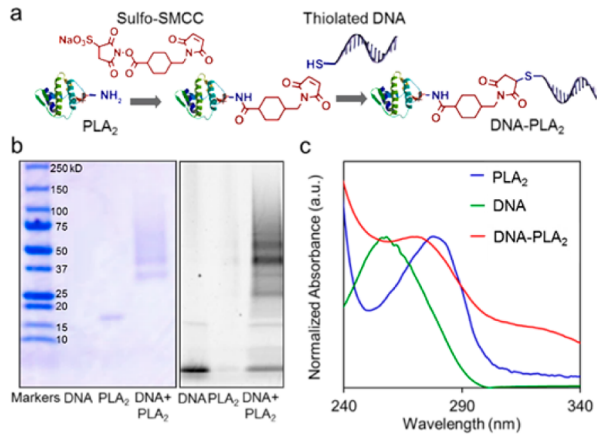


Figure 2. (a) Conjugation chemistry of DNA and PLA_2 using the heterobifuntional linker sulfo-SMCC. (b) Protein-staining (left) and fluorescence (right) images of native PAGE gel (4–20% gradient) for DNA–PLA_2 conjugates. (c) UV–vis spectra of PLA_2, DNA, and DNA–PLA_2 conjugates.

chemistry of the DNA–PLA_2 conjugate, SDS-PAGE gel electrophoresis was applied, followed by fluorescence and photographic imaging. For this experiment, Coomassie Brilliant Blue was used to stain the free PLA_2 and the concentrated but not fully purified DNA–PLA_2 conjugate. The thiolated DNA was modified with fluorescein so that the free DNA and DNA–PLA_2 conjugate could be fluorescently imaged. As shown in Figure 2b, the DNA–PLA_2 conjugate exhibited multiple bands that migrated slowly in both the photograph image (left) and fluorescent image (right), while the free DNA and free PLA_2 migrated faster than the DNA–PLA_2 conjugate. The molecular weight of the free PLA_2 is ~18 kDa, which is in agreement with the location of the band. After conjugating with ~7 kDa DNA strands, the molecular weight of the conjugates showed multiple bands around ~25–50 kDa, suggesting one or multiple DNA strands binding to PLA_2. The UV absorption spectrum of purified DNA–PLA_2 conjugate is the sum of those of free DNA and PLA_2, further confirming the successful formation of the DNA–PLA_2 conjugates (Figure 2c). Further enzyme activity studies confirmed that the formed DNA–PLA_2 conjugates retained ~50% enzymatic activity of free PLA_2 (Figure S1a,b).

The formulation and hydrolysis of the stimuli-responsive liposome is shown in Figure 3a. The PEG modification on the surface increases the surface area interacting with PLA_2 and therefore increases the rate of the hydrolysis reaction.[27] Uranin

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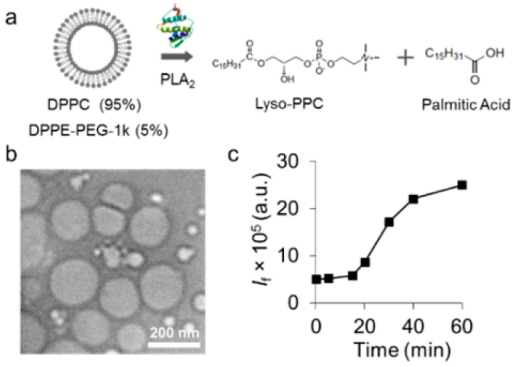

Figure 3. (a) Formulation of stimuli-responsive liposome and the hydrolysis reaction of liposome catalyzed by PLA_2 enzyme. (b) Cryo-EM micrograph of stimuli-responsive liposomes. (c) Kinetic curve of the release of uranin molecules inside liposomes at different incubation times under 16 nM PLA_2 concentrations.

and gadopentetic acid (Gd-DTPA) were encapsulated into liposomes as signaling agents. To elucidate the size and morphology of stimuli-responsive liposome, cryo-electron microscopy (cryo-EM) experiments were performed. The obtained liposomes were spherical, with an average size of around ~160 nm (Figure 3b). The liposomes were further characterized by dynamic light scattering (DLS) (Figure S2). The liposomes showed an average hydrodynamic diameter of ~150 nm, which is consistent with the Cryo-EM images. When the PLA_2 was added into liposome solution, the average hydrodynamic diameter increased to >200 nm after 30 min incubation, indicating the hydrolysis of liposomes. The monodispersity of the liposomes is comparable to those reported in the literature.[28,29] Better monodispersity would increase the precision of the sensing results.

