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Hydrogels

# Stabilization of Polymer-Hydrogel Capsules via Thiol–Disulfide Exchange

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***P**olymer hydrogels are used in diverse biomedical applications including drug delivery and tissue engineering. Among different chemical linkages, the natural and reversible thiol–disulfide interconversion is extensively explored to stabilize hydrogels. The creation of macro-, micro-, and nanoscale disulfide-stabilized hydrogels commonly relies on the use of oxidizing agents that may have a detrimental effect on encapsulated cargo. Herein an oxidization-free approach to create disulfide-stabilized polymer hydrogels via a thiol–disulfide exchange reaction is reported. In particular, thiolated poly(methacrylic acid) is used and the conditions of polymer crosslinking in solution and on colloidal porous and solid microparticles are established. In the latter case, removal of the core particles yields stable, hollow, disulfide-crosslinked hydrogel capsules. Further, a procedure is developed to achieve efficient disulfide crosslinking of multilayered polymer films to obtain stable, liposome-loaded polymer-hydrogel capsules that contain functional enzymatic cargo within the liposomal subcompartments. This approach is envisaged to facilitate the development of biomedical applications of hydrogels, specifically those including fragile cargo.*

**Keywords:**
- capsosomes
- crosslinkers
- hydrogels
- layer-by-layer assembly
- poly(methacrylic acid)

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## 1. Introduction

Polymer hydrogels are unique materials that are widely used in various biomedical applications from drug delivery to tissue engineering.[1] For live-cell immobilization, polymer hydrogels offer unhindered diffusion of nutrients and other small molecules to and from the cells and provide a structural matrix for cell attachment. In drug delivery, hydrogels allow for controlled release of therapeutics and targeted delivery of cargo to the desired organ and tissues–two features that revolutionized the field of biomedicine. Both macroscopic gels and micro- and nanoscale-sized gel particles find use in diverse applications, and in each case controlled structural stability plays a pivotal role in their design. Physical and chemical crosslinking methods are available to create hydrogels, and both approaches offer their own advantages. In the latter case, degradable linkages are employed to create reversibly cross-linked gels, specifically to obtain biodegradable constructs.

Thiol–disulfide interconversion is a particularly attractive biologically relevant chemistry that combines the specificity of reactions with mild reaction conditions. It has been used to

create macro-, micro-, and nanoscale-sized gels for varied applications from manufacturing of optical lenses[2] to the creation of micropatterned structures for sensing and nanoscale drug carriers.[3–5] Of particular importance for drug delivery is that the overall redox potential of the blood is oxidative, thus favoring the existence of disulfides, and the intracellular compartments are often reductive, which leads to deconstruction of disulfide linkages upon cellular internalization.[6–8]

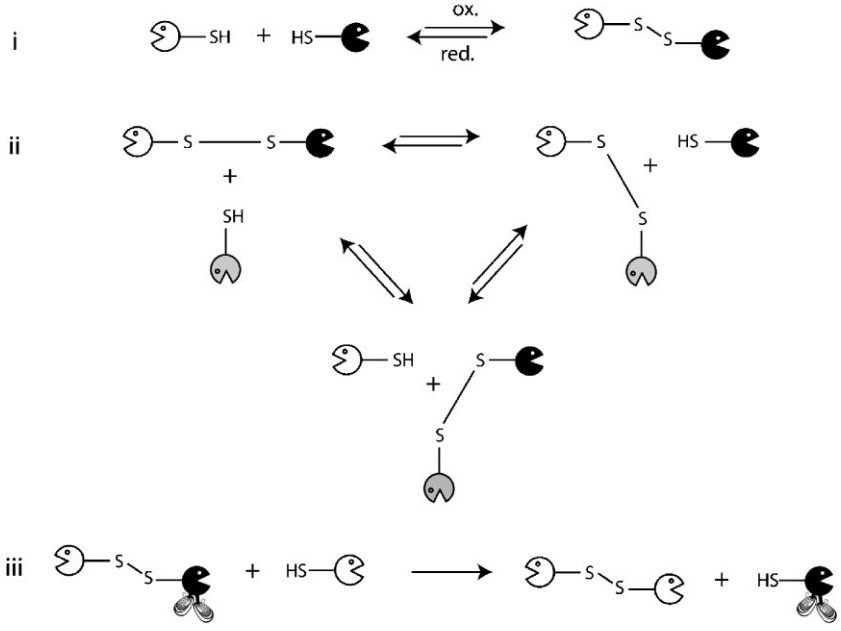
Recently, we established an approach to prepare disulfide-stabilized micrometer- and submicrometer-sized monodisperse polymer-hydrogel capsules via the layer-by-layer (LbL) technique.[9] The method employs thiolated poly(methacrylic acid), $PMA_{SH}$, and poly(vinylpyrrolidone), PVPON. The two polymers are sequentially deposited onto colloidal template particles in acidic environment to form a hydrogen-bonded polymer multilayered film. The as-formed film spontaneously disassembles when placed into media with pH above 6.5, the $pK_a$ of PMA, due to ionization of the carboxylic acid groups and deconstruction of the hydrogen bonds. Conversion of the thiol groups in the multilayered film into bridging disulfide linkages yields polymer capsules that release PVPON at pH above 6.5. These single-component PMA-hydrogel capsules owe their stability solely to the biodegradable disulfide linkages. We have gained control over the wall thickness, permeability, stability, and degradability of the capsules, and have developed drug- and reagent-loading techniques to accommodate for the encapsulation of single- and double-stranded DNA,[10,11] intact proteins,[12] and oligopeptides,[13] as well as oil-solubilized hydrophobic drugs.[14] The structural stability of these capsules also makes them well-suited for the creation of microreactors[15] and artificial organelles.[16] Our primary assembly protocol to date involves the controlled oxidization of thiol groups into disulfide linkages with the use of chloramine T (CaT).[9] While non-damaging in most cases, the use of an oxidizing agent may harm the encapsulated material and cause a corresponding loss of its functional activity. Therefore, we sought an alternative, non-oxidative approach to convert the $PMA_{SH}$ thiol groups into disulfides.

In this article, we introduce a novel method to create disulfide–crosslinked polymer hydrogels that are obtained through the controlled interaction of the constituent polymers via thiol–disulfide exchange, a thiol-specific reaction that can be conducted in a variety of conditions. The presented approach is shown to be applicable to crosslink the polymers in solution as well as on colloidal solid and porous particles. We further report on a novel technique to obtain disulfide-stabilized polymer hydrogel capsules and demonstrate that these are comparable to their CaT-oxidized counterparts in structural stability and in their ability to serve as carriers for liposomal subcompartments. The developed approach is expected to have impact beyond the field of LbL-derived materials and will prove useful for a variety of applications that are based on disulfide-stabilized gels.

