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Capsosomes with Multilayered Subcompartments: Assembly and Loading with Hydrophobic Cargo**

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Abstract:

Therapeutic artificial cells or organelles are nanoengineered vehicles that are expected to substitute for missing or lost cellular function. The creation of capsosomes, polymer carrier capsules containing liposomal subcompartments, is a promising approach towards constructing such therapeutic devices using the layer-by-layer assembly method. Herein we report on the assembly of intact, non-agglomerated capsosomes containing multiple liposome layers. We further demonstrate that Thiocoraline, a hydrophobic model peptide with antitumor activity, can be efficiently loaded into the membrane of the liposomal subcompartments of the capsosomes. Cell viability assays verify the activity of the trapped antitumor cargo. We also show that pristine capsosomes do not display inherent cytotoxic effects. The ability to tune the number of liposome layers and hence the drug loading in capsosomes as well as their non-cytotoxicity provide new opportunities for the creation of therapeutic artificial cells and organelles.

1. Introduction

Addressing challenges in medical care requires access to smart, multifunctional nanoengineered biomedical platforms. Artificial cells^[1] and organelles are an approach which involves the retrieval of missing or lost cellular function and by doing so, is expected to provide a convenient (long-term) therapeutic solution for chronic diseases. Designing these high-end therapeutic vehicles holds many challenges, including the requirement for a structurally stable semipermeable scaffold which entraps the machinery where the (enzymatic) reactions can be performed. Additionally, selective access from (bio)molecules to the entrapped enzymes and their subsequent release are key prerequisites in order to allow for a specific therapeutic effect to occur.

Liposomes^[2-4] and polymer capsules^[5,6] have proven to be successful as simple carriers for a large variety of biomolecules^[7-10] and drugs,^[11,12] and they also have the potential to accommodate triggered encapsulated reactions.^[7,13-18] To establish a multifunctional advanced therapeutic platform, we recently introduced capsosomes, polymer carrier capsules containing liposomes.^[19,20] This combination is a promising new approach which exploits the benefits of both systems while improving the flexibility in their design and application. We reported on capsosomes containing different types of liposomes embedded within either nondegradable^[19] or (bio)degradable polymer carrier capsules.^[20] We also introduced a novel anchoring concept to non-covalently adsorb liposomes onto polymer films by using cholesterol-modified polymers.^[20] The capsosome functionality was verified by encapsulating the enzyme β -lactamase into the liposomal subcompartments and triggering the enzymatic reaction by disintegrating the liposomes.^[20] By doing so, we confirmed capsosomes as a promising approach towards the creation of artificial cells and organelles. However, in order to substitute for cellular function, the reactions need to occur repetitively in a

cascade manner and thus require the encapsulation of subcompartments containing different enzymes. Additionally, not only the entrapped molecules, but also the activity and selectivity of moieties associated with the lipid cell membrane are involved in the functionality of biological cells, i.e., specific gates between the cell interior and exterior. Mimicking cellular activity will require the implementation of selective functionality into the membrane of the subcompartments.

With the goal to implement additional functionality into capsosomes to develop an active artificial cell, herein we report on (i) the encapsulation of multiple layers of liposomes separated by polymer layers within a (bio)degradable polymer membrane, (ii) the loading and quantification of a small hydrophobic cargo molecule within the lipid membrane of the liposomes in the capsosomes, (iii) the preservation of cargo functionality, and (iv) the absence of intrinsic capsosome cytotoxicity by cell viability assays. Control over the number and position of the liposomal subcompartments within the capsosomes is essential for the co-encapsulation of different cargo within the same capsosome for conducting enzymatic cascade reactions. Additionally, the incorporation of a small hydrophobic model peptide into the membrane of the liposomes is a first step in equipping capsosomes with functionality in the membrane of the subcompartments. The peptide is expected to give a first insight into the possibility to incorporate transmembrane proteins that have the ability to act as specific gates. Thiocoraline, an antitumor cyclic thiodipeptide isolated from the mycelium of a marine actinomycete (*Micromonospora* sp. L-13-ACM2-092),^[21] was chosen as the hydrophobic model cargo. It has demonstrated approximately equal cytotoxic activity at nanomolar concentrations on a panel of 12 human cancer cell lines by unwinding negatively supercoiled double-stranded DNA and binding to DNA by bisintercalation.^[22] The cytotoxic activity can confirm both the presence and functionality of the cargo.

Moreover, since Thiocoraline shows emission at 547 nm (in chloroform) this molecule acts as a convenient indicator of the amount of loaded hydrophobic cargo within the capsosomes. Finally, the absence of inherent cytotoxicity of capsosomes is a crucial attribute when using these vehicles in biomedical applications, including as artificial cells and organelles.