The release profile of stimuli-responsive liposome was first tested to optimize the experimental conditions in pH 7.4 HEPES buffer. The liposomes containing uranin were treated with PLA_2 of various concentrations at room temperature for 15 min and 1 h, respectively (Figure S3a,b). The liposome treated with Triton-X and heat, which is known to rupture the liposome completely, was used as a positive control (red dot). The liposome incubated without PLA_2 was used as negative control (green dot). The fluorescence profile and photograph image were monitored. The results suggested that higher concentrations of PLA_2 resulted in increasing green color and higher fluorescence intensity. After incubation, the liposome sample treated with 16 nM PLA_2 started reaching a plateau, and in the presence of 64 nM PLA_2, the liposome sample showed ~80% fluorescence intensity of the positive control (Figure S3b). The kinetics of uranin-loaded stimuli-responsive liposome releasing profile was further studied in the presence of 16 nM PLA_2 (Figure 3c). The results showed that stimuli-responsive liposome began to rupture after 15 min and started reaching a plateau after 40 min. These studies suggested that even low-nanomolar changes in PLA_2 concentration can potentially release enough uranin to generate detectable fluorescence signal.

To demonstrate the proof-of-concept of the liposome responsive to small molecule targets, cocaine was selected as the model target, as cocaine is an important diagnostic target and cocaine aptamer is a well-studied DNA aptamer. As shown in Figure 4a, the DNA–PLA_2 conjugates hybridize with the cocaine aptamer and biotin–DNA. The DNA strands were

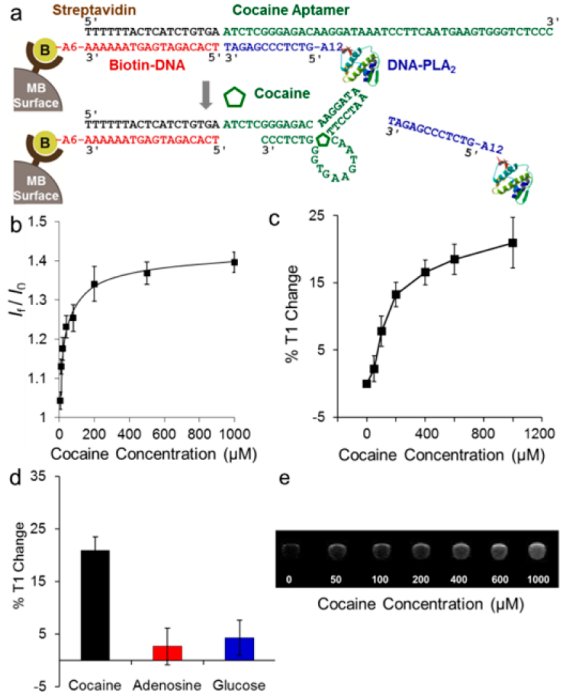

Figure 4. Design and performance of multimodal fluorescence and MRI detection of cocaine. (a) Cocaine-induced release of DNA–PLA_2 conjugates. (b) Calibration curve of the fluorescence detection of cocaine using uranin liposome samples. I_f/I_0 is the relative fluorescence intensity using no cocaine control as reference. (c) Calibration curve of the % T1 change of liposome samples in the presence of different cocaine concentrations. (d) Selectivity of the MRI detection of cocaine over adenosine and glucose plotting with % T1 change. The significant change of T1 relaxation time can only be observed with the existence of cocaine. (e) T1-weighted images of Gd-DTPA liposome samples treated with cocaine of different concentrations.