## 2. Results and Discussion

A disulfide bond is a covalent linkage formed by two sulfur atoms that is typically obtained via the controlled coupling of two thiol groups. The main strategies to achieve this include i) the oxidization of two thiol groups,[17] ii) thiol–disulfide exchange between a thiol and an existing disulfide bond (disulfide reshuffling),[18] and iii) disulfide reshuffling with the aid of a mixed disulfide formed with a sulfur-containing good leaving group[19–23] (Scheme 1). The latter method is singled out here from thiol–disulfide exchange solely due to the shifting of the equilibrium toward the products. The former method is typically employed in the creation of various materials, including macrogels and polymer films. It relies on the use of oxidizing agents, such as air oxygen,[17] dimethyl sulfoxide (DMSO),[24,25] hydrogen peroxide,[26,27] and other oxidizing reagents. This method is rather well-established, however, it suffers from a range of shortcomings. First, the oxidation is a pH-sensitive and time-consuming reaction, which can lead to over-oxidation of thiols. Furthermore, the use of oxidizing agents can have a detrimental effect on (bio)molecules and cells. In this regard, the formation of disulfides via thiol–disulfide exchange appears to offer significant advantages, yet to the best of our knowledge, application of this concept for the creation of polymer hydrogels remains largely unexplored.

In this work, we aimed to investigate the utility of thiol–disulfide exchange, in particular with use of activated thiol groups, to crosslink polymer chains and form polymer hydrogels. Our primary target was to develop a method to




**Scheme 1.** Schematic illustration of the three main strategies to form disulfide bonds: i) reversible oxidization of two thiol groups into a disulfide linkage; ii) disulfide reshuffling, which involves an existing disulfide linkage and a free thiol group; and iii) disulfide reshuffling in which one of the thiol groups is a good leaving group.

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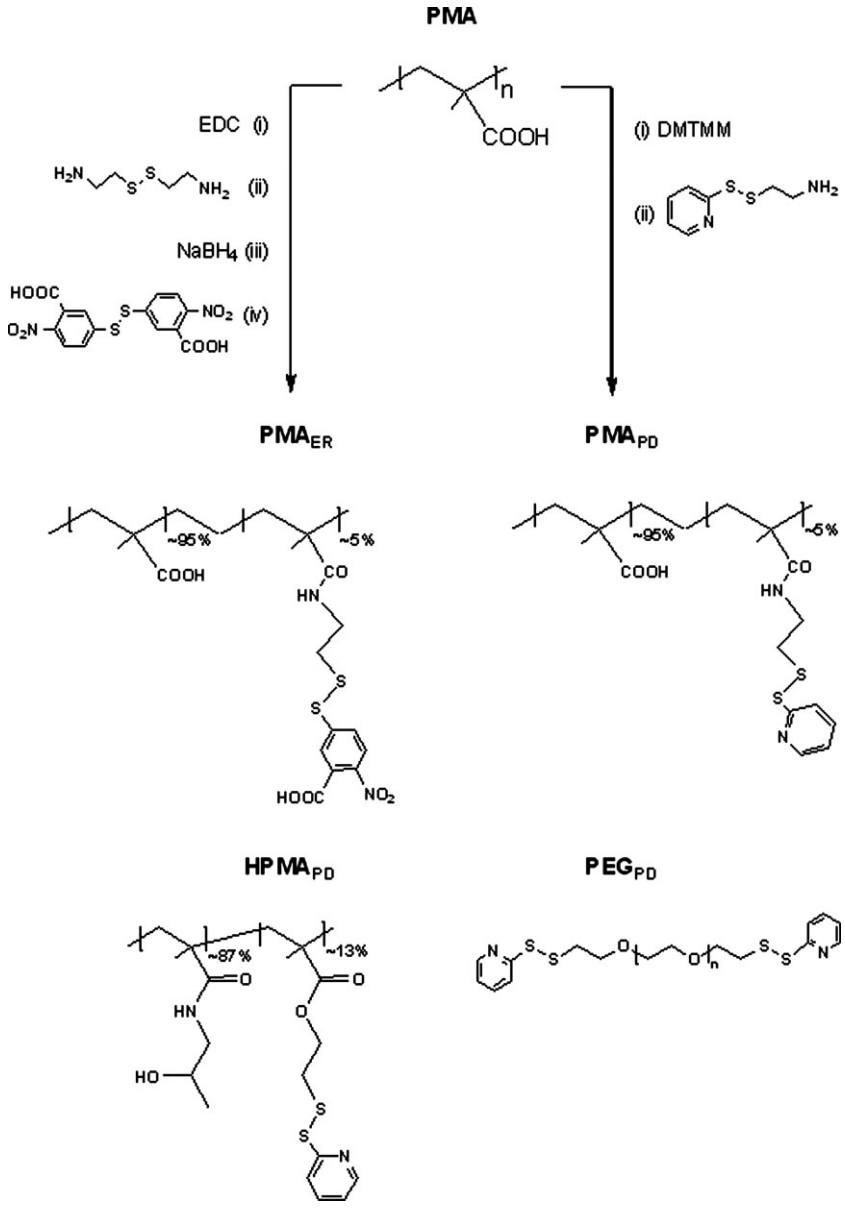
obtain disulfide-stabilized PMA-hydrogel capsules, and for this reason we used this polymer in all further experiments.

### 2.1. Preparation of $PMA_{ER}$ and $PMA_{PD}$ Crosslinkers

We first elaborated the synthesis of PMA samples with thiol groups activated towards thiol–disulfide exchange and considered the two activating groups that are most often employed for similar applications in the field of bioconjugation, namely 3-carboxy-4-nitrobenzene sulfide (Ellman's reagent, ER), $PMA_{ER}$, and pyridine-2-sulfide (PD), $PMA_{PD}$. Throughout the paper, the PMA samples with activated thiol groups (i.e., $PMA_{PD}$ and $PMA_{ER}$) are referred to as crosslinking polymers.

