Degradable, Surfactant-free, Monodisperse Polymer-Encapsulated Emulsions as Anti-Cancer Drug Carriers**

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Keywords: Emulsions, Drug delivery, Polymer capsules, Layer-by-Layer assembly, Anti-cancer drugs

Particulate drug carriers can shield the drug from degradation, increase drug bioavailability, and facilitate targeted delivery.^[1-4] In the case of lipophilic drugs, the use of a carrier can improve solubility and increase the payload of the therapeutic.^[4-6] Emulsion-based systems are attractive, yet production of emulsions for biomedical applications presents a number of challenges, such as controlling the size, composition and dispersity of the emulsion droplets, as well as toxicity issues associated with the use of stabilizing surfactants.^[5,7,8] Herein, we report a general and versatile surfactant-free approach to produce highly stable, monodispersed, submicron-sized polymer-encapsulated emulsions of lipophilic drugs. *In vitro* drug release studies demonstrated controlled release under intracellular conditions and

incubation with human colorectal cancer cells triggered cell death with greater efficiency (up to 10⁶-fold) than the free drug. These emulsions represent novel, degradable drug carriers for the effective delivery of lipophilic drugs.

Delivery of lipophilic drugs for cancer therapy and treatment of other diseases relies on the use of a drug carrier that improves the solubility of the therapeutic substance, prolongs the drug circulation time in the body, and allows for targeting and controlled release of the drug. Among the various drug delivery vehicles such as liposomes,^[9] niosomes,^[10] organogels,^[11,12] nanoparticles,^[13] and micro/nanocapsules,^[14] emulsion-based delivery systems are particularly attractive because of their ability to solubilize high payloads of lipophilic drugs and their potential biocompatibility. Yet to date, the significant limitation in the use of emulsions lies in that current production methods often yield droplets of uncontrollable size and/or with sizes typically larger than 2 μm .^[15,16] Monodispersity and size are two important features of drug-loaded colloidal carriers: monodisperse systems permit the reliable and precise dosage of drugs, while carriers <1 μm in diameter avoid capillary blockage or filtration, leading to effective uptake by cells. This size range can also exploit the leaky nature of tumor blood vessels (380–780 nm), providing a means for uptake by cancer cells *in vivo*.^[17,18] A recent study demonstrates that bacterially derived minicells of size ~400 nm can be effectively used for targeting cancer cells.^[19] Larger particles (up to ~ 5 μm in diameter) have also been administered intratumorally and subcutaneously for cancer drug delivery applications.^[20,21] Microemulsions, which are submicron-sized, single-phase colloidal oil-in-water or water-in-oil emulsions that form in ternary mixtures containing a water phase, an oil phase and surfactant/co-surfactants, are widely used as drug delivery vehicles: however, the formation of microemulsions typically requires a high concentration of surfactants (20-40%), which can lead to significant side effects such as acute hypersensitivity reactions and peripheral neuropathy.^[8,22] Another limitation of microemulsions is that they are thermodynamically stable, which means that solubilizing a high drug payload can render the

droplets unstable.^[7,22] Although some previous studies^[23,24] report the preparation of oil-filled nanocapsules, they have not demonstrated control over monodispersity, size, intracellular deconstruction of the delivery vehicle to release encapsulated drugs, and generality in terms of materials to control the composition of emulsion droplets. All of these parameters are of importance in designing and advancing next-generation delivery vehicles.

Recently, we reported an approach to produce colloidally stable, monodispersed emulsions of a predetermined droplet size (1-10 μm) by filling hollow polymer capsules with an oil phase (liquid crystal, silicon oil, and paraffin oil).^[25] The capsules were formed via layer-by-layer deposition of polymers on monodispersed sacrificial colloidal particles followed by subsequent removal of the core.^[26,27] In this work, we report the preparation of degradable, surfactant-free, micron- to submicron-sized polymer-encapsulated emulsions loaded with lipophilic drugs (doxorubicin and 5-fluorouracil) and demonstrate their *in vitro* drug release and anti-cancer effect on the human colorectal cancer cell line LIM1215 (**Scheme 1**).