Capsosome formation is based on the layer-by-layer (LbL) assembly technique^[23-26] using poly(L-lysine) (PLL) as a precursor layer before the immobilization of either zwitterionic DOPC (L^{zw}) or negatively charged DOPC/DOPS liposomes (L^-). In contrast to previously reported LbL multilayers of cationic and anionic organic-inorganic hybrid liposomes (cerasomes),^[27,28] we assemble charged and zwitterionic liposomes, and thus require a polymer separation layer (either PLL, poly(methacrylic acid)-co-(cholesteryl methacrylate) (PMA_c) or both polymers) prior to the second liposome adsorption step. This assembly was capped with a layer of PMA_c and the membrane of the carrier capsule consisting of poly(*N*-vinyl pyrrolidone) (PVP) and thiol-modified poly(methacrylic acid) (PMA_{SH}) was built up via hydrogen bonding (Scheme 1). These two polymers were chosen as building blocks for the carrier capsule because disulfide-stabilized PMA capsules^[29-31] are non-toxic,^[32] (bio)degradable^[33] and have been successfully applied to encapsulate genes,^[8,34] peptides,^[31] a monolayer of intact enzyme-loaded liposomes,^[20, 35] and drug-loaded oil droplets.^[12] When the thiols in the polymer film are cross-linked and the silica template is removed, capsosomes containing multiple layers of liposomes are obtained. Together with our previous findings (i.e., the encapsulation of hydrophilic cargo) this study further progresses the establishment of capsosomes as a multifunctional vehicle towards the creation of novel therapeutic artificial cells and organelles.

2. Results and Discussion

To achieve a high level of liposome loading within the polymer films, we examined film assemblies that enabled the deposition of multiple liposome layers on planar and colloidal substrates. The focus was to identify suitable polymer separation layer(s) between the liposome layers. The optimized capsosome assemblies were chosen for the encapsulation and quantification of the hydrophobic compound Thiocoraline within the lipid membrane of the liposomes. The activity of the hydrophobic cargo of these capsosomes, which showed the highest Thiocoraline loading and negligible cytotoxicity of the pristine capsosomes, was then confirmed by an MTT cell viability assay using a colorectal cancer-derived cell line, LIM1899.

2.1. Adsorption of Multiple Liposome Layers on Planar Substrates

A high concentration of hydrophobic or hydrophilic cargo in the capsosomes requires the efficient loading of liposomes into polymer films. To increase the loading capacity of capsosomes in a defined manner, we identified the polymer layers required between the first and second liposome adsorption steps on planar substrates using Dual Polarization Interferometry (DPI).

The use of PLL as a precursor layer has previously been shown to facilitate the adsorption of unsaturated zwitterionic DOPC liposomes (L^{zw}) or unsaturated negatively charged DOPC/DOPS liposomes (L^-).^[20] DPI confirms that both types of liposomes adsorb with a similar thickness onto PLL ($L^{zw} = 31 \pm 8$ nm, $L^- = 41 \pm 6$ nm), forming the first layer of liposomes (Figure 1a).

A polymer separation layer is required to enable the adsorption of additional liposomes. Three different types of polymer separation layers, namely PLL, PMA_c or both PMA_c/PLL (details in Table S1, Supporting Information), were chosen since we

previously found that these polymers are suitable to stably incorporate liposomes into polymer films.^[20] Figures 1b and 1c show the thickness increase of assemblies consisting of PLL/L^{zw} and PLL/L⁻, respectively, after the second incubation with liposomes for each of the three different separation layers. An increase in thickness of ~32 nm or ~40 nm for L^{zw} and L⁻, respectively, indicates the addition of a similar amount of liposomes to the films as the first liposome layer. Depending on whether the first layer was L^{zw} or L⁻, different separation layers favored the adsorption of additional liposomes. When L^{zw} was used in the first layer, only PMA_c facilitated the additional adsorption of L^{zw} (gray bars, Figure 1b), while both PMA_c and PMA_c/PLL allowed for the immobilization of L⁻ (dark gray bars, Figure 1b), indicating that the electrostatic interaction between PLL and the liposomes alone was not sufficient, but cholesterol as a non-covalent anchor was required to achieve a high liposome loading. On the other hand, a film assembly starting with (PLL/L⁻) was found to be insensitive to the type of separation layer and allowed the adsorption of additional L⁻ in all three cases (dark gray bars, Figure 1c) by electrostatic interaction and/or cholesterol anchoring. In addition, adsorbing PMA_c to a (PLL/L⁻) assembly facilitated the addition of L^{zw} (gray bars, Figure 1c). On the whole, we identified seven assembly combinations (Table S2, Supporting Information) that enabled the controlled, serial immobilization of liposomes on planar substrates.

