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Cellular Association and Cargo Release of Redox-Responsive Polymer Capsules Mediated by Exofacial Thiols

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Controlling the release of drugs from carriers is a critical issue in advanced drug delivery, and is closely linked to effective and optimized therapeutic applications.[1] Recently, significant effort has been focused on the development of responsive controlled release systems that are capable of releasing drugs based on the presence of specific stimuli.[2] A significant advantage of triggered release delivery systems over conventional therapies is the ability to regulate temporal and spatial drug profiles for maximum therapeutic benefits.

In recent years, layer-by-layer (LbL)-assembled polymer capsules aimed at therapeutic applications have attracted increasing attention.[3,4] These capsules can be assembled from a range of functional polymers and can encapsulate a variety of cargo to achieve targeted and triggered release.[3,4] There are several mechanisms that have been exploited to trigger cargo release from LbL polymer capsules, including changes in pH and temperature, enzymatic degradation, and redox-activated cleavage.[2-4] Due to the reversible nature of thiol-disulfide chemistry, redox-responsive LbL-assembled polymer capsules are particularly attractive for triggered release. Disulfide bonds can be incorporated into either the polymer backbone[5,6] or crosslinkers used to stabilize the capsules.[7] We have reported the use of thiolated poly(methacrylic acid) (PMA_{SH}) to prepare single-component polymer capsules stabilized by disulfide bonds ($PMA_{SH(disulfide)}$).[6] These disulfide-bonded capsules are stable under oxidizing conditions (such as the bloodstream) but degrade in reducing environments (such as the cytoplasm of cells), thus triggering cargo release and improving cargo bioavailability.[6] Recent studies using $PMA_{SH(disulfide)}$ capsules have shown that encapsulated peptides and proteins can be delivered to dendritic cells,[8] and encapsulated small chemotherapeutic drugs, including doxorubicin, 5-FU and paclitaxel,[9] drug-loaded liposomes,[10] or siRNA can be delivered to cancer cells.[11] More recent studies have demonstrated that $PMA_{SH(disulfide)}$ capsules are readily taken up by various types of cells,[12] and that the internalized capsules become localized in membrane-enclosed compartments, such as lysosomes.[9b] The presence

of the disulfide bonds in $PMA_{SH(disulfide)}$ capsules has been associated with effective cargo release from these systems.[9a] However, the role of disulfide bonds has yet to be confirmed and the detailed mechanism of cargo release remains unclear. Understanding the cellular processing of these capsules is of particular importance for further development and optimization of these systems.

Herein, we report the first investigation on the influence of thiols and disulfide linkages of LbL-assembled PMA capsules on cell entry and cargo release. To address this, we have combined two recent developments: (i) mesoporous silica (SiO_2) templated-encapsulation of a fluorescent hydrophobic cargo (1,1'-dioctadecyl-3,3,3',3'-tetramethindocarbocyanine perchlorate, DiI) in PMA capsules to follow cargo release;[9c] and (ii) a versatile crosslinking approach to generate a series of PMA-containing capsules to evaluate the specific roles of thiols and disulfide bonds.[13] Three types of PMA microcapsules have been used in this study: redox-cleavable, thiolated PMA capsules ($PMA_{SH(disulfide)}$); non-cleavable, thiolated PMA capsules ($PMA_{SH(thioether)}$); and non-cleavable, non-thiolated PMA capsules ($PMA_{(amide)}$) **(Scheme 1)**. The ability of DiI to cross lipid bilayers allows evaluation of the cargo release from PMA capsules based on the intracellular distribution of DiI. When DiI is released from the capsules, DiI can further traffic through the endocytic membranes during capsule internalization, resulting in a wide intracellular distribution along cellular hydrophobic structures.[14] In the current study, we demonstrate that the disulfide bonds are crucial for effective intracellular release of small hydrophobic cargo. Such redox-activated cargo release is mediated by a mechanism that involves thiols associated with cell surface proteins (hereafter referred to as exofacial thiols). Further, we investigate the role of the free thiols in PMA_{SH} capsules on cellular association. These experiments suggest that thiolation of PMA capsules enhances cellular interactions upon cell entry. The results provide further key insights into the mechanisms of redox-activated release, which will aid in the rational design of these and related thiol-disulfide-containing delivery systems for biomedical applications.

