A virus-mimicking, endosomolytic liposomal system for efficient, pH-triggered intracellular drug delivery

*Siyuan Chen, Rongjun Chen**

Department of Chemical Engineering, Imperial College London, South Kensington Campus, London, SW7 2AZ, United Kingdom

KEYWORDS: pseudo-peptides; liposomes; pH-responsive; endosomal escape; bridging effect; drug delivery.

ABSTRACT

A novel multifunctional liposomal delivery platform has been developed to resemble the structural and functional traits of an influenza virus. Novel pseudo-peptides were prepared to mimic the pH-responsive endosomolytic behavior of influenza viral peptides through grafting a hydrophobic amino acid, L-phenylalanine, onto the backbone of a polyamide, poly(L-lysine isophthalamide), at various degrees of substitution. These pseudo-peptidic polymers were employed to functionalize the surface of cholesterol-containing liposomes that mimic the viral envelope. By controlling the cholesterol proportion, as well as the concentration and amphiphilicity of the pseudo-peptides, the entire payload was rapidly released at endosomal pHs whilst there was no release at pH 7.4. A pH-triggered, reversible change in liposomal size was observed and the release mechanism was elucidated. In addition, the virus-mimicking nanostructures efficiently disrupted the erythrocyte membrane at pH 6.5 characteristic of early endosomes, whilst showed negligible cytotoxic effects at physiological pH. The efficient intracellular delivery of the widely used anticancer drug doxorubicin (DOX) by the multifunctional liposomes was demonstrated, leading to significantly increased potency against HeLa cancer cells over the DOX-loaded bare liposomes. This novel virus-mimicking liposomal system, with the incorporated synergy of efficient liposomal drug release and efficient endosomal escape, is favorable for efficient intracellular drug delivery.

1. INTRODUCTION

Despite continuous advancements in delivery systems, the development of methods for efficient delivery of therapeutic agents to the intracellular site of action, such as the cytoplasm or the nucleus, still remains a major issue.¹ Viral vectors have high transfection efficiency, but enthusiasm for their clinical use is dampened by concerns regarding serious safety issues and difficulties in large-scale production.^{2,3} Increasing interest is focused on rational design of non-viral vectors with improved safety and ease to synthesize.⁴⁻⁶ Liposomal drug-delivery systems have been demonstrated as one of the most promising non-viral vectors with great clinical potential since the first nanomedicine (Doxil®) approved by FDA in 1995 is based on the use of liposomes.⁷ However, conventional liposomes usually exhibit low colloidal stability, difficulties in achieving controlled drug delivery/release, fast elimination, and a lack of targeting effect, which have greatly limited their clinical applications.⁸

There is an increasing demand to develop multifunctional liposomes for targeted, controllable and efficient cytoplasmic drug delivery. Many viruses have evolved anionic peptide sequences in their protein coats, which can be transformed to a membrane-destabilizing state upon acidification in endosomes, enabling release of their nucleic acid cargo into the cell cytoplasm.⁹ With this increased understanding of the mechanisms of viral infection, researchers have been motivated to adapt viral structures and functions towards the design of pH-sensitive liposomes via coating the liposomal membrane surface with fusogenic peptides.¹⁰ However, safety concerns and difficulties in large-scale production of the naturally derived peptides have potentially limited their clinical application.¹¹ While synthetic peptides have been developed, they are far less efficient at membrane destabilization and are difficult to manufacture.¹²

A class of novel biocompatible pseudo-peptides has been recently developed to mimic the structure and pH-responsive membrane-destabilizing activity of influenza viral peptides. The parent pseudo-

peptide is a metabolite-derived linear polyamide, poly(L-lysine *iso*-phthalamide) (PLP)^{13,14}, and the hydrophobic amino acids present in influenza viral peptides were grafted onto its pendant carboxylic groups to manipulate its structure and amphiphilicity.^{15,16} The bio-inspired anionic polymers are easy-to-synthesize and can undergo the desirable coil-to-globule change of conformation upon reduction of pH below their pK_a, leading to efficient membrane destabilization at endosomal pHs.^{17,18} PP75 (75% stoichiometric degree of substitution with L-phenylalanine on the PLP backbone) displayed the vastly superior membrane activity at early endosomal pH, 35-fold more efficient than the antimicrobial peptide melittin, but essentially non-lytic at neutral pH.¹⁸ The pseudo-peptidic polymers can efficiently traverse across extracellular matrix to reach individual cells in three-dimensional multicellular spheroid tumor models.¹⁹ The polymers have been successfully used to deliver small-molecule model drugs^{18,20} and bioactive macromolecules (e.g. therapeutic protein and siRNA)^{21,22} into the cell interior for efficient *in vitro* and *in vivo* cancer treatment. These characteristics make the pseudo-peptides an ideal candidate for the liposomal surface functionalization to develop novel pH-sensitive liposomes.