Apart from the precursor and separation layers, the choice of the polymer capping layer is crucial since it should efficiently anchor the liposomes to the polymer film and enable the subsequent assembly of polymer multilayers while avoiding liposome rupturing or displacement. We previously demonstrated that PMA_c is well suited to be used as a capping layer.^[20] PMA_c not only allowed the anchoring of the liposomes via cholesterol, it also facilitated the subsequent polymer membrane

assembly of PVP and PMA_{SH} via hydrogen bonding. In order to assemble the polymer membrane of the carrier capsule, we sequentially adsorbed PVP and PMA_{SH}, as depicted in Figure 2 and Figure S1 (Supporting Information). We observed a similar linear build up of PVP and PMA_{SH} on an assembly of PLL/L^{zw}/PMA_c/PLL/L⁻/PMA_c (····■····) and on bare silica (····●····), indicating that the presence of the liposomes does not interfere with the polymer multilayer film assembly.

2.2. Adsorption of Multiple Liposome Layers onto Colloidal Supports

The formation of capsosomes requires the assembly of liposomes and polymers on colloidal supports followed by removal of the template particles. With the goal to correlate the findings on planar and colloidal substrates, we assembled the same 12 combinations previously used on planar substrates onto colloids. The fluorescence intensity of the colloids due to the adsorbed (fluorescent) liposomes was monitored by flow cytometry. The fluorescence reading monitored after the adsorption of the first layer of liposomes was set to 100%. Figure 3 shows the measured increase in fluorescence intensity due to the adsorption of liposomes after the second liposome deposition step for different PLL/liposome/(separation layer) assemblies. The polymer/liposome combinations which favored the adsorption of liposomes on colloidal and planar substrates were largely similar. However, there were also some differences, as often observed when assemblies are transferred from planar to colloidal substrates. In particular, independent of the first layer of liposomes, a PMA_c/PLL separation layer enabled the additional immobilization of L^{zw} on colloidal substrates, a feature not observed on planar substrates. Also, PLL as a separation layer did not facilitate the adsorption of liposomes during the second deposition step on colloidal supports. In contrast to planar supports, PMA_c was found to be a less successful separation layer on

colloids when L^- was used in both adsorption steps. Otherwise, the same assemblies allowed for the multiple adsorption of liposome layers, confirming the highest liposome loadings for PLL/ L^{zw} /PMA_c/PLL/ L^- (Figures 1b and 3a), PLL/ L^- /PMA_c/PLL/(L^{zw} or L^-) and PLL/ L^- /PMA_c/ L^{zw} (Figures 1c and 3b). In summary, seven out of the twelve polymer/liposome combinations were found to facilitate high liposome loadings on planar surfaces, and eight out of twelve on colloidal substrates (Table S2, Supporting Information). Although the details for differences in assembly behavior on planar and colloidal supports are not currently clear, we chose those six combinations that enabled the adsorption of multiple liposome layers on both substrates for all further experiments. These six assemblies included all four combinations using PMA_c as a separation layer in addition to PLL/(L^{zw} or L^-)/PMA_c/PLL/ L^- . Again, on colloidal as well as on planar surfaces, the use of the cholesterol-modified polymers in the separation layers is essential to allow the deposition of multiple liposome layers.

To obtain structurally stable, intact, non-agglomerated capsosomes with a high liposome loading, the polymer/liposome film was capped with 5 bilayers of PMA_{SH}/PVP. The thin films were subsequently cross-linked (converting the thiols to disulfides) and the silica cores were removed to yield capsosomes. The obtained capsosomes were characterized under physiological conditions (HEPES buffer) using optical and confocal microscopy (Figure 4). Differential interference contrast (DIC) images demonstrated that the capsosomes were intact and non-agglomerated, and preserved the spherical shape of the template silica particles (Figure 4a). Upon increasing the pH from 4 to 7.4, the capsosomes swelled by about 20% (Figure S2, Supporting Information). A negatively stained (PLL/ L^- /PMA_c/PLL/ L^- /PMA_c)-capsosome imaged by TEM revealed a homogeneous, intact polymer membrane without any large pores (Figure 4b). A comparison of the fluorescence intensity of

(PLL/L^{zw}_{NBD}/PMA_c)-capsosomes and (PLL/L^{zw}_{NBD}/PMA_c/PLL/L⁻_{NBD}/PMA_c)-

capsosomes by fluorescence microscopy indicates a higher liposome loading of the latter carrier capsules (Figures 4c and 4d). Confocal laser scanning microscopy (CLSM) images of (PLL/L⁻_{NBD}/PMA_c/PLL/L⁻_{NBD}/PMA_c)-capsosomes using NBD-labeled liposomes (Figure 4e) demonstrated that the liposomes were homogenously distributed along the polymer membrane (shown by the regular green fluorescence). CLSM also showed that the AF-633-labeled disulfide-stabilized PMA film homogenously coated the liposomes, and that stable carrier capsules are formed, as demonstrated by the regular red fluorescence associated with the capsosomes (Figure 4f).