further immobilized onto MBs by streptavidin–biotin binding. The target-specific structure switching of the aptamer in the presence of cocaine can release the DNA–PLA_2 conjugate to solution and hydrolyze the liposomes containing uranin fluorophores and Gd-DTPA MRI contrast agents, resulting in an increase of the outputs for both signals. The fluorescence detection of cocaine with the concentration from 5 to 1000 μM is shown in Figures 4b and S4. Upon the addition of as low as 10 μM cocaine and subsequent removal of the MBs, the spectrum showed >10% increased fluorescence intensity compared to that of a sample containing no cocaine (Figure S4). The calibration curve was plotted using relative fluorescence intensity. In the presence of cocaine with a concentration ranging from 5 to 1000 μM, the increase of cocaine concentration resulted in the increase of the relative fluorescence intensity, which started reaching a plateau when the cocaine concentration was higher 200 μM (Figure 4b). Moreover, the result in Figure 2b suggests one or multiple DNA strands binding to PLA_2. As a result, it may take more than one equivalent cocaine molecule to release the DNA–PLA_2 conjugate, which may affect the sensitivity of the system. The sensitivity can be improved further by developing conjugation approaches that can achieve high yield of 1:1 DNA to PLA_2 conjugates.

To demonstrate the generality of the stimuli-responsive liposome platform for other detection modalities besides fluorescence, Gd-DTPA, a widely used T1-weighted MRI

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contrast agent, was loaded into stimuli-responsive liposomes for MRI detection of cocaine. Different concentrations of cocaine samples (0−1000 μM) were added to trigger the target specific structure switching of the aptamer to release the DNA−PLA_2 conjugate into solution. The released PLA_2 was mixed with Gd-liposomes and incubated at room temperature for 1 h. The PLA_2-treated Gd-liposomes were centrifuged, and the supernatant was collected for MRI data by using an Allegra 3T MRI scanner (Figure S5).

The T1-weighted MRI images obtained at different inversion times were used to estimate T1 relaxation time for samples of different cocaine concentrations. Quantitative analysis was achieved by plotting T1 relaxation time with corresponding cocaine concentration. The addition of cocaine decreased the T1 relaxation time from ∼2430 ms in the presence of 50 μM cocaine to ∼1970 ms in the presence of 1000 μM cocaine (Figure S6a). The percentage of T1 change profile indicated that there was a ∼20% average T1 change after the treatment of 1000 μM cocaine (Figure 4c). The selectivity of cocaine detection using this stimuli-responsive liposome platform was tested by measuring the T1 response toward 1000 μM cocaine, adenosine, and glucose (Figures 4d and S6b). The addition of cocaine decreased the average T1 by ∼21% (from ∼2490 to ∼1970 ms), while the addition of the same concentration of adenosine and glucose only changed T1 by ∼2.8% (to ∼2420 ms) and ∼4.4%,(to ∼2380 ms), respectively. Although this cocaine aptamer has shown good selectivity over adenosine and glucose, it has been reported that this cocaine aptamer may bind quinine derivatives.[30,31] This poor selectivity can be improved by performing SELEX employing "negative selection" to remove sequences that bind quinine derivatives. Despite this issue, the sensing system design can be applied to detect other targets selectively, as long as the corresponding aptamer displays the required selectivity. Furthermore, at an inversion time of 800 ms and repetition time of 2000 ms, the T1-weighted MRI of different samples under increased cocaine concentration from left to right changed from dark (left: no cocaine) to brighter (in the presence of 1000 μM of cocaine), showing the contrast of the T1-weighted images increased with the addition of different concentrations of cocaine (Figure 4e). The observation is consistent with the trends of the T1 change, suggesting that a ∼20% T1 change can provide a significant contrast change for potential diagnostic applications.

CONCLUSIONS

In conclusion, we have developed a stimuli-responsive liposome platform that is responsive to external small molecular stimuli by using aptamer and PLA_2 enzyme. The use of DNA−PLA_2 conjugate provided a link between the target recognition ability from aptamer and the signal production ability from liposome. As a proof-of-concept, fluorescence and MRI detection were demonstrated by loading uranin and Gd-DTPA into liposomes as signaling agents. The liposome platform can achieve low-micromolar sensitivity for fluorescence detection and a ∼50 μM detection limit for MRI detection, which is comparable to previously reported methods,[21,32,33] indicating the compatibility of this platform for different detection modalities. Aptamers that have much stronger affinity for other targets can be employed,[34−36] which will allow this system with much higher sensitivity. Furthermore, because the aptamer−PLA_2 trigger-release mechanism is compatible with different functional DNAs, the target-responsive liposome system can be easily applied to other aptamers and DNAzymes. The broad scope of this reported strategy to various small molecular targets as well as imaging agents would allow a wide range of potential diagnostic and therapeutic applications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b04031.

Experimenal details; additional figures (PDF)

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Notes

The authors declare no competing financial interest.

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