To obtain hollow capsule templates, poly(*N*-vinylpyrrolidone), (PVPON), and thiolated poly(methacrylic acid), (PMA), were sequentially deposited via hydrogen bonding on sacrificial silica particles. The PMA chains within the layers were cross-linked via disulfide linkages followed by the removal of the core particles. PVPON was released from the capsules via disruption of interpolymer hydrogen bonds, which resulted in single-component PMA capsules stabilized by disulfide linkages.^[28,29] We previously showed that these capsules exhibit excellent colloidal stability.^[29] Moreover, they can be used to achieve uniform loading of material such as oligonucleotides^[28] and plasmid DNA,^[29] and deconstruct in the presence of intracellular concentrations of glutathione, GSH, to release encapsulated substances.

The PMA capsules (0.5 μm and 1 μm in diameter) were dehydrated in ethanol and dispersed in a drug/oleic acid mixture (0.1 mg/ml drug concentration) to allow infiltration of

the oil phase through the semi-permeable walls of the polymer capsules and filling of the capsules. FDA-approved oleic acid was chosen as an oil phase to solubilize two model chemotherapy drugs: doxorubicin (Dox) and 5-fluorouracil (5-FU). The excess drug/oleic acid mixture was removed by centrifugation followed by repetitive washing with phosphate buffer of pH ~7.2. This final preparation was dispersed in phosphate buffer of pH ~7.2 as monodispersed drug-loaded oleic acid emulsions stabilized within polymer capsules. Fluorescence microscopy images of Dox/oleic acid-loaded PMA capsules (1 μm) show a uniformly distributed red fluorescence signal arising from the Dox molecules, which confirms the loading of Dox in the capsules (**Figure 1**). The corresponding bright field optical image is shown as an inset in Figure 1. Sizing of these capsules from microscopy images yielded an average diameter of $1.0 \pm 0.1 \mu\text{m}$ in 10 mM phosphate buffer of pH ~4. Similar size ranges were observed for the capsules in 10 mM PBS buffer of pH ~7.2. We note that emulsions of sub-100 nm in size can be also potentially prepared by templating nanocapsules.^[30-32] The amount of Dox loaded into the PMA capsules was determined using flow cytometry and UV-visible spectrophotometry (see Experimental Section). We deduce that the drug loading in each capsule of size 1 μm and 0.5 μm are approximately 4×10^{-8} ng and 0.5×10^{-8} ng, respectively. The relative loading amounts are consistent with the expected volume difference between 1 μm and 0.5 μm capsules.

For a drug-delivery vehicle to be highly effective, it is desirable that it should not degrade readily in the blood stream. However, it should be easily degraded and release its cargo after reaching the target cells. The use of thiol-disulfide chemistry as a platform for drug delivery is based on the difference in the red-ox potential across the cellular membrane.^[33] While the overall potential in the blood stream is oxidative, the intracellular potential is reductive, which is largely achieved by a markedly higher concentration of the reduced form of glutathione (GSH), over its oxidized counterpart, GSSG. Disulfide linkages are known to deconstruct intracellularly, hence rendering the capsules (bio)degradable.^[33] We

previously showed that disulfide-stabilized PMA capsules remain stable at and above pH 7 in the absence of reducing agents and deconstruct in the presence of an intracellular concentration of GSH (5 mM) within 4-6 hours. In the current work, in the absence of GSH, the Dox/oleic acid-loaded capsules released negligible amounts of Dox (< 5% of the total Dox loaded, ~ 0.02 ng) when incubated in 100 mM PBS solution at 37°C over 24 h (**Figure 2**). In contrast, in the presence of 5 mM GSH, a near linear release of Dox was observed over 6 h with no initial burst phase (Figure 2). This highlights the ability of encapsulated emulsions to retain the vast majority of drug (>90% after 5 hours) within the capsule, which could potentially limit systemic toxicity and allow redox-responsive drug release in the presence of intracellular reducing agents. These PMA capsules remain colloidally stable (i.e., do not aggregate) in the presence of whole human blood (over at least 48 h).^[34]