We incorporated a small hydrophobic cytotoxic model molecule, Thiocoraline, into the lipid membrane of the liposomes. The amount of Thiocoraline incorporated into the core-shell particles was determined by fluorescence spectrophotometry after the first and second liposome adsorption steps using the six different assembly approaches (all four combinations using PMA_c as a separation layer and PLL/(L^{zw} or L⁻)/PMA_c/PLL/L⁻) that yielded the adsorption of multiple liposome layers on planar substrates and colloids) (Figure 5). When Thiocoraline-loaded liposomes were used, only two assemblies, namely those containing PMA_c/PLL as a separation layer, allowed for the additional adsorption of liposomes during the second deposition step, as demonstrated by the measured increase in fluorescence from entrapped Thiocoraline. We assume that the incorporation of Thiocoraline into the lipid membrane is likely to have changed the lipid membrane morphology, and thereby affected the adsorption behavior of the liposomes. The lipid membrane would potentially become less flexible and the integration of the cholesterol molecules may have been hindered, making PMA_c only unsuitable as separation layer. Importantly, subsequent polymer multilayer assembly

and silica core removal using HF did not affect the amount of encapsulated Thiocoraline (results not shown).

Based on our findings on planar and colloidal substrates, the two capsosome assemblies that yield the highest liposome loading, namely (PLL/L⁻/PMA_c/PLL/L⁻/PMA_c) and (PLL/L^{zw}/PMA_c/PLL/L⁻/PMA_c), were used for all further studies.

2.3 Functionality of Thiocoraline-Loaded Capsosomes

The functionality of the small hydrophobic anti-cancer cargo incorporated within the capsosomes was demonstrated by monitoring the cytotoxicity of Thiocoraline-loaded capsosomes on LIM1899 colon cancer cells using MTT assays. Figure 6 shows the results of the normalized cell viability assays performed with Thiocoraline-loaded capsosomes (**3**: (PLL/L^{zw/TC}/PMA_c/PLL/L^{-TC}/PMA_c)-capsosomes and **5**: (PLL/L^{-TC}/PMA_c/PLL/L^{-TC}/PMA_c)-capsosomes), the corresponding empty capsosomes (**2**: (PLL/L^{zw}/PMA_c/PLL/L⁻/PMA_c)-capsosomes and **4**: (PLL/L⁻/PMA_c/PLL/L⁻/PMA_c)-capsosomes) and capsules with free Thiocoraline (**6**: (PLL/TC/PMA_c/PLL/TC/PMA_c)-capsosomes). As shown in Figure 6, the Thiocoraline-loaded capsosomes (**3**: 34% reduction in viability and **5**: 43% reduction in viability) had a significant effect on cell viability. Importantly, the pristine capsosomes themselves did not exhibit cytotoxic properties, making them well-suited for biomedical applications. The requirement of liposomes for the successful encapsulation of Thiocoraline was proven by the absence of the Thiocoraline peak in the fluorescence spectrophotometry analysis when free Thiocoraline was directly adsorbed onto the polymer films (results not shown) and the fact that such capsules did not affect cell viability (**6** in Figure 6). CLSM images suggest the uptake of the empty fluorescently labeled capsosomes by the cancer cells (Figure S3, Supporting Information).

3. Conclusions

We have reported the incorporation of Thiocoraline-loaded liposomes into a (bio)degradable polymer carrier capsule. Depending on the properties of the liposomes, (e.g., charge or fluidity of the membrane), different polymer separation layers favored additional liposome adsorption. Cholesterol-modified polymers are key building blocks for creating capsosomes efficiently loaded with liposomes. The highest Thiocoraline loading was achieved when PLL/PMA_c were used as separation layers between L^{zw/TC} or L^{-TC} and L^{-TC}. Cell studies using colon cancer cells confirmed the functionality of such capsosomes via the cytotoxic activity of the incorporated peptide Thiocoraline. Importantly, the pristine capsosomes did not affect cell viability. Taken together, our studies demonstrate that capsosomes provide a powerful nanoengineered biomedical platform for the encapsulation of hydrophobic and hydrophilic^[20] moieties within liposomal subcompartments. Although the assembly of functional artificial cells and organelles is the primary goal, our results highlight the potential of capsosomes as drug delivery vehicles for small hydrophobic drugs.