To evaluate the role of disulfide bonds on cargo release, two types of DiI-loaded capsules were prepared (redox-cleavable $PMA_{SH(disulfide)}$ and non-cleavable $PMA_{SH(thioether)}$) through LbL assembly on mesoporous SiO_2 particles. The particles were then loaded with DiI by adsorption from solution. Poly(N-vinylpyrrolidone) (PVPON) and thiolated poly(methacrylic acid) (PMA_{SH}) were then sequentially deposited through hydrogen bonding interactions onto the mesoporous SiO_2 particles, as reported previously.[6] The resulting nanoscale polymer films were crosslinked by either oxidation of thiol groups on the PMA polymer to create disulfide linkages, or with a bifunctional crosslinker (1,8-bismaleimidodiethyleneglycol) to create a non-cleavable thioether bond with the thiol groups.[13] Subsequently, the DiI-loaded PMA_{SH} capsules were produced by dissolution of the porous silica template, and release of non-crosslinked PVPON in solution at pH 7.4.[6,13] The capsules were analyzed with confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM). As shown in **Figure 1**, the encapsulated DiI was associated with the capsule wall (Fig. 1a-c). The TEM images confirmed that the encapsulated hydrophobic cargo formed small clusters (Fig. 1d, e). HeLa cells were treated with either DiI-loaded $PMA_{SH(disulfide)}$ or DiI-loaded $PMA_{SH(thioether)}$ capsules using a capsule-to-cell ratio of 100:1 at 37 °C for 24 h to allow cellular uptake. The intracellular distribution of DiI was visualized by CLSM, which showed that DiI was widely distributed throughout the cytoplasm (evidenced as small fluorescent spots). As DiI is a lipophilic dye used for staining membranes, presumably the released DiI in the cytoplasm is associated with intracellular hydrophobic membrane structures (**Fig. 2**a-c). In contrast, DiI remained as micrometer-sized spots when DiI was delivered by $PMA_{SH(thioether)}$ capsules, where a noncleavable thioether bond crosslinker replaced the disulfide bridge (Fig. 2d-f). These results suggest that DiI was effectively released from $PMA_{SH(disulfide)}$ capsules, but not from $PMA_{SH(thioether)}$ capsules during endocytosis of the capsules, which indicates that the disulfide linkage plays an important role in cargo release.

Studies on cellular entry of viruses and disulfide-based conjugates have shown that disulfide reduction can occur at cell membranes.[15] Typically, these processes involve exofacial thiols and cell surface-associated proteins with thiol-disulfide interchange activity, such as protein disulfide isomerase (PDI). Emerging evidence suggests that the exofacial thiols are a critical regulator for cellular redox potential and that their status has a significant effect on various cellular functions.[16] To explore the role of exofacial thiols in the redox-activated cargo release of $PMA_{SH(disulfide)}$ capsules, a membrane impermeable reagent (2,2'-dinitro-5,5'-dithiobenzoic acid, DTNB) was used to block exofacial thiols and hence inhibit disulfide-thiol exchange at the cell surface. Firstly, we evaluated the effects of DTNB on cell viability and on inhibition of exofacial thiols. Propidium iodide staining showed a negligible effect on cell viability when DTNB (1 mM) was incubated with HeLa cells for 24 h (Fig. S1). Importantly, treatment with DTNB (1 mM) resulted in a significant decrease of exofacial thiols in HeLa cells, as determined by staining with an Alexa Fluor (AF) 647 (maleimide) dye, followed by flow cytometry analysis (Fig. S2).

We next investigated hydrophobic cargo release from $PMA_{SH(disulfide)}$ capsules when the exofacial thiols were blocked. Following treatment of HeLa cells with DiI-loaded $PMA_{SH(disulfide)}$ capsules in the presence of DTNB (1 mM) at 37 °C for 24 h, the intracellular distribution of DiI visualized by CLSM was seen as micron-sized bright spots (Fig. 2g-i), similar to that seen with non-cleavable DiI-loaded $PMA_{SH(thioether)}$ capsules (Fig. 2d-f). Given the possibility that the remaining thiols in $PMA_{SH(disulfide)}$ capsules could also be modified by DTNB during incubation with cells, we further examined cargo release of DTNB pretreated DiI-loaded $PMA_{SH(disulfide)}$ capsules. Experiments on cells incubated with DTNB (5 mM) pretreated DiI-loaded $PMA_{SH(disulfide)}$ capsules showed that DiI was released from the capsules and broadly distributed in the cytoplasm (Fig. S3a-c). In contrast, blocking exofacial thiols with DTNB prevented the release of DiI from these capsules (Fig. S3d-f), indicating that exofacial thiols are a major contributor to redox-activated cargo release in the $PMA_{SH(disulfide)}$

system. It has been demonstrated that protein disulfide isomerase (PDI), which also possesses reduced thiols that can be inhibited by DTNB, plays an important role in the reduction of disulfide bridges for virus entry and insulin resistance.[15a,17] To determine whether PDI could regulate the release of DiI from $PMA_{SH(disulfide)}$ capsules, a neutralizing anti-PDI monoclonal antibody was used to specifically inhibit PDI.[16,17] Even at very high concentrations (up to 40 μg mL^{-1}), which has been shown to be active in other studies,[17,18] the anti-PDI monoclonal antibody had no significant inhibitory effect on cargo release (data not shown), suggesting that PDI is not operative in the disulfide reduction of the capsules. Further studies will be required to elucidate the molecular nature of the exofacial thiols, which potentially involves membrane-associated proteins that have the capacity to catalyze thiol-disulfide exchange reactions.