Uptake of Dox/oleic acid-loaded PMA capsules (1 μm) by LIM1215 human colorectal cancer cells was investigated at 100 capsules per cell and visualized using confocal laser scanning microscopy (CLSM) after 15 h of incubation at 37°C. These observations revealed that nearly all cells contained at least several capsules (**Figure 3**) internalized into the intracellular compartments. The cytotoxicity of drug/oleic acid-loaded PMA capsules in LIM1215 colorectal cancer cells was investigated using the MTT cell viability assay (**Figure 4**). Two different drug molecules, Dox and 5-FU, were employed in these studies to show the general applicability of this method. LIM1215 cells were incubated with 0.5 μm and 1 μm drug/oleic acid-loaded polymer capsules (Dox and 5-FU) at 100 capsules per cell, along with several controls (E – M) for 16 h at 37°C. Naked oleic acid emulsions (K) and drug-free oleic acid-loaded PMA capsules of 0.5 μm (L) and 1 μm (M) did not significantly affect the cell viability, suggesting that free oleic acid and PMA capsules are biocompatible and do not cause any noticeable cytotoxicity. Conversely, treatment of LIM1215 cells with Dox/oleic acid-loaded PMA capsules of size 1 μm (B) and 0.5 μm (C), and 5-FU/oleic acid-loaded capsules of 0.5 μm (D) resulted in a significant decrease in the number of viable cells. Similar

results were obtained using a trypan blue cell viability assay (data not given). The 0.5 μm diameter drug/oleic acid-loaded capsules (C and D) were found to be more effective in eradicating the cancer cells ($> 85\%$ cell death) than the 1 μm drug/oleic acid-loaded capsules (B) ($< 50\%$ cell death). Moreover, Dox/oleic acid-loaded capsules of 0.5 μm (C) and 5-FU/oleic acid-loaded capsules of 0.5 μm (D) were found to be more effective in eradicating LIM1215 cells than free Dox (E and F) and 5-FU (G), respectively. This is of paramount importance, taking into account the drug loading in capsules (10^8 capsules per 10^6 cells) of size 1 and 0.5 μm are 4 ng and 0.5 ng respectively, which represents $10^5 - 10^6$ fold less drug than the concentration of free drug used (E and G). Moreover, the free Dox (5 ng/mL concentration (H)) and the supernatant solution of Dox/oleic acid-loaded 0.5 μm PMA capsules in cell media (J) did not significantly affect the cell viability. Thus, using emulsions encapsulated within degradable PMA capsules, it becomes possible to use an incommensurable amount of a toxic therapeutic, confine the drug within the capsule with limited passive release and achieve a considerably greater therapeutic effect in eradicating tumor cells. Furthermore, non-degradable Dox/oleic acid-loaded PSS/PAH capsules of 0.5 μm (I) did not significantly affect the cell viability, suggesting that PMA capsules deconstruct intracellularly and release the Dox. We have determined the IC₅₀ value for free Dox to be $\sim 1.5 \times 10^{-6}$ M, and the IC₅₀ value for the 0.5 μm PMA-encapsulated Dox-loaded oleic acid emulsions as $\sim 10^{-14}$ M (data not shown). This further confirms that Dox/oleic acid-loaded PMA capsules are considerably more effective in LIM1215 cell killing than the free Dox. Detailed cytotoxicity studies will be reported in a forthcoming manuscript.

In conclusion, a general and versatile method, based on the encapsulation of drug-loaded oleic acid emulsions within 0.5 and 1 μm diameter PMA capsules is reported. These capsules, held together by disulfide linkages, are redox-responsive, as demonstrated by the *in vitro* release of encapsulated Dox under reducing conditions. The polymer-encapsulated emulsions were internalized by cells and viability assays showed their effectiveness in

eradicating tumor cells, which was more pronounced with the 0.5 μm drug-loaded capsules than the 1 μm capsules or free drug. This system presents a novel drug carrier system for lipophilic drugs, which would otherwise have restricted accessibility to tumors when injected in the aqueous blood stream. *In vivo*, the drug molecules would be expected to be released only after capsules are internalized within the target cells, eliminating any systemic toxicity. Moreover, significantly higher amounts and more than one type of drug can, in principle, be loaded in these systems in a controlled manner. In addition, the functional groups present in the outer polymer layer can be tailored for easy conjugation of targeting moieties^[35,36] to target drug/oil-loaded capsules to various tumors. Our current studies are focusing on PEGylation of the emulsion droplets and the application of these PEGylated oil droplets in tumor targeting.