Herein, we report our work on the development of a novel biocompatible multifunctional liposomal platform, which resembles the structural and functional traits of an influenza virus for safe, efficient and controllable intracellular drug delivery (Scheme 1). In this virus-mimicking system, the novel endosomolytic pseudo-peptides mimic fusogenic peptides in the viral spikes, the self-assembled liposomal structure mimics the viral envelope, and the encapsulated drug payload mimics the viral genome. The polymer-mediated, pH-responsive drug release profiles and the effects of key parameters (e.g. amphiphilicity and concentration of the polymer and proportion of cholesterol) were examined. The reversible pH-triggered size change was evaluated and the mechanism of polymer-liposome interaction was proposed. The endosomal escape ability of the virus-mimicking nanostructures was further explored and their cytotoxicity was assessed. Doxorubicin (DOX), a widely used anticancer

drug, was encapsulated into the novel multifunctional liposomes and their potency against HeLa cancer cells was compared to free DOX and conventional liposomes.

Scheme 1. Schematic of the virus-mimicking, pH-sensitive liposome and its endocytic pathway. The nanocarrier consists of the pH-responsive, endosomolytic pseudo-peptide mimicking the fusogenic peptide in the viral spikes, the liposome mimicking the viral envelope, and the drug payload mimicking the viral genome.

2. MATERIALS AND METHODS

2.1. Materials

L- α -phosphatidylcholine from egg yolk (EPC), cholesterol, *iso*-phthaloyl chloride, *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), 6-aminofluorescein, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Dulbecco's phosphate buffered saline (D-PBS), penicillin, calcein and doxorubicin (DOX) were purchased from Sigma-Aldrich (Dorset, UK). Dimethyl sulfoxide (DMSO), triethylamine, *N,N*-dimethylformamide (DMF), 4-dimethylaminopyridine (DMAP), sodium chloride, Hoechst 33342, alamar blue and Pierce™ LDH Cytotoxicity Assay Kit, LysoTracker® Red DND-99, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE) and Lissamine™ rhodamine B 1,2-

dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Rh-PE) were purchased from Fisher Scientific (Loughborough, UK). Anhydrous ethanol, acetone, hydrochloric acid, d_6 -DMSO, potassium carbonate, sodium hydroxide, chloroform, methanol, diethyl ether, sodium citrate dehydrate and sodium phosphate were obtained from VWR (Lutterworth, UK). L-lysine methyl ester dihydrochloride, L-phenylalanine methyl ester hydrochloride, *N,N'*-dicyclohexylcarbodiimide (DCC) and triton[®] X-100 were purchased from Alfa Aesar (Heysham, UK). Defibrinated sheep red blood cells were obtained from TCS Biosciences Ltd (Buckingham, UK).

2.2. Synthesis and characterization of pseudo-peptidic polymers

PLP ($M_w = 35,700$, $M_n = 17,900$, polydispersity = 1.99) was synthesized and then grafted with different amounts of L-phenylalanine (Phe) to prepare the pseudo-peptidic PP polymers (e.g. PP25, PP50 and PP75) according to the previously published methods.^{13,16} The numbers (e.g. 25, 50 and 75) denote to the stoichiometric molar percentages of Phe relative to pendant carboxylic acid groups on the backbone of PLP. The FT-IR spectra of PLP, PP25, PP50 and PP75 in their acid forms were recorded using a Spectrum 100 FT-IR Spectrometer (Perkin Elmer, USA) (Figure S1), with characteristic peaks located at 3301 cm^{-1} (N–H str and O–H str), 1726 cm^{-1} (C=O acid str), 1636 cm^{-1} (amide band I), and 1527 cm^{-1} (amide band II). The actual degrees of grafting of PP25, PP50 and PP75 were 25.2, 46.8 and 63.4 mol% respectively, which were calculated according to the previously published method¹⁶ from their ¹H-NMR spectra (Figure S2) recorded in d_6 -DMSO at room temperature using a 400 MHz NMR