In other thiol-containing delivery systems, such as thiolated polymer conjugates, studies have shown that the introduction of thiols is associated with higher cellular uptake facilitated by exofacial thiols.[15c,19] Firstly, we confirmed the presence of free thiols in PMA_{SH} capsules. We chose to examine free thiols in redox-noncleavable $PMA_{SH(thioether)}$ capsules because the number of free thiols in $PMA_{SH(disulfide)}$ capsules could vary depending on the nature of disulfide bonds (oxidative form as disulfide bonds or reductive form as free thiols) influenced by the exofacial thiols. AF488-labeled $PMA_{SH(thioether)}$ capsules were incubated with the thiol-reactive AF647 (maleimide) dye. After the reaction, the capsules displayed fluorescence in both FL1 and FL5 channels, as detected by flow cytometry (Fig. S4). Pretreating AF488-labeled $PMA_{SH(thioether)}$ capsules with DTNB (5 mM) resulted in significantly less coupling to AF647 (Fig. S4), suggesting that some thiols in the $PMA_{SH(thioether)}$ capsules have been irreversibly consumed. Following confirmation of the presence of thiol groups on the PMA_{SH} capsules, we next sought to investigate the effect of these free thiols on cellular association. HeLa cells were incubated with three types of capsules individually: AF488-labeled $PMA_{SH(disulfide)}$, AF488-labeled $PMA_{SH(thioether)}$ and AF488-labeled $PMA_{(amide)}$ capsules, at a

capsule-to-cell ratio of 100:1 at 37 °C for 24 h. It was shown that ~77% of cells were associated with $PMA_{SH(disulfide)}$ capsules, and ~63% cells were associated with $PMA_{SH(thioether)}$ capsules, whereas significantly less (~27%) cells were associated with $PMA_{(amide)}$ capsules (**Fig. 3**), despite the size and morphology of all three types of capsules being similar, as observed by TEM (Fig. 1e and Fig. S5). These results suggest that the free thiols on the capsules enhance cellular contact, leading to increased cellular association. Involvement of the intrinsic thiol groups on the capsules in cellular association was further evaluated by modification of the thiols by DTNB. The $PMA_{SH(disulfide)}$ and $PMA_{SH(thioether)}$ capsules were both pretreated with 5 mM DTNB and incubated with cells at 37 °C for 24 h. Consumption of the free thiols on both capsules resulted in a decrease of cellular association compared to untreated capsules (Fig. 3a-d), confirming the role of the free thiols on the thiolated capsules in cellular association.

Next, we tested the exofacial thiol-dependent cellular association of the thiol-consumed PMA_{SH} capsules. Both AF488-labeled $PMA_{SH(disulfide)}$ and AF488-labeled $PMA_{SH(thioether)}$ capsules were pretreated with DTNB (5 mM), followed by incubation with cells at a capsule-to-cell ratio of 100:1 at 37 °C for 24 h in the presence of DTNB. Flow cytometric analysis showed that there was a further decrease in cellular association following blocking of the exofacial thiols in the treatment with thiol-consumed $PMA_{SH(disulfide)}$ capsules (Fig. 3a, b). In contrast, the thiol-consumed $PMA_{SH(thioether)}$ capsules showed no significant difference in cellular association following blocking of the exofacial thiols (Fig. 3c, d). Similarly, blocking of exofacial thiols with DTNB also had no significant effect on cellular association of AF488-labeled $PMA_{(amide)}$ capsules that lack free thiols (Fig. 3e, f). These results suggest that the interaction between the exofacial thiols and the disulfide bonds of $PMA_{SH(disulfide)}$ further facilitate cellular association of the capsules, which is consistent with previous reports that $PMA_{SH(disulfide)}$ capsules can be readily internalized by various cells.[8a,9b,12] Taken together, the

thiolation of the PMA polymers not only offers a reversible pathway to stabilize the capsules via disulfide bonds, but also enhances the interactions with cells via the exofacial thiols.

In conclusion, we have demonstrated the influence of thiols and disulfide bonds of the thiolated PMA capsules upon cell entry and cargo release. Our data show that the free thiols in the capsules enhance cellular association and the disulfide bonds play an important role in cellular cargo release. Additionally, we have identified a mechanism that involves the exofacial thiols for the reduction of disulfide bonds leading to the cargo release. The presence of the thiols/disulfide bonds in the capsules has been shown to enhance cellular contact by interacting with exofacial thiols, which may facilitate cell membrane wrapping, leading to internalization of the capsules and cargo release. Given the current interest in the use of redox-responsive delivery systems, it is essential to unravel both the mechanisms and sites of cellular interaction and reduction to guide optimized delivery.

Experimental

Confocal Laser Scanning Microscopy: HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. HeLa cells were plated at 5×10^4 cells/well into 8-well Lab-Tek I chambered coverglass slides (Thermo Fisher Scientific, Rochester) and allowed to adhere overnight. Cells were then incubated with DiI-loaded $PMA_{SH(disulfide)}$, or DiI-loaded $PMA_{SH(thioether)}$ capsules at a capsule-to-cell ratio of 100:1 for 24 h (37 °C, 5% CO_2) individually. For inhibition of exofacial thiols, cells were pretreated with 1 mM DTNB for 15 min, and incubated with DiI-loaded $PMA_{SH(disulfide)}$ capsules for 24 h (37 °C, 5% CO_2). Following the treatment, cells were washed with phosphate buffered saline (PBS) three times and fixed with 4% paraformaldehyde for 30 min at room temperature. Fluorescence images and optical sections were collected with a Leica laser-scanning confocal unit (TCS SP2; Leica, Germany).