To synthesize $PMA_{ER}$, we used a sample of thiolated PMA obtained from pristine PMA and cystamine via carbodiimide-mediated coupling. The polymer was then treated with excess sodium borohydride to convert all disulfide linkages into thiol groups, and the latter were reacted with ER (Scheme 2). This procedure is typically performed to quantify the number of thiol groups on the polymers,[28] that is, it is a quantitative reaction to obtain the desired product, $PMA_{ER}$. To prepare $PMA_{PD}$, we used 2-aminoethyl 2-pyridyl disulfide (PDA) and directly coupled it with PMA using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) as a coupling reagent[29] (Scheme 2). For both polymeric crosslinkers, the samples with a high degree of thiol substitution (i.e., 12 mol% thiol groups) had limited solubility in buffered aqueous solutions with a pH below the $pK_a$ of PMA, pH 6.5. This practical aspect limited us to use PMA crosslinking samples with a content of activated thiols not exceeding 5 mol%.




**Scheme 2.** Schematic illustration of the chemical synthesis of $PMA_{ER}$ and $PMA_{PD}$ and the chemical formulae of $HPMA_{PD}$ and $PEG_{PD}$.

### 2.2. Reaction of the Crosslinkers with $PMA_{SH}$ in Solution

First, we aimed to compare the PMA crosslinking polymers with the two different activating groups, ER and PD, in their ability to react with $PMA_{SH}$ in solution. Both leaving groups have distinct absorption maxima in their UV–Vis spectra (412 nm for 2-nitro-thiobenzoic acid released from $PMA_{ER}$ and 343 nm for 2-pyridinethione released from $PMA_{PD}$), providing a convenient way to quantify each reaction. In both cases, the 100% conversion was ascertained by the use of dithiothreitol (DTT), a potent reagent that cleaves disulfide linkages into the constituent thiols.

The two parameters expected to govern the reaction kinetics are the reactivity of the thiol groups towards disulfide exchange and the interaction of the PMA chains in solution (association via hydrogen bonding or electrostatic repulsion). While thiol–disulfide exchange is known to be a pH-dependent process with reaction rates accelerated upon an increase in pH to a value close to the $pK_a$ of the thiol groups (≈8.5 for aliphatic thiols), the pH increase is also expected to enhance the repulsion of the PMA chains, $pK_a$ 6.5. For $PMA_{ER}$, the former factor seems to dominate, leading to a higher reaction conversion when the pH was increased (Figure 1). The reactivity of the PD activating group at acidic pH is known to be higher than the reactivity of ER due to the protonation of pyridyl nitrogen.[30] In agreement with this, at pH 4 the reaction between $PMA_{SH}$ and $PMA_{PD}$ reaches a markedly higher conversion compared to those with $PMA_{ER}$. Moreover, in the case of $PMA_{PD}$, the reaction conversion does not appear to be pH-dependent, which provides a wide

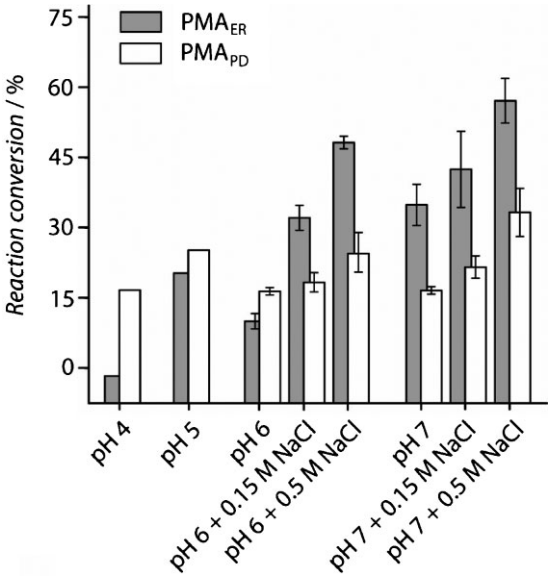



**Figure 1.** Conversion for the thiol–disulfide exchange between $PMA_{ER(PD)}$ and $PMA_{SH}$ in solutions with different pH and ionic strength achieved after 30 min of incubation. Reaction conversion was ascertained via UV–Vis spectrophotometry used to monitor the release of PD ($\lambda_{max} = 343$ nm) and ER ($\lambda_{max} = 412$ nm) leaving groups.

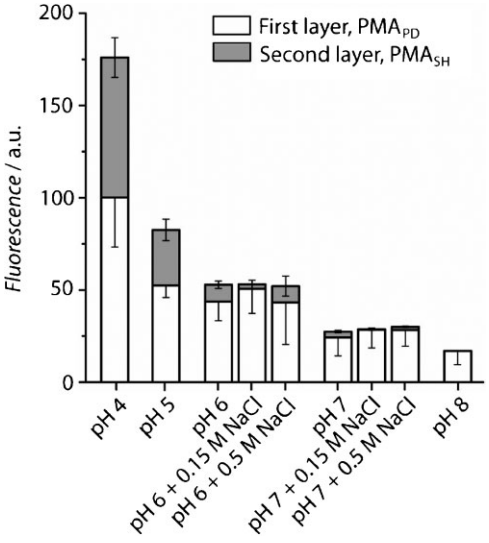



**Figure 2.** Fluorescence intensity of amine-functionalized 3-μm particles achieved upon electrostatic adsorption of fluorescently labeled $PMA_{PD}$ polymer (white) and the subsequent addition of fluorescently labeled $PMA_{SH}$ polymer (gray) via a disulfide exchange reaction in solutions of a different pH and ionic strength. The fluorescence intensity was quantified using flow cytometry.

range of possible reaction conditions to achieve the formation of disulfide-linked hydrogels. For both activating groups, the reaction reached higher conversion degrees when the solution was supplemented with NaCl. The low-molecular-weight electrolyte decreases the electrostatic repulsion between the charged PMA chains and in doing so facilitates the reaction between $PMA_{SH}$ and $PMA_{ER(PD)}$.

### 2.3. Reaction of the Crosslinkers with $PMA_{SH}$ on Particles

Our primary research focus relates to surface-tethered hydrogels, and specifically to colloidal particles for the creation of hydrogel capsules for drug-delivery applications. In our next experiment we monitored the reaction between $PMA_{SH}$ and its activated counterparts on the surface of 3-μm silica particles. Here, we used fluorescently labeled polymers and employed flow cytometry to quantify the fluorescence of each individual particle in flow.

The first polymer layer, $PMA_{PD}$, was electrostatically adsorbed onto the surface of amine-functionalized, positively charged solid silica particles (Figure 2, white bars). Lower pH values favored the adsorption of a higher amount of polymer. This is readily attributed to the alteration of ionization state and conformation of the polymer chains at different pH values: at high pH, the ionized chains are adsorbed in an extended conformation forming a thin layer of polymer on the surface; at pH 4, the chains are adsorbed in their coiled conformation, resulting in a higher polymer mass per unit area. Similar trends are well-documented in the literature for a multitude of polyelectrolytes with pH-sensitive properties and were observed for both pristine PMA and $PMA_{SH}$ (data not shown).