Experimental

Materials: Poly(L-lysine) (PLL, 40-60 kDa), poly(*N*-vinyl pyrrolidone) (PVP, 10 kDa), 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid (HEPES), sodium chloride (NaCl), sodium acetate (NaOAc), 2-(*N*-morpholino)ethanesulfonic acid (MES), 4-nitrophenol chloroformate (NPC, 95%), triethylamine (99%), chloramine T, hydrofluoric acid (HF) and MTT media (0.5 mg mL⁻¹) were obtained from Sigma-Aldrich. Silica particles (3.25 μm) were obtained from Microparticles GmbH, Germany. Micro-90[®] was obtained from Cole-Parmer, USA. Poly(methacrylic acid) (PMA, 15 kDa) was obtained from Polysciences (USA). RPMI 1640 media (with 10% fetal bovine serum, 10.8 μg mL⁻¹ α-

thioglycerol, 0.025 U mL insulin, 1 $\mu\text{g mL}^{-1}$ hydrocortisone, 60 $\mu\text{g mL}^{-1}$ penicillin, and 12.6 $\mu\text{g mL}^{-1}$ streptomycin) was obtained from Invitrogen. Chloroform was obtained from Chem Supply. Alexa Fluor 633 maleimide was obtained from Molecular Probes. Zwitterionic lipids, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, phase transition temperature -4 °C), negatively charged lipids, 1,2- dioleoyl-*sn*-glycero-3-(phospho-L-serine) (DOPS, phase transition temperature -4 °C), and fluorescent lipids 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycero-3 phosphocholine (NBD-PC) were obtained from Avanti Polar Lipids, USA. Thiocoraline was isolated and purified by PharmaMar, S.A. (Colmenar Viejo, Madrid, Spain), as described previously.^[21]

Three types of buffers were used throughout all of the experiments: HEPES buffer consisting of 10 mM HEPES and 150 mM NaCl (pH 7.4), NaOAc buffer consisting of 20 mM NaOAc (pH 4) and MES buffer consisting of 50 mM MES buffer (pH 6). The buffer solutions were made with ultrapure water (Milli-Q gradient A 10 system, resistance 18 M Ω cm, TOC < 4 ppb, Millipore Corporation, USA).

Unilamellar liposomes were prepared by the evaporation of the chloroform (2.5 mg lipids dissolved in chloroform) under nitrogen for 1 h followed by hydration into 1 mL HEPES buffer, and extrusion through 50 nm filters (31 times). For fluorescently labeled and Thiocoraline-loaded liposomes, 1% (w/w) of NBD-PC or 0.1 mg Thiocoraline dissolved in chloroform was added to the lipid solution, respectively.

Thiol-modified poly(methacrylic acid) (PMA_{SH}, 15 kDa),^[30,34] its fluorescently labeled counterpart (PMA_{SH}-AF-633),^[33] and poly(methacrylic acid)-*co*-(cholesteryl methacrylate) (PMA_c, 12.4 kDa)^[20] were synthesized according to previously published protocols.

Dual Polarization Interferometry (DPI): Optimization of the polymer/liposome assembly was conducted using an Analight BIO200 dual polarization interferometer (Farfield Scientific Ltd., U.K.). DPI is an evanescent field-based optical technique that uses changes in interference patterns (birefringes) from a reference and sensing beam to independently measure thickness and refractive index of layers adsorbed to the chip. A more detailed description of the instrument can be found elsewhere.^[36,37] All experiments were carried out at $T = 24 \pm 0.002$ °C and unmodified silicon oxinitride chips (FB80 DPI, Farfield) were used. The chips were cleaned by soaking them in Piranha solution (70:30 v/v sulphuric acid/ hydrogen peroxide, *Caution! Piranha solution is highly corrosive. Extreme care should be taken when handling Piranha solution and only small quantities should be prepared.*) for 48 h, extensively rinsing with Milli-Q water and drying before loading into the flow chamber of the instrument. The recyclable Kalrez gasket was sonicated in a 1% Micro-90 cleaning solution for 10 min, rinsed with copious amounts of water, and followed by a final rinse with isopropanol and drying. The gaskets were used a maximum of 10 times before disposal. After obtaining a stable water baseline, the chips were calibrated with respect to their optical properties using an 80:20 w/w ethanol/water mixture according to the manufacture protocol. The sensing waveguide thickness and refractive index was calculated using the known refractive index change from 80% w/w ethanol to water. To enable maximum measurement accuracy, a chip linearization was carried out in order to reduce any effects from inhomogeneities of the chip surface. Then the running buffer was switched to HEPES buffer for the experiment and its refractive index was calculated. PLL (1 mg mL^{-1} HEPES, 15 min incubation) was used as precursor layer for all experiments. The liposomes were adsorbed by passing a solution (1.25 mg mL^{-1} HEPES) over the chip for 1 h. The film assembly was continued by adding the different

polymer separation layers (1 mg mL^{-1} , 15 min incubation). Subsequently, the second layer of liposomes (1.25 mg mL^{-1} , 1 h incubation) was adsorbed. Finally, the assembly was capped with a layer of PMA_c (1 mg mL^{-1} , 15 min incubation). After changing from HEPES to NaOAc buffer, the polymer layering was continued by alternately depositing PVP and PMA_{SH} (1 mg mL^{-1} , 10 min incubation) until a total of 5 bilayers were deposited. The chip was rinsed with the corresponding buffer for 10 min between each adsorption step.

The data were analyzed using the AnaLight Explorer software (Farfield Scientific) applying $dn/dc \approx 0.139 \text{ cm}^3 \text{ g}^{-1}$ for the polymer layers and $dn/dc \approx 0.135 \text{ cm}^3 \text{ g}^{-1}$ ^[38] for the liposome layers. Mass and density values were calculated to obtain an approximate value for comparison between the different systems under investigation.

Capsosome Formation: Capsosomes were assembled via the layer-by-layer (LbL) technique. Silica particles (5 wt %) were washed three times in HEPES buffer (1060 g, 30 s). The particles were suspended in a PLL solution (1 mg mL^{-1} , 15 min) washed three times, followed by the adsorption of the first layer of liposomes (1.25 mg mL^{-1} , 60 min), washed 3 times in HEPES buffer (1060 g, 30 s), and then the separation layer(s) were adsorbed (1 mg mL^{-1} , 15 min). After three washes in HEPES buffer (1060 g, 30 s), the second layer of liposomes was deposited (1.25 mg mL^{-1} , 60 min), washed 3 times in HEPES buffer (1060 g for 30 s) and subsequently the PMA_c capping layer was adsorbed (1 mg mL^{-1} , 15 min). The particles were then washed twice in HEPES buffer (1060 g for 30 s) and once in NaOAc buffer. The polymer membrane was assembled by the sequential deposition of five bilayers of PVP and PMA_{SH} (1 mg mL^{-1} , 10 min). The thiols within the polymer layers were cross-linked using the oxidizing agent chloramine T (2.5 mM in MES buffer (50 mM, pH 6), 1 min). The silica core was dissolved using a

2 M hydrofluoric acid solution for 2 min, followed by several washing cycles in NaOAc buffer (4500 g, 3 min). The PVP was released from the capsosomes by washing them into HEPES buffer, which resulted in liposome-loaded PMA hydrogel capsules stabilized via disulfide linkages.

PMA capsules with free Thiocoraline were assembled in a similar way as the regular capsosomes, but a solution containing only Thiocoraline (dried and rehydrated mimicking the liposomes formation process) was used.

Flow Cytometry: A Becton Dickson FACS Calibur flow cytometer using an excitation wavelength of 488 nm was used for all of the flow cytometry experiments. At least 20 000 particles were analyzed in each experiment.

Differential Interference Contrast (DIC) and Fluorescence Microscopy: Differential interference contrast (DIC) and fluorescence images were taken on an inverted Olympus IX71 microscope equipped with a DIC slider (U-DICT, Olympus), the corresponding filter sets and a 60 × oil immersion objective (Olympus UPFL20/0.5NA, W.D. 1.6). Confocal Laser Scanning Microscopy (CLSM) images were taken on a Leica TCS SP2 SE (Leica Microsystems Pty Ltd, Australia) equipped with an argon laser ($\lambda = 488$ nm), a He-Ne laser ($\lambda = 633$ nm) and a 63 × oil immersion objective.

Transmission Electron Microscopy (TEM): All TEM grids were plasma treated for 10 s prior to use. 5 μ L of sample was adsorbed onto a carbon-coated Formvar film mounted on 300 mesh copper grids (ProSciTech, Australia) for 3 min. The grids were blotted and dipped into a 1.5% aqueous uranyl acetate solution for 15 s for negative staining. Investigations were undertaken using an FEI Company Tecnai TF30 (FEI-Company,

Eindhoven, The Netherlands) operated at 200 kV and fitted with a Gatan US1000

2k×2k CCD camera (Pleasanton, Ca, USA).

Fluorescence Spectrophotometry: Thiocoraline was quantified by fluorescence measurements using an excitation wavelength of 365 nm and an emission wavelength of 547 nm. The Thiocoraline entrapped within a known number of capsosomes (determined by flow cytometry) was extracted with chloroform prior to the assay. The concentration of Thiocoraline was determined by correlation with a calibration curve (Figure S4, Supporting Information). The experiments were carried out using a Fluorolog-3 Model FL3-22 spectrofluorometer (Jobin Yvon Inc., USA) equipped with a HgXe lamp.

MTT Assay: Cell proliferation was measured by the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), which corresponds to viable cell number and metabolic activity.^[39] Human colon cancer cells, LIM1899 cells,^[40] were seeded into 96-well plates at a density of 1×10^4 cells/well and allowed to attach overnight at 37 °C, 5% CO₂ and 95% relative humidity in complete RPMI 1640 media supplied with 10% fetal bovine serum, 10.8 $\mu\text{g mL}^{-1}$ α -thioglycerol, 0.025 U mL insulin, 1 $\mu\text{g mL}^{-1}$ hydrocortisone, 60 $\mu\text{g mL}^{-1}$ penicillin, and 12.6 $\mu\text{g mL}^{-1}$ streptomycin. The cells were then exposed to different types of capsosomes and incubated for 48 h. After incubation, the media was removed and the cells were incubated with 200 μL fresh media containing MTT (0.5 mg mL^{-1}) for 2 h. The resulting blue formazan was solubilized in 150 μL of acidified isopropanol (0.04 M HCl) and the absorbance was measured at 560 nm using a plate reader (Multiskan Ascent, Thermo Scientific). The values of the untreated cells were taken as 100%, and

the cell viability was expressed as a percentage of the untreated cells. The data were obtained from four independent experiments performed in triplicate and the results were statistically analyzed (one-way ANOVA coupled to Bonferroni multiple comparison test).

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Figure 1. Liposome incorporation into polymer layers characterized by DPI. **(a)**

Increase in thickness after the adsorption of negatively charged liposome (L^-) and unsaturated zwitterionic liposomes (L^{zw}) to a PLL precursor layer. Increase in thickness upon liposome deposition to a (PLL/L^{zw}) assembly **(b)** or a (PLL/L^-) assembly **(c)** using three different polymer separation layers (PLL, PMA_c/PLL or PMA_c) between the

liposome layers. The red line “monolayer” refers to the same increase in thickness as measured after the first liposome deposition step.

Figure 2. Sequential assembly of PVP and PMA_{SH} to a PLL/L^{zw}/(PLL/PMA_c)/L⁻/PMA_c assembly (··· ■ ···) and a bare chip (··· ● ···), as monitored by DPI.

Figure 3. Liposome adsorption onto colloidal supports. Normalized fluorescence intensity measured after the second liposome deposition step onto silica particles pre-coated with PLL/ L^{zw} (a) or PLL/ L^- (b) using different separation layers (PLL, PMA_c/PLL and PMA_c). The red line “monolayer” refers to the fluorescence intensity measured after the first liposome deposition step.

Figure 4. Structural integrity of (PLL/L⁻/PMA_c/PLL/L⁻)-capsosomes under physiological conditions (HEPES buffer): **(a)** DIC and **(b)** TEM images. Fluorescence microscopy images of capsosomes assembled using one **(c, PLL/L^{zw}_{NBD}/PMA_c)** or two **(d, PLL/L^{zw}_{NBD}/PMA_c/PLL/L^{zw}_{NBD})** liposome deposition steps. CLSM images of (PLL/L⁻/PMA_c/PLL/L⁻)-capsosomes with NBD-labeled liposomes **(e)** loaded within an AF-633-labeled disulfide-stabilized PMA carrier capsule **(f)**.

Figure 5. Thiocoraline (TC) entrapment within capsosomes: **(a)** The amounts of TC per particle after the first (light gray bars) and second (dark gray bars) liposome adsorption steps are shown. Only two assembly combinations, namely those with both PMA_c and PLL as separation layers (1 and 2), exhibit an increase in Thiocoraline content upon the second liposome deposition step. **(b)** **(i)** Fluorescence microscopy and **(ii)** DIC image of (PLL/L^{-TC}/PMA_c/PLL/L^{-TC})-capsosomes.

Figure 6. Cell viability assay of LIM1899 colon cancer cells after 48 h, measured by MTT assays: untreated (**1**), and after incubation with different capsosomes which contain empty liposomes (**2**, **4**), liposomes with TC (**3**, **5**), or free TC (**6**). The results are expressed as the percentage compared to untreated cells, and represent the mean $\pm$ standard deviation of four independent experiments. (*) $P < 0.001$.

Capsosomes, polymer capsules containing liposomes, were engineered to contain multiple liposome layers, thus providing a means to tune the liposome loading. Drug-loaded capsosomes were formed by entrapping the antitumor hydrophobic peptide Thiocoraline within the lipid membrane of the liposomal subcompartments. The drug-loaded capsosomes showed anti-tumor activity, as verified by a cell viability assay using a cancer cell line.

Keywords: Capsosomes, Liposomes, Polymer multilayers, Hydrophobic Cargo

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Capsosomes with Multilayered Subcompartments: Assembly and Loading with Hydrophobic Cargo

Column Title: *L. Hosta-Rigau et al.*/ Drug-Loaded Multilayered Capsosomes

Supporting Information:

Capsosomes with Multilayered Subcompartments: Assembly and Loading with Hydrophobic Cargo**

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Table S1. Thickness increment, mass and density after the first (i) and second (ii)

liposome adsorption steps as a function of the polymer separation layer. (iii) The same increments for the separation layers themselves adsorbed onto (a) L^{zw} or (b) L^- .

i)

1 st liposome layer	Δ Thickness [nm]	Δ Mass [ng mm ⁻²]	Δ Density [g cm ⁻³]
L^{zw}	26.9	5.5	-0.2
L^-	53.2	14.3	-0.6

ii)

a)

1 st liposome layer: L^{zw} 2 nd liposome layer: L^{zw}			
Separation Layer	Δ Thickness [nm]	Δ Mass [ng mm ⁻²]	Δ Density [g cm ⁻³]
PLL	2.3	0.4	0.008
PMA _c / PLL	3.1	1.1	0.005
PMA _c	32.6	0.4	0.008

b)

1 st liposome layer: L^{zw} 2 nd liposome layer: L^-			
Separation Layer	Δ Thickness [nm]	Δ Mass [ng mm ⁻²]	Δ Density [g cm ⁻³]
PLL	22.7	8.2	0.05
PMA _c / PLL	70.5	23.7	0.03
PMA _c	37.8	8.2	0.05

c)

1 st liposome layer: L^- 2 nd liposome layer: L^{zw}			
Separation Layer	Δ Thickness [nm]	Δ Mass [ng mm ⁻²]	Δ Density [g cm ⁻³]
PLL	21.5	4.9	0.02
PMA _c / PLL	0.9	0.6	0.002
PMA _c	65.7	20.8	0.04

d)

1 st liposome layer: L^- 2 nd liposome layer: L^{zw}			
Separation Layer	Δ Thickness [nm]	Δ Mass [ng mm ⁻²]	Δ Density [g cm ⁻³]
PLL	63.6	17.2	0.01
PMA _c / PLL	65.5	24.8	0.003
PMA _c	70.2	14.7	0.02

iii)

a)

1 st liposome layer: L ⁻			
Separation Layer	Δ Thickness [nm]	Δ Mass [ng mm ⁻²]	Δ Density [g cm ⁻³]
PLL	-2.4	-0.5	-0.2
PLL onto PMA _c	-5.6	4.6	0.09
PMA _c	-7.8	2.3	0.7

b)

1 st liposome layer: L ^{zw}			
Separation Layer	Δ Thickness [nm]	Δ Mass [ng mm ⁻²]	Δ Density [g cm ⁻³]
PLL	0.7	0.4	0.004
PLL onto PMA _c	-5.9	8.1	0.1
PMA _c	17.8	-1.4	-0.08

Table S2. Ticks: Polymer/liposome combinations which showed additional liposome adsorption after the second liposome deposition step on planar **(a)** or colloidal **(b)** substrates. Crosses: Assemblies that did not show additional liposome adsorption during the second liposome adsorption step.

a) Planar Substrates

Separation	1 st	2 nd	
Layer	liposome	liposome	
	layer	layer	
PLL	L ^{zw}	L ^{zw}	✗
		L ⁻	✗
	L ⁻	L ^{zw}	✗
		L ⁻	✓
PMA _c / PLL	L ^{zw}	L ^{zw}	✗
		L ⁻	✓
	L ⁻	L ^{zw}	✗
		L ⁻	✓
PMA _c	L ^{zw}	L ^{zw}	✓
		L ⁻	✓
	L ⁻	L ^{zw}	✓
		L ⁻	✓

b) Colloidal Supports

Separation	1 st	2 nd	
Layer	liposome	liposome	
	layer	layer	
PLL	L ^{zw}	L ^{zw}	✗
		L ⁻	✗
	L ⁻	L ^{zw}	✗
		L ⁻	✗
PMA _c / PLL	L ^{zw}	L ^{zw}	✓
		L ⁻	✓
	L ⁻	L ^{zw}	✓
		L ⁻	✓
PMA _c	L ^{zw}	L ^{zw}	✓
		L ⁻	✓
	L ⁻	L ^{zw}	✓
		L ⁻	✓

Figure S1. Sequential assembly of PVP and PMA_{SH} onto a PLL/L^{zw}/(PLL/PMA_C)/L⁻/PMA_C assembly (---▲---) and a bare chip (---●---).

Figure S2. Swelling behavior of capsosomes (PLL/L^{zw}/PMA_c/PLL/L^{zw}/PMA_c) upon increasing the pH from 4 to 7.4.

Figure S3. Fluorescence arising from the internalization of (PLL/L^{ZW}/PMA_c/PLL/L⁻)-capsosomes by LIM1899 colon cancer cells after 48 h incubation. **(a)** NBD-labeled liposomes, **(b)** AF-633-labeled PMA_{SH} carrier capsule, and **(c)** the green, red and DIC merged signals.

Figure S4. Fluorescence calibration curve for Thiocoraline. Different concentrations of Thiocoraline were excited at a wavelength of 365 nm and the fluorescence intensity was recorded at an emission wavelength of 547 nm. The measurements were taken in chloroform.

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