Flow Cytometry: HeLa cells were plated at 5×10^4 cells/well into 48-well plates and allowed to adhere overnight. Cells were pretreated with 1 mM DTNB for 15 min, then incubated with AF488-labeled $PMA_{SH(disulfide)}$, AF488-labeled $PMA_{SH(thioether)}$, DTNB-pretreated AF488-labeled $PMA_{SH(disulfide)}$ or AF488-labeled $PMA_{(amide)}$ capsules individually at a capsule-to-cell ratio of 100:1 for 24 h (37 °C, 5% CO_2) either in the presence or absence of DTNB (1 mM). After incubation, the cells were washed with PBS three times, resuspended in PBS, and analyzed by flow cytometry (Partec Cyflow Space). Cells were identified based on their scatter characteristics, and the percentage of cells associated with capsules was determined by acquisition of AF488 (FL1).

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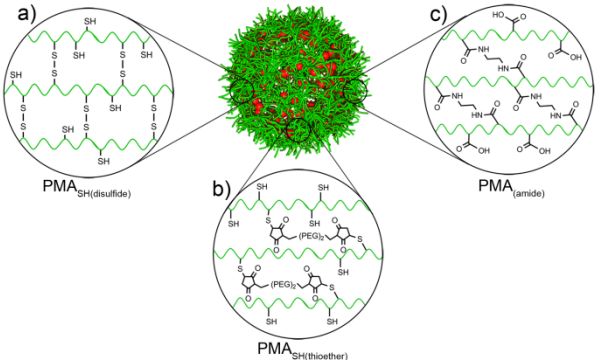

Scheme 1. Schematic illustration of various crosslinking approaches to stabilize PMA capsules loaded with DiI. a) Oxidation of thiolated-PMA to form disulfide bonds. b) Crosslinking thiolated-PMA with a bifunctional thiol-reactive reagent containing diethylene glycol. c) Crosslinking PMA with ethylenediamine, a bifunctional carboxyl-reactive reagent in the presence of the catalyst 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). Each capsule only contains one type of crosslinker.

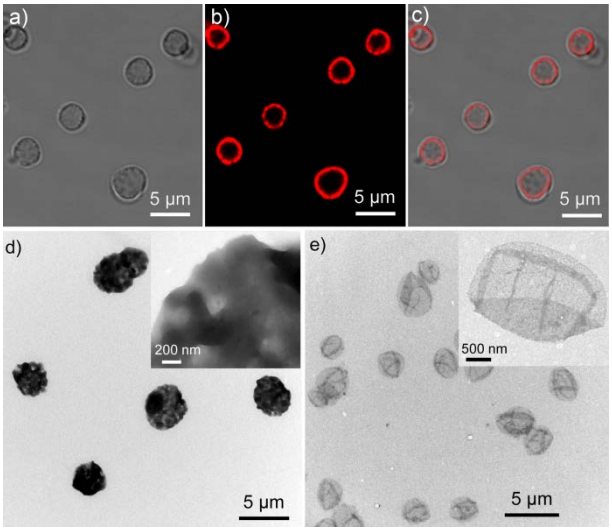

Figure 1. CLSM images of the DiI-loaded $PMA_{SH(disulfide)}$ capsules (a-c). a) Brightfield image. b) Fluorescence image. c) The overlay image of a) and b). TEM images of DiI-loaded $PMA_{SH(disulfide)}$ capsules (d) and empty $PMA_{SH(disulfide)}$ capsules (e). The insets in (d) and (e) correspond to higher magnification TEM images of the DiI-loaded and empty capsules, respectively.

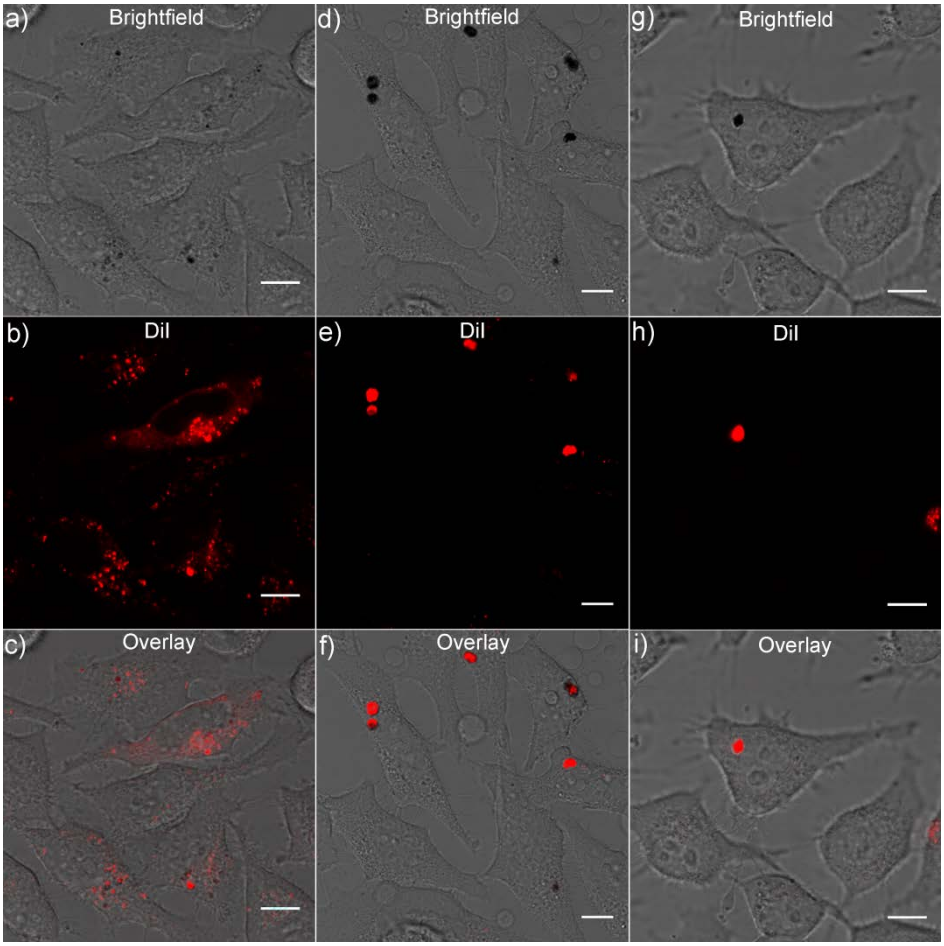

Figure 2. CLSM images of the intracellular distribution of DiI in HeLa cells. Cells were treated with DiI-loaded $PMA_{SH(disulfide)}$ capsules (a-c), or DiI-loaded $PMA_{SH(thioether)}$ capsules (d-f), or DiI-loaded $PMA_{SH(disulfide)}$ capsules in the presence of 1 mM DTNB (g-i) at 37 °C, 5% CO_2 for 24 h. The images correspond to brightfield images of cells (a, d, g, gray), intracellular distribution of DiI (b, e, h, red), and the overlay images (c, f, i). Scale bars, 10 μm.