Particles (3-μm) with an adsorbed layer of $PMA_{ER(PD)}$ were incubated in a solution of $PMA_{SH}$. The full coverage of $PMA_{ER(PD)}$ on the particles does not allow adsorption of $PMA_{SH}$, and the increase in particle fluorescence intensity indicates a successful chemical reaction between the introduced polymer and the surface via thiol–disulfide exchange (Figure 2, grey bars). Contrary to expectations, the particles with adsorbed $PMA_{PD}$ exhibited minimal increase in fluorescence at high pH, that is, negligible reaction with the introduced $PMA_{SH}$ at pH 6 and above, regardless of the presence of NaCl. That is, while these conditions were found to be optimal for the reaction between both components in solution, they did not sustain any reaction when one of the reacting polymers was adhered to the surface. Furthermore, in the case of colloids coated with $PMA_{ER}$, none of the conditions used led to a reaction with $PMA_{SH}$. On the other hand, the reaction of $PMA_{SH}$ with the surface-attached $PMA_{PD}$ was possible and most pronounced at pH 4, conditions where the electrostatic interaction of PMA chains is suppressed and the activity of the PD group is increased. Within 60 min, the amount of $PMA_{SH}$ reacting onto the surface reached 70% of the first layer.

Interestingly, when porous silica particles were used as templates, the reaction between $PMA_{ER}$ and $PMA_{SH}$ did proceed at pH 4 and 5 (and to a lesser extent at higher pH values). At pH 4, the second layer (the product of the reaction) comprised almost 80% of the first (adsorbed) polymer layer. Stable capsules were obtained upon template core removal, confirming the successful reaction between the two polymers. While the complete explanation for this marked change in the reactivity of the polymers requires further experiments, a plausible reason relates to a drastically greater concentration of the reactive groups attained within the confines of mesopores.

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We next attempted to assemble multilayered polymer films via the LbL technique by sequentially depositing $PMA_{SH}$ and its activated counterparts onto solid colloidal particles using the previously described chemical reaction. A similar approach has been described for various polymers using click-chemistry reactions[31–33] and thiol–disulfide exchange.[34] However, after deposition of three layers of thiolated PMA through the formation of covalent disulfide linkages, the amount of polymer deposited significantly diminished (see Supporting Information). Similar behavior was previously reported for the sequence-specific assembly of DNA-modified micelles.[35a] A plausible reason for this is the increase in the charge-repulsion forces between a surface with an increased amount of deposited polymer and its counterpart in solution. Importantly, we note that the attained thickness was sufficient to provide structural stability to obtain stable hollow capsules upon removal of the template particles (data not shown). These can prove useful for the encapsulation of macrosolutes when fast and easy exchange of biomolecules across the polymer membrane is required.

## 2.4. Disulfide-Stabilized Polymer-Hydrogel Capsules

In further pursuit of a facile technique to obtain disulfide-stabilized polymer capsules without the use of oxidizing agents, we devised an alternative strategy based on the assembly of a polymer thin film by the sequential deposition of $PMA_{SH}$ and PVPON via hydrogen bonding. This system is well-established and was previously reported in a number of our previous publications. Among the attractive features of this system is a linear build-up of polymers,[9] that is, a linear incremental increase in the thickness of the film with the number of deposited polymer bilayers. We also showed that subsequent core removal does not lead to a detectable loss of material from the capsule wall. Combined, these two features allow for easy control over the capsule properties, such as wall thickness and permeability.[35b]

In order to obtain capsules that are stable at physiological conditions (pH 7), the thiol groups within the film need to be converted into bridging disulfide linkages. In this work, we infiltrated $PMA_{PD}$ as a polymeric crosslinker into the preassembled film, either on colloidal supports or after removal of the template particles, referred to as pre- and post-crosslinking, respectively. For these experiments, we used the optimized crosslinking protocol that afforded well-dispersed, non-aggregated, and robust capsules, namely 15 kDa $PMA_{PD}$ (1 g $L^{-1}$ for pre-crosslinking and 0.5 g $L^{-1}$ for post-crosslinking in 20 mM NaOAc buffer, pH 4). Under the crosslinking conditions used, 6 h of incubation has proven to be sufficient to obtain stable capsules, however, for practical reasons in all further experiments, crosslinking was performed overnight (15–20 h). Fluorescently labeled $PMA_{SH}$ was used to visualize the capsules using a fluorescence microscope and ascertain the size of the capsules at pH 7. The degree of swelling of a hydrogel and hydrogel capsules in particular[36] can be correlated to the crosslinking density. This feature was used to monitor the success of thiol-to-disulfide conversion.

For all the capsules studied, as few as two bilayers of $PMA_{SH}$/PVPON deposited onto 1-μm colloidal particles gave rise to robust capsules that were stable at pH 7 after conversion of thiol groups into disulfide linkages (Figure 3). The CaT-treated capsules exhibited similar swelling, regardless of the number of deposited polymer bilayers. This was also true for $PMA_{PD}$-crosslinked capsules obtained via pre- or post-crosslinking. However, these capsules exhibited greater swelling than their CaT-treated counterparts. While the latter observation suggests a lower degree of crosslinking in the capsules obtained via the disulfide reshuffling method, the isolated capsules exhibited excellent colloidal stability and their monodispersity makes them well-suited for diverse biomedical applications.

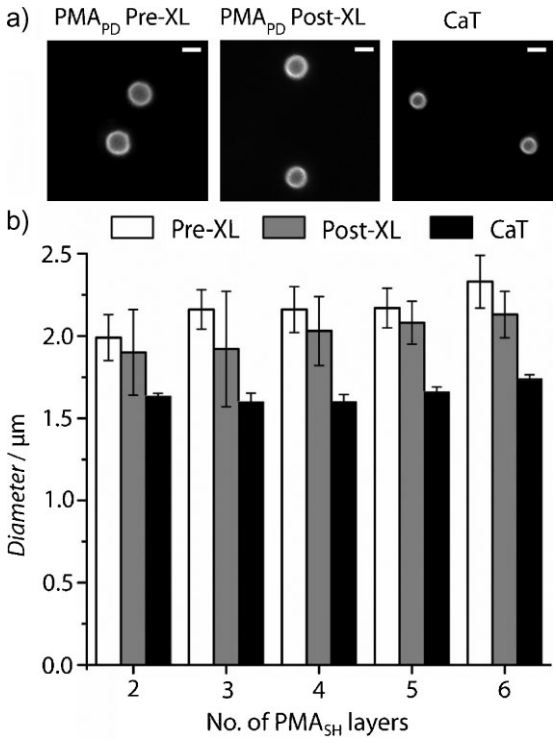


**Figure 3.** Fluorescence microscopy images of PMA hydrogel capsules (a) and diameters of PMA hydrogel capsules as a function of the number of $PMA_{SH}$/PVPON bilayers deposited onto 1-μm silica particles (b) pre- or post-crosslinked with $PMA_{PD}$ or crosslinked with CaT. Microscopy images (a) show hydrogel capsules obtained from $PMA_{SH}$/PVPON films with five deposited bilayers. All images and readings were taken at pH 7. All scale bars are 5 μm.

In an effort to optimize crosslinking, we tested a range of polymeric crosslinkers bearing PD-activated thiol groups, varied experimental conditions, and considered the PMA hydrogel capsules of two sizes that we have previously successfully used as drug carriers[13] and microreactors[15] (1- and 3-μm template particles, respectively). The optimized reaction conditions are summarized in Table 1, and the size of capsules obtained using these conditions is presented in Figure 4. To achieve efficient crosslinking, the introduced polymer with activated thiol groups should be able to infiltrate into and throughout the multilayered polymer film. Shorter polymer chains are expected to have an increased capacity to do so, and to test this we used the samples of $PMA_{PD}$ with lower molecular weights (4 and 7 kDa). Contrary to our expectations, the use of these polymers did not afford stable capsules and led to severe sample aggregation (data not shown).

  

**Table 1.** Summary of the crosslinking polymers examined, optimized crosslinking conditions and the resulting capsule sizes at pH 7.

| | | $PMA_{PD}$ | | $HPMA_{PD}$ | | $PEG_{PD}$ |
|---|---|---|---|---|---|---|
| $M_w$ | 4 kDa | 7 kDa | 15 kDa | 33 kDa | 2 kDa | 10 kDa |
| thiolation mol% | ≈5% | ≈5% | ≈5% | ≈13% | 2 functional groups | |
| | | | Concentrations (g $L^{-1}$) | | | |
| pre-XL | 0.25–2 | 0.25–2 | 1 | 1 | 1–10 | 3 |
| post-XL | 0.25–2 | 0.25–2 | 0.5 | 1 | 1–10 | 1–10 |
| | | | Diameter of PMA capsules [μm] prepared from 3 μm template | | | |
| pre-XL | Aggregation | Aggregation | 5.29 ± 0.23 | 4.35 ± 0.30 | Aggregation | 5.45 ± 0.44 |
| post-XL | Aggregation | Aggregation | 5.01 ± 0.46 | 4.26 ± 0.35 | Aggregation | Non-uniform[a] |
| | | | Diameter of PMA capsules [μm] prepared from 1 μm template | | | |
| pre-XL | Aggregation | Aggregation | 2.17 ± 0.16 | 1.80 ± 0.12 | Aggregation | 2.64 ± 0.2 |
| post-XL | Aggregation | Aggregation | 2.08 ± 0.14 | 1.75 ± 0.13 | Aggregation | Non-uniform[a] |

[a] Refers to polydisperse capsules with slight aggregation.

Nonionic, low-fouling polymers are attractive candidates as crosslinking polymers due to potentially enhanced permeability into the polymer multilayers. To test this, we used the two polymers that are widely used in biomedical applications, poly(ethylene glycol) (PEG) and poly(hydroxypropyl methacrylamide) (HPMA), both equipped with PD-activated thiol groups. In the case of PEG, we employed commercially available divalent homobifunctional PEG linkers ($PEG_{PD}$), which are typically used in bioconjugation applications and contain two terminal thiol groups activated with PD. The synthesized sample of $HPMA_{PD}$ had a molecular weight of 33 kDa and 13 mol% repeat units bearing PD groups (i.e., ≈26 PD groups per chain).

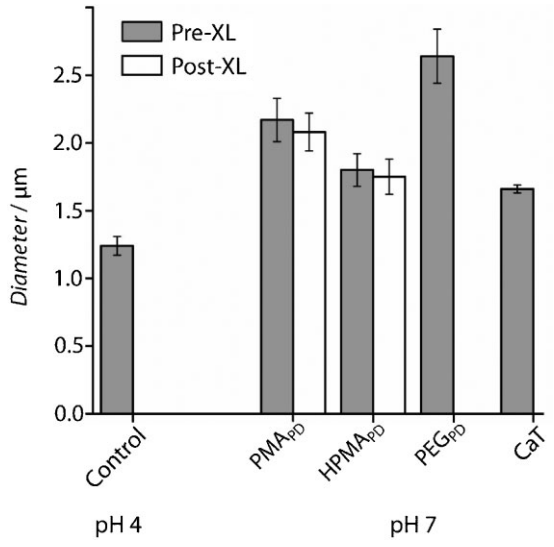


**Figure 4.** Diameters of PMA hydrogel capsules pre- or post-crosslinked either by $PMA_{PD}$, $HPMA_{PD}$, $PEG_{PD}$, or CaT. The capsules were derived from 1-μm silica particles and their diameters were ascertained by fluorescence microscopy at pH 7. At pH 4, the size of the hydrogel capsules was independent of the crosslinker and is represented as a control.

Surprisingly, the use of a 2 kDa $PEG_{PD}$ yielded unstable and non-uniform PMA capsules for both pre- and post-crosslinking methods. This was also true when 10 kDa $PEG_{PD}$ was used for post-crosslinking, yet in this case pre-crosslinking afforded robust, well-dispersed hydrogel capsules. We believe that the effectiveness of $PEG_{PD}$ can be increased by the use of multi-arm polymer samples with an increased number of active sites, and this hypothesis is supported by the data obtained with the use of $HPMA_{PD}$ (Figure 4). The use of this multivalent crosslinker afforded well-dispersed capsules with an increased degree of crosslinking, that is, smaller size compared to the capsules obtained with the use of $PMA_{PD}$. Moreover, these capsules were very similar in size to their CaT-crosslinked counterparts, which when coupled with the successful history of biomedical applications of HPMA, makes this crosslinking reagent attractive to obtain PMA-hydrogel capsules and disulfide-crosslinked hydrogels in general.

## 2.5. Formation of Enzyme-Loaded Capsosomes

Recently, we used disulfide-stabilized PMA-hydrogel capsules as the structural construct of capsosomes, liposome subcompartmentalized polymer capsules.[16] These vehicles combine the benefits provided by the structural stability and controlled permeability of the polymer membrane and the sealed nature of thousands of liposomal subcompartments that protect and trap small fragile biomolecules. These capsosomes are attractive candidates to create advanced therapeutic vessels such as artificial cells or organelles. The use of an oxidization-free approach as described above to stabilize the polymer carrier capsules is particularly important in the creation of capsosomes because the use of CaT can lead to a loss of function of the enzymatic cargo within the liposomes. However, the incorporation of 50–200-nm large soft objects such as intact liposomes within a polymer film can drastically change its properties including its crosslinking density. Aiming to characterize the effect of the liposomes on the structural integrity of the polymer carrier capsule, we used the polymeric crosslinkers to obtain capsosomes and compared their swelling

  

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to the CaT-derived capsosomes as well as to pristine PMA capsules.

The design of capsosomes includes the adsorption of a cholesterol-modified poly(L-lysine) ($PLL_c$) precursor layer onto the 3-μm silica core particles prior to the immobilization of the enzyme-loaded liposomes (Scheme 3). The enzyme-loaded liposomes are stably anchored to the surface by capping them with poly(methacrylic acid)-*co*-(cholesteryl methacrylate) ($PMA_c$), a polymer that also enables the transition to the polymer multilayer assembly of PVPON and $PMA_{SH}$. Finally, the thiols in the polymer multilayer film were crosslinked using $PMA_{PD}$, $HPMA_{PD}$, or CaT either before or after removal of the silica template.

All tested crosslinking approaches yielded colloidally stable 3-μm $PMA_{AF488}$ capsules (Figure 5a) and enzyme-loaded ($\beta$-$lactamase_{AF488}$) capsosomes (Figure 5b) in both assembly and physiological conditions (AF488 refers to Alexa Fluor 488 labeling). In the absence of liposomes, the 3-μm capsules were well-crosslinked with the use of both $PMA_{PD}$ and $HPMA_{PD}$, evidenced by their swelling behavior, which was found to be similar to the CaT-oxidized capsules (Figure 5c, white and gray bars). Furthermore, in the particular case of $HPMA_{PD}$, the capsules appear to have a high crosslinking density (almost no swelling upon increasing the pH), which may have a significant impact on their permeability characteristics. For capsosomes, the use of $PMA_{PD}$ gave rise to somewhat lower crosslinked samples with a higher radius as compared to parent PMA-hydrogel capsules. In contrast, $HPMA_{PD}$ afforded capsosomes with swelling characteristics similar to those obtained with the use of CaT, suggesting a high degree of crosslinking. Importantly, the use of polymeric crosslinkers preserved the enzymatic activity of the proteins within the liposomal subcompartments and yielded functional capsosomes,[16] an important step on the way to creating novel therapeutic vehicles such as artificial organelles.

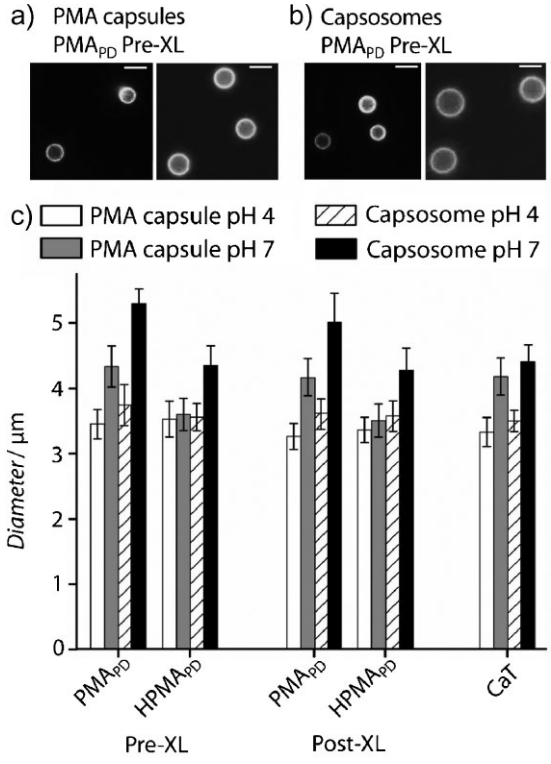
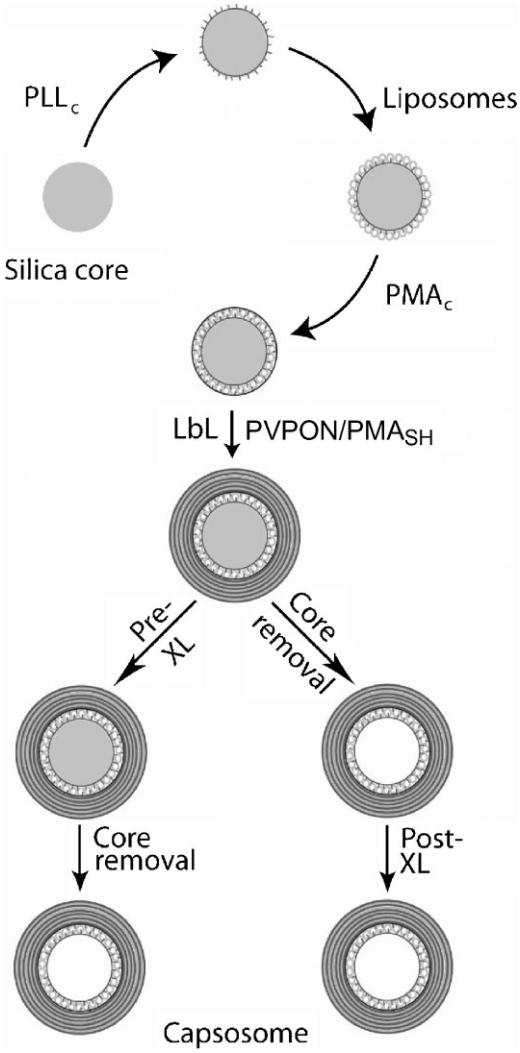



**Figure 5.** a) Fluorescence microscopy images of $PMA_{AF488}$ hydrogel capsules and b) enzyme-loaded ($\beta$-$lactamase_{AF488}$) capsosomes, pre-crosslinked with $PMA_{PD}$ at pH 4 (left) and pH 7 (right). All scale bars are 5 μm. c) Diameters of PMA capsules and capsosomes, depending on different crosslinking methods at both assembly (pH 4) and physiological (pH 7) conditions.




**Scheme 3.** Schematic illustration of capsosome assembly. Silica particles were coated with $PLL_c$, liposomes, and $PMA_c$, followed by the subsequent polymer layering of $PMA_{SH}$/PVPON. The thiol groups in the polymer film were crosslinked prior to core removal (pre-XL), while post-XL refers to converting thiols to bridging disulfide linkages after dissolution of the template core to yield capsosomes (liposome-loaded polymer hydrogel capsules).

## 3. Conclusion

In pursuit of benign methods to obtain disulfide-stabilized PMA-hydrogel capsules for biomedical applications, we have characterized and optimized a polymer crosslinking concept

based on thiol–disulfide exchange. We have investigated the synthesis of $PMA_{PD}$ and $PMA_{ER}$, two polymers activated towards thiol–disulfide exchange, and optimized their reaction with $PMA_{SH}$ both in solution and on colloidal particles. Our attempts to assemble multilayered polymer thin films via sequential deposition of $PMA_{SH}$ and $PMA_{PD(ER)}$ were met with partial success: the process has proven to be ineffective beyond deposition of three polymer layers, yet this was sufficient to produce stable polymer-hydrogel capsules upon removal of the template particles. To obtain crosslinked, multilayered polymer hydrogels, we developed a strategy based on infiltration of crosslinking polymers into the preformed $PMA_{SH}$/PVPON multilayers on colloidal supports and considered three different crosslinking polymers, namely $PMA_{PD}$, $HPMA_{PD}$, and $PEG_{PD}$. We demonstrated that under different conditions all three polymers give rise to stable disulfide-crosslinked polymer-hydrogel capsules. $HPMA_{PD}$ was shown to be the most effective candidate and produced hydrogel capsules with a high crosslinking density, similar to that attained using the oxidizing agent CaT. The developed crosslinking concept was also successfully implemented to obtain capsosomes, liposome-loaded hydrogel capsules, with functional enzymatic cargo contained within liposomal subcompartments. Taken together, the presented data establish a novel approach to create polymer hydrogels and hydrogel capsules for a variety of biomedical applications from drug delivery to tissue engineering and biosensing.

## 4. Experimental Section

*Materials:* All materials and chemicals, unless stated otherwise, were purchased from Sigma–Aldrich and used as received without purification. $SiO_2$ particles of 1- and 3-μm diameter were purchased from MicroParticles GmbH as a 5 wt% suspension and were used as received. PMA sodium salt, $M_w = 15$ kDa, was purchased from Polysciences (USA). Ethylenediaminetetraacetic acid (EDTA), 2-(*N*-morpholine)ethane-sulfonic acid (MES), 3-morpholinopropane-1-sulfonic acid (MOPS), sodium acetate (NaOAc), and 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) were purchased from Merck. PDA was purchased from Speed-Chemical Corp. (China). Phosphate-buffered saline (PBS) was purchased from Invitrogen. High-purity water with a resistivity greater than 18 MΩ cm was obtained from an in-line Millipore RiOs/Origin system (MilliQ water). $PLL_c$ (40–60 kDa) and $PMA_c$ (11.6 kDa) were synthesized according to previously published protocols.[37] Recombinant β-lactamase was expressed in *Escherichia coli* from pUC19 gene cloned into a pET28a expression vector. Zwitterionic lipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, phase transition temperature 24 °C) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, phase transition temperature 41 °C) were purchased from Avanti Polar Lipids (USA). Stock solutions of the lipids (50 g $L^{-1}$) were prepared in chloroform and stored in the freezer. Unilaminar liposomes were prepared by the evaporation of the chloroform (2.5 mg lipids) under nitrogen (1 h) followed by hydration with 48 μg enzyme dissolved in 30 μL PBS buffer. This solution was diluted into 1 mL HEPES buffer (150 mM NaCl, 10 mM HEPES, pH 7.4) and extruded through 50 nm filters. 2,2′-azobisisobutyronitrile (AIBN) was purchased from Aldrich and recrystallized twice from methanol prior to use. High purity Argon (Linde gases) was used for purging the HPMA polymerization solution. Pyridyldisulfide ethylmethacrylate was synthesized according to the procedure reported elsewhere.[20] 4-(Cyanopentanoic acid)-4-dithiobenzoate (CPADB) was synthesized according to the previously reported procedure[38] and used as a reversible addition–fragmentation chain transfer (RAFT) agent. HPMA was synthesized according the method described elsewhere.[39]

*Methods:* Absorbance measurements were performed using an Agilent 8453 diode-array UV–Vis spectrophotometer. Flow cytometry was performed on a CyFlow Space (Partec GmbH) flow cytometer using an excitation wavelength of 488 nm. In each case at least 25 000 events were analyzed. Particles were imaged on an Olympus IX71 digital wide-field fluorescence microscope with a fluorescein filter cube.

*Preparation of $PMA_{SH}$:* Samples of PMA with 5 and 12 mol% thiol groups were synthesized from PMA and cystamine dihydrochloride via carbodiimide coupling as described in a previous published protocol.[9] The thiol content in the resulting polymer was characterized using ER and a cystamine standard curve.[28]

*Preparation of $PMA_{ER}$:* $PMA_{SH}$ with 5 mol% thiol groups was dissolved in water (10 g $L^{-1}$). To this solution sodium borohydride was added to a 1 M final concentration and the reaction mixture was incubated at room temperature for 2 h. Excess borohydride was neutralized by concentrated hydrochloric acid. Subsequently, the mixture was supplemented with PBS to 0.1 M concentration and the pH was adjusted to 8 using HCl and NaOH. To this solution, excess ER was added and the reaction was allowed to proceed for 30 min. The reaction mixture was purified via size exclusion chromatography (SEC) using NAP-25 desalting columns (2×) and freeze-dried to obtain a light yellow powder of $PMA_{ER}$.

*Preparation of $PMA_{PD}$:* In a typical experiment, a PMA solution (200 mg of 30 wt% solution) was diluted into 2.5 mL of phosphate buffer (0.1 M, pH 7.2). The resulting solution was charged with DMTMM (31 mg) and the mixture was stirred for 5 min. Subsequently, PDA (6.2 mg, target modification = 5 mol%) was added to the mixture and the reaction was allowed to proceed overnight. The reaction mixture was purified via SEC using NAP-25 desalting columns (2×) and freeze-dried to obtain a white powder of $PMA_{PD}$.

*Preparation of $HPMA_{PD}$:* Monomers HPMA (0.4 g, $2.8 \times 10^{-3}$ mol) and 2-(2-pyridyldisulfide)ethylmethacrylate (PDSM) (47.5 mg, 0.186 mmol), RAFT agent CPADB (2.79 mg, 10 μmol), and initiator AIBN (0.55 mg, 3.35 μmol) were dissolved in 3-mL dimethylacetamide (DMAc). The flask was sealed and purged with argon for 1 h. The flask was then immersed in a preheated oil bath at 70 °C. The polymerization was quenched after 7.5 h by immersing the flask in liquid nitrogen. After thawing, the polymer solution was analyzed by $^1$H NMR spectroscopy in DMSO-$d_6$ to determine the monomer conversion. The polymerization solution was precipitated in diethyl ether (3×). The yielding polymer (pink powder) was dried in a vacuum oven for 24 h. The polymer was characterized by $^1$H NMR spectroscopy and SEC. The number average molecular weight and polydispersity index were 33 000 g $mol^{-1}$ and 1.26, respectively. $^1$H NMR (300 MHz, DMSO-$d_6$, δ):

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8.46 (bd, 1H, aromatic proton ortho-N), 7.73–7.90 (m, 2H, aromatic proton meta-N and para-N), 7.24 (m, 1H, aromatic proton, ortho-disulfide linkage), 7.16 (m, 1H, amide proton), 4.66 (bd, 1H, −OH), 4.12 (bd, 2H, $-OCH_2CH_2-SS-$), 3.65 (bd, 1H, $HO-CH(CH_3)-$), 3.07 (bd, 2H, $-OCH_2CH_2-SS-$), 2.89 (bd, 2H, $-NH-CH_2-CH=$), 1.09 (bd, 3H, $CH_3-CH(-OH)-$), 0.8–2.0 (bd, 10 H, methyl and methylene protons of backbone). The HPMA/PDSM mole ratio in the resultant copolymer was 87:13, as determined by NMR spectroscopy.

*Reaction of $PMA_{SH}$ and crosslinkers in solution:* $PMA_{SH}$ with 12 mol% thiol groups (1.5 mg) was dissolved in PBS (0.1 M, pH 6.5) at 50 g $L^{-1}$ concentration. To this solution sodium borohydride was added to a 1 M final concentration and the reaction mixture was incubated for 1 h. The solution was then neutralized with an equal amount of hydrochloric acid and divided into six samples. To each sample was added a total volume of 450 μL of respective buffers and 5 μL of crosslinkers (10 g $L^{-1}$). The mixtures were left to react for 1 h and analyzed using a UV–Vis spectrophotometer.

*Reaction of $PMA_{SH}$ and crosslinkers on solid silica particles:* In a typical experiment, 50 μL of a 5 wt% suspension of 3 μm $SiO_2^+$ particles were washed (3×) and dispersed into 25 μL of buffer. To this suspension an equal volume of fluorescently labeled crosslinker (2 g $L^{-1}$, 15 min) was added. The particles were then washed (3×), dispersed in 25 μL of buffer, and subsequently allowed to interact with an equal volume of fluorescently labeled $PMA_{SH}$ (2 g $L^{-1}$, 30 min). The fluorescence intensity of the particles was analyzed via flow cytometry.

*Reaction of $PMA_{SH}$ and crosslinkers on porous $SiO_2^+$ particles:* In a typical experiment, 40 μL of 3-μm porous particles were washed (3×) and dispersed into 20 μL of NaOAc buffer (20 mM, pH 4). To this suspension an equal volume of Alexa Fluor 633-labeled $PMA_{SH}$ (2 g $L^{-1}$, 20 min) was added. The particles were then washed (3×) and dispersed in 20 μL of NaOAc buffer. Subsequently, an equal volume of Alexa Fluor 488-labeled $PMA_{ER}$ (2 g $L^{-1}$, 20 min) was added to the solution. The fluorescence intensity of the particles was analyzed via flow cytometry.

*Assembly of $PMA_{SH}$/PVPON capsules:* Stock solution of PVPON (100 g $L^{-1}$) was prepared in MilliQ water. A stock solution of $PMA_{SH}$ (50 g $L^{-1}$) was prepared using 0.5 M DTT in MOPS buffer (20 mM, pH 8) and incubated for 15 min prior to the assembly of the multilayer films to convert mixed disulfides into thiol groups. In a typical experiment, 250 μL of a 5 wt% suspension of 1 μm $SiO_2^+$ particles were washed (3×) and dispersed into 125 μL of NaOAc buffer. To this suspension an equal volume of $PMA_{SH}$ (2 g $L^{-1}$, 15 min) was added and the adsorption was allowed to proceed with constant shaking. The particles were then washed via centrifugation/redispersion cycles (3×) using fresh buffer and finally dispersed in 125 μL of NaOAc buffer. The outlined procedure is for the assembly of a single layer. This process was repeated until the desired number of layers was assembled. After completion of the multilayer assembly, the particles were crosslinked with the different polymer crosslinkers before or after removal of the template core in aqueous HF. *Caution! Hydrofluoric acid and ammonium fluoride are highly toxic. Extreme care should be taken when handling HF solution and only small quantities should be prepared*. The obtained capsules were washed via multiple centrifugation/NaOAc buffer washing cycles and stored as a suspension in NaOAc buffer. Control samples were assembled under the same conditions and crosslinked with CaT (2.5 mM in MES buffer, 20 mM, pH 6) for 1 min, followed by two washing cycles with MES buffer solution (10 mM, pH 6) and one wash with NaOAc buffer prior to core removal.

*Assembly of capsosomes:* Polymer capsules with incorporated liposomes were assembled via the LbL technique. A 5 wt% suspension of 3-μm $SiO_2$ particles were washed (3×) in HEPES buffer. The particles were suspended in a solution of $PLL_c$ (1 g $L^{-1}$ in HEPES buffer, 15 min) and washed (3×). They were then allowed to interact with DMPC/DPPC liposomes (1.25 g $L^{-1}$ in HEPES buffer, 60 min) and washed (3×), followed by the adsorption of a $PMA_c$ capping layer (1 g $L^{-1}$ in HEPES buffer, 15 min) and washed again (3×). The buffer was changed to NaOAc and five bilayers of alternating PVPON (1 g $L^{-1}$, 10 min) and $PMA_{SH}$ (1 g $L^{-1}$, 10 min) were sequentially adsorbed. The core–shell particles were then crosslinked with the different polymer crosslinkers before or after the removal of the template core. Capsosomes were formed by dissolving the silica core using a 2 M HF/8 M $NH_4F$ solution for 2 min, followed by multiple centrifugation/buffer washing cycles.

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