*Experimental**Materials*

Poly (methacrylic acid) (PMA, M_w 15 kDa) was purchased from Polysciences (USA). Poly (*N*-vinylpyrrolidone) (PVPON, M_w 10 kDa), hydrofluoric acid, oleic acid, sodium acetate, sodium hydrogen phosphate, doxorubicin, 5-fluorouracil, glutathione, trypan blue and acetic acid were purchased from Sigma-Aldrich and used as received. Silica particles were purchased from Microparticles GmbH and were used as received. Thiolated PMA with 12 mol% thiol groups was synthesized as described elsewhere [28]. LIM1215 cells are derived from human colorectal carcinoma and were grown in RPMI 1640 medium supplemented with ADDS (α -thioglycerol ($10.8 \mu\text{g mL}^{-1}$), insulin ($0.025 \mu\text{g mL}^{-1}$), hydrocortisone ($1 \mu\text{g mL}^{-1}$), penicillin ($60 \mu\text{g mL}^{-1}$) and streptomycin ($12.6 \mu\text{g mL}^{-1}$)) and 10% heat-inactivated fetal calf serum (FCS) at 37°C in a 5% CO_2 humidified atmosphere. MTT cell viability kits were purchased from Invitrogen. An inline Millipore RIOs/Synergy system was used to produce high-purity water.

Preparation of PMA capsules

PMA capsules were prepared as described in detail elsewhere [37]. Briefly, solutions of PVPON and thiolated PMA were used for the build-up of a multilayered polymer film on colloidal silica particles of 500 nm or 1 μm diameter. The films of (PVPON/PMA_{SH})₅/PVPON composition were treated with a solution of chloramine T to achieve conversion of thiol groups into disulfide linkages. The template particles were removed using aqueous hydrofluoric acid (5 M) and the PMA capsules were isolated via centrifugation / redispersion cycles (in phosphate buffer of pH ~ 7.2).

Preparation of Drug/Oleic acid-loaded PMA Capsules

PMA capsules dispersed in phosphate buffer (pH ~7.2) were centrifuged and the supernatant was removed. The pellet was redispersed in ethanol (0.5 mL) and centrifuged at 4500 g for 5 min. This procedure was repeated. The resulting pellet containing PMA capsules was dispersed with drug/oleic acid (0.1 mL, 0.1 mg mL⁻¹) and the mixture was incubated for 24 hours at 22 °C. The drug/oleic acid-loaded PMA capsules were centrifuged (~2000 g) for 2 min and washed three times with phosphate buffer of pH ~7.2 to remove excess drug/oleic acid from the capsule walls. To determine the amount of Dox loaded into the PMA capsules, we examined the capsules using flow cytometry and UV–visible spectrophotometry. The number of Dox/oleic acid-loaded PMA capsules was determined by flow cytometry. The Dox/oleic acid-loaded PMA capsules were then exposed to ethanol to dissolve the Dox, and the Dox absorbance in the supernatant was measured. By using a UV–visible absorbance calibration curve, we deduced that the drug loading in each capsule of size 1 µm and 0.5 µm is approximately 4×10^{-8} ng and 0.5×10^{-8} ng, respectively.

In-vitro Assay

The release of Dox from Dox/oleic acid-loaded PMA capsules was monitored in PBS (0.5 mL, 100 mM, pH 5.0 and 7.2) in the presence or absence of glutathione (5 mM) at 37°C. At specified time points, the capsules were centrifuged and supernatant was taken for UV-vis analysis. The drug release studies were performed under sink conditions. The total amount of drug released from the capsules is 40 ng, which is much lower than the solubility of Dox under experimental conditions (solubility of Dox in water is 1mg/mL).