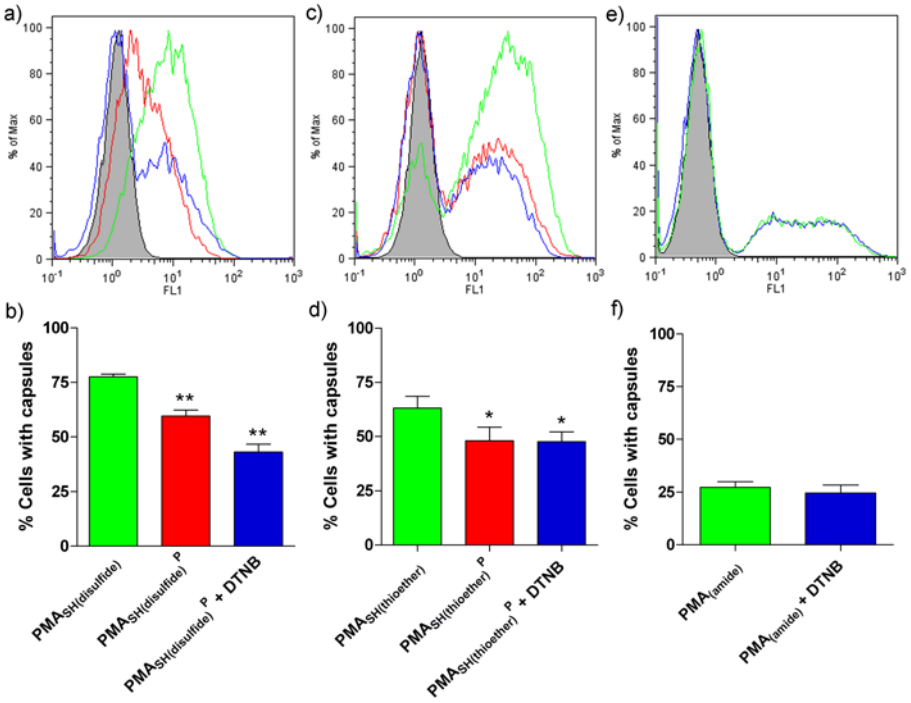

Figure 3. Effect of thiol groups on the capsules and exofacial thiols on the cell membrane on cellular association of AF488-labeled PMA capsules. Capsules investigated were: $PMA_{SH(disulfide)}$, $PMA_{SH(disulfide)}^{P}$ (i.e., $PMA_{SH(disulfide)}$ capsules pretreated with 5 mM DTNB) (a, b), $PMA_{SH(thioether)}$, $PMA_{SH(thioether)}^{P}$ (i.e., $PMA_{SH(thioether)}$ capsules pretreated with 5 mM DTNB) (c, d), and $PMA_{(amide)}$ (e, f). Cells were incubated with these capsules individually at a capsule-to-cell ratio of 100:1 in the absence or presence of 1 mM DTNB at 37 °C, 5% CO_2 for 24 h. Representative fluorescence intensity histograms of cells incubated with capsules were shown as untreated cells (a, c, e, filled), $PMA_{SH(disulfide)}$ (a, green), $PMA_{SH(disulfide)}^{P}$ (a, red), $PMA_{SH(disulfide)}^{P}$ in the presence of 1 mM DTNB (a, blue), $PMA_{SH(thioether)}$ (c, green), $PMA_{SH(thioether)}^{P}$ (c, red), $PMA_{SH(thioether)}^{P}$ in the presence of 1 mM DTNB (c, blue), $PMA_{(amide)}$ (e, green), and $PMA_{(amide)}$ in the presence of 1 mM DTNB (e, blue). Percentage of cells associated with capsules was evaluated by the flow cytometrical analysis (b, d, f). Data are the mean ± the standard error of three independent experiments, each measured 15 000 cells. (**) $p < 0.005$, (*) $p < 0.05$ (*t*-test).

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Redox-responsive polymer capsules stabilized by disulfide bonds and loaded with hydrophobic cargo are prepared by templating mesoporous silica particles via layer-by-layer assembly. The thiols and disulfide bonds in the capsules interact with the exofacial thiols on the cell membrane, which regulate cellular association and cargo release from the capsules.

Keyword: Stimuli-Responsive Materials

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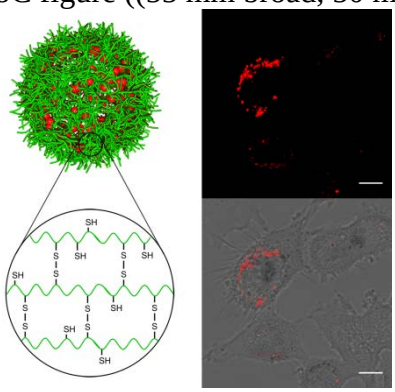

Supporting Information

Materials: 1,1'-dioctadecyl-3,3,3',3'-tetramethindocarbocyanine perchlorate (DiI), poly(methacrylic acid) (PMA, Mw 15 000 g mol^{-1}), poly(N-vinylpyrrolidone) (PVPON, Mw 10 000 g mol^{-1}), ethylenediamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), dimethyl sulfoxide (DMSO), and 2,2'-dinitro-5,5'-dithiobenzoic acid (DTNB) were purchased from Sigma-Aldrich. 1,8-bismaleimidodiethyleneglycol homobifunctional linker was obtained from Pierce and used without purification. Thiolated PMA (PMA_{SH}) with 12 mol% of thiol groups was synthesized from PMA and cystamine dihydrochloride via carbodiimide coupling, as described previously [12]. Anti-PDI monoclonal antibody was purchased from Abcam. Alexa Fluor 488, Alexa Fluor 647, and Propidium Iodide (PI) were purchased from Invitrogen.

DiI Loading and Capsule Preparation: The water-insoluble dye (DiI) was firstly loaded in the mesoporous silica particles (2-4 μm in diameter, surface area 630 m^2 g^{-1}, pore volume 1.7 cm^3 g^{-1}). In this process, 5 mg of the freshly dried mesoporous silica particles were dispersed in 30 mL of hexane solution saturated with DiI. The suspension was then shaken at ambient temperature for 2 h. After centrifugation (500 *g* for 1 min) and removal of the supernatant, the pellets were dried in vacuum overnight. The dried particles were resuspended in acetate buffer (20 mM, pH 4) at a concentration of 5 mg mL^{-1}. As depicted in Scheme 1, DiI-loaded PMA_{SH} microcapsules with two types of linkers (i.e., $PMA_{SH(disulfide)}$, and $PMA_{SH(thioether)}$) were prepared. In the preparation of the thiolated PMA capsules ($PMA_{SH(disulfide)}$ and $PMA_{SH(thioether)}$), suspension of the DiI-loaded mesoporous silica particles was firstly incubated in a 1.0 g L^{-1} solution of PVPON in acetate buffer (pH 4) for 10 min. After being washed three times in acetate buffer (pH 4), the particles were suspended in a 1.0 g L^{-1} solution of PMA_{SH} for 10 min. PVPON and PMA_{SH} were added sequentially until 10 layers had been deposited. The disulfide-stabilized capsules were crosslinked through treating with a

2.5 mM chloramine T solution in MES buffer (50 mM, pH 6) for 90 s, followed by two washing steps with MES buffer (50 mM, pH 6) and redispersion in NaOAc buffer (50 mM, pH 4). The thioether-stabilized capsules were crosslinked using thiolmaleimide chemistry through incubating the multilayer coated particles in 0.3 g L^{-1} of 1,8-bismaleimidodiethyleneglycol solution in MES buffer (50 mM, pH 6) over night. After that, the samples were washed with MES buffer (50 mM, pH 6) and 10 vol% of DMSO and redispersed in pH 4 buffer. In the preparation of the amide bond-linked PMA capsules ($PMA_{(amide)}$), the same procedures were used in the polymer multilayer assembly with the exception that the unmodified PMA was used. The amide-stabilized capsules were crosslinked through treating with 10 µL of 2.5 mM ethylenediamine in 50 mM MES buffer (pH 5) and 500 µL of 5 mM EDC in 50 mM MES buffer (pH 5.5) for 1 h, followed by two washing steps with MES buffer (50 mM, pH 5.5) and redispersion in NaOAc buffer (50 mM, pH 4). After the PMA multilayer was stabilized via different crosslinking methods, the mesoporous silica template was dissolved by adding 500 µL of 2 M HF. The resulting capsules were washed three times with acetate buffer (pH 4) and twice with MES buffer (pH 6) by centrifugation (1500 *g* for 2 min) and finally dispersed in phosphate buffered saline (PBS, pH 7.4).

Cell Viability: Cell viability was measured by Propidium Iodide (PI) nucleic acid stain using flow cytometry. After the incubation of cells with 1 mM DTNB for 24 h, HeLa cells were washed with PBS, trypsinized, collected by centrifugation and resuspended in PBS containing 3 µM PI at room temperature for 15 min. After the staining, FL2 fluorescence intensity of cells were analysed by flow cytometry in the presence of the dye (Partec Cyflow Space). The percentage of FL2 positive cells was quantified.

Flow Cytometric Analysis of Exofacial Thiols: The exofacial thiols were detected based on their reactivity with maleimide. HeLa cells were incubated with Alexa Fluor (AF) 647 (maleimide) dye (5 μM) in cold PBS for 20 min on ice. After the treatment, cells were then washed with cold PBS three times, trypsinized and resuspended in PBS for flow cytometry analysis (Partec Cyflow Space). Cells were identified based on their scatter characteristics, and the AF647 fluorescence intensity (FL5) was acquired.

Flow Cytometric Analysis of Free Thiols of AF488-labeled $PMA_{SH(thioether)}$ Capsules: The free thiols in capsules were evaluated based on the ability of the capsules to react covalently with maleimide. AF488-labeled $PMA_{SH(thioether)}$ capsules were incubated with AF647 (maleimide) dye in PBS (pH 7.4) for 30 min at room temperature. Capsules were then washed with PBS twice, and analysed by flow cytometry (Partec Cyflow Space). The capsules were identified based on their FL1 fluorescence intensity, and the AF647 fluorescence intensity (FL5) was acquired.

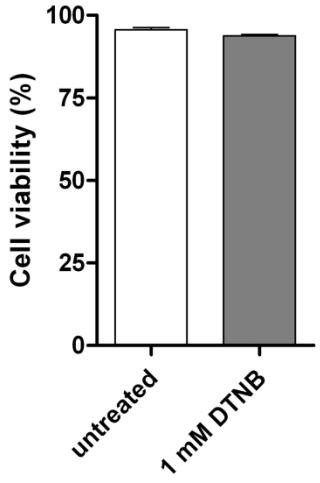

Figure S1. Effect of DTNB on cell viability, evaluated using a propidium iodide (PI) nucleic acid stain by flow cytometry. Cells were treated with 1 mM DTNB for 24 h at 37 °C, 5% CO_2. The values were normalized to that of untreated cells, which was set at 100%. Data are the mean ± the standard error of three independent experiments. Each experiment measured 20 000 cells.

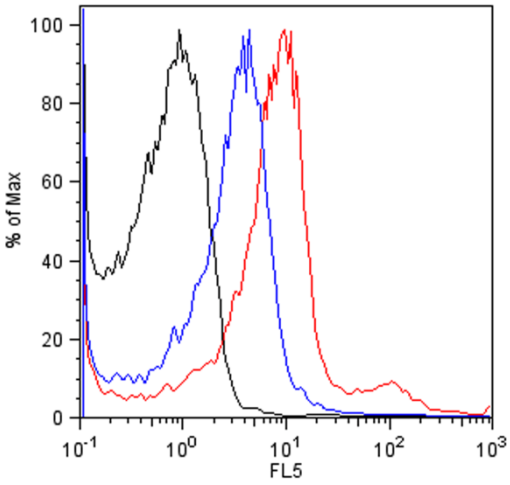

Figure S2. Evaluation of exofacial thiols using the AF647 maleimide dye by flow cytometry. Cells were treated with 1 mM DTNB for 24 h at 37 °C, 5% CO_2, and subsequently incubated with AF647 maleimide for 20 min on ice to stain the exofacial thiols. Fluorescence intensity histograms of cells treated with DTNB (blue) compared to untreated (red). Unstained and untreated cells as control (black). Each experiment measured 20 000 cells.

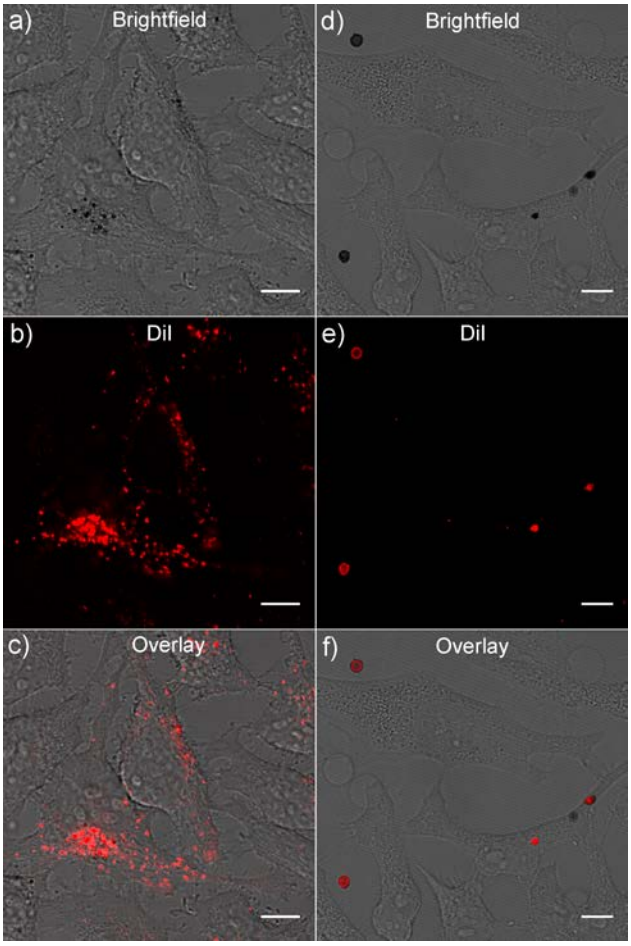

Figure S3. CLSM images of the intracellular distribution of DiI in HeLa cells. DiI-loaded $PMA_{SH(disulfide)}$ capsules were pretreated with 5 mM DTNB. Cells were then incubated with the pretreated capsules in the absence (a-c) or the presence of 1 mM DTNB (d-f) at 37 °C, 5% CO_2 for 24 h. The images correspond to brightfield images of cells (a, d, grey), intracellular distribution of DiI (b, e, red), and the overlay images (c, f). Scale bars, 10 µm.

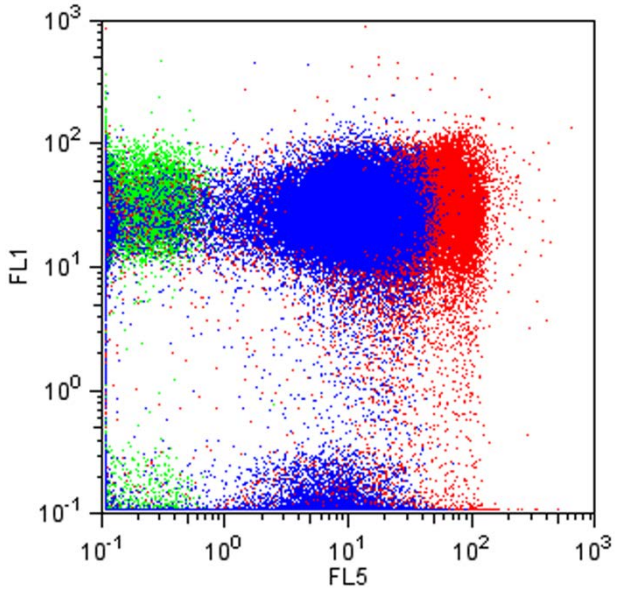

Figure S4. Evaluation of free thiols in AF488-labeled $PMA_{SH(thioether)}$ capsules using the AF647 maleimide dye by flow cytometry. Capsules were treated with 5 mM DTNB in PBS (pH 7.4) for 1.5 h at 37 °C, and subsequently incubated with AF647 maleimide for 30 min at room temperature to stain the free thiols. Dot plot of fluorescent intensity (FL5 versus FL1) of capsules treated with DTNB (blue) compared to untreated (red), and unstained capsules as control (green).

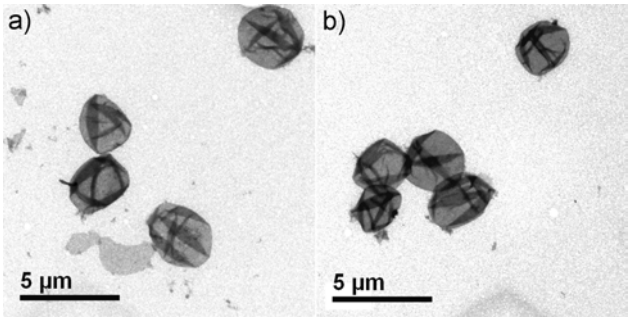

Figure S5. TEM images of $PMA_{SH(thioether)}$ capsules (a) and $PMA_{(amide)}$ capsules (b).