MTT Assay

LIM1215 cells were seeded in 6-well plates at a density of 10^6 cells/well. Following overnight incubation at 37°C in a 5% CO₂ humidified atmosphere, the medium was replaced with fresh medium (2 mL) without FCS, and capsules (50 µL, 2×10^6 particles µL⁻¹) containing either oleic acid or oleic acid with doxorubicin were added to the cells. As controls, cells were incubated with free doxorubicin and 5-FU at final concentrations (0.1 mg mL⁻¹). The cells were incubated with the capsules for ~ 15 h with continuous shaking in a 37°C, 5% CO₂ humidified incubator. After incubation, the cells were detached from the wells by incubation with 300 µL of 10 mM EDTA in PBS at 37°C in a 5% CO₂ humidified atmosphere for 10 min. Detached cells were transferred to Eppendorf tubes and centrifuged at 400 g for 3 min. The pellet was resuspended in RPMI (100 µL) supplemented with ADDS and MTT (10 µL, 12 mM) solution was added to each tube. As a negative (blank) control, MTT was added to medium (100 µL) alone. The tubes were incubated for 2 h at 37°C and then centrifuged at 1500 g for 3 min. The supernatant was then removed, leaving medium (25 µL), and DMSO (175 µL) was added. The tubes were vortexed and incubated at 37°C for 10 min. Absorbance at 570 nm (with blank subtracted) was measured for each sample. After resuspension in RPMI (100 µL) supplemented with ADDS as described above (and before addition of MTT), cells (10 µL) were removed and observed under CLSM to observe the uptake of capsules by cells.

Typhan Blue Exclusion Assay

After resuspension in RPMI (100 µL) supplemented with ADDS as described above (and before addition of MTT), cells (10 µL) were removed and added to medium (40 µL). Trypan blue (50 µL of 0.4%) was added and allowed to incubate with the cells for ~10 min. Using a haemocytometer to obtain a uniform concentration of cells, images of the cells from

each sample were taken. Live and dead cells per image were counted and averaged over 10 images.

Instrumentation

Fluorescence and bright field images were taken using an Olympus IX71 inverted microscope (60x objective). Confocal laser scanning microscopy (CLSM) images were taken with a Leica TSC SP2 confocal unit. Flow cytometry was performed on a Partec CyFlow Space using an excitation wavelength of 488 and 633 nm. A HP 8453 UV-vis spectrophotometer (Agilent, Palo Alto, CA) was used to determine the concentration of Dox in the capsules A ND-1000 nanodrop spectrophotometer (Thermo Fisher Scientific) was used to determine the release of Dox from the capsules in *in vitro* assays.

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Scheme 1. Schematic representation of the preparation of PMA encapsulated drug-loaded emulsions and their uptake by cells.

Figure 1. Fluorescence microscopy image of 1 μm Dox/oleic acid-loaded PMA capsules. Inset is a bright field optical image of the same capsules.

Figure 2. Release of Dox as a function of time from 1 μm Dox/oleic acid-loaded PMA or PSS/PAH capsules. The capsules were incubated (at 37 $^{\circ}\text{C}$) in 100 mM PBS buffer (pH $\sim$ 5.0 or 7.2) with or without glutathione (5 mM). A negligible amount of Dox is released from Dox/oleic acid-loaded PMA capsules without glutathione at pH 5.0 (data not shown).

Figure 3. Confocal images of the uptake of 1 μm Dox/oleic acid-loaded PMA capsules by LIM1215 cells. a) Bright field image. b) Fluorescence image. c) Overlaid image of bright field and fluorescence image.

Figure 4. MTT assay results of samples A-M. (A - Living cell control, B - Dox/oleic acid-loaded 1 μ m PMA capsules, C - Dox/oleic acid-loaded 0.5 μ m PMA capsules, D - 5FU/oleic acid-loaded 0.5 μ m PMA capsules, E - Free Dox in water (0.1 mg/ml), F - Free Dox in water (0.05 mg/ml), G - Free 5FU in water (0.1 mg/ml), H - Free Dox in water (5 ng/ml), I - Dox/oleic acid-loaded 0.5 μ m PSS/PAH capsules, J - Supernatant solution of Dox/oleic acid-loaded 0.5 μ m PMA capsules in cell media, K - naked oleic acid emulsions, L - oleic acid-loaded 0.5 μ m PMA capsules, and M - oleic acid-loaded 1 μ m PMA capsules). Note: The cell viability from the MTT assay has been normalized by setting the viability of the cell control at 100%.

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The preparation of degradable, surfactant-free, micron- to submicron-sized polymer-encapsulated emulsions loaded with lipophilic drugs (doxorubicin and 5-fluorouracil) is reported. In vitro drug release studies demonstrates controlled release under redox conditions and incubation with human colorectal cancer cells triggers cell death with greater efficiency ($\sim 10^6$ fold) than the free drug.

Keywords: Biomedical materials, Polymeric materials, Drug delivery, Emulsions

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LIM1215 cells with
drug/oil-loaded
PMA capsules

● - Drug/oil-loaded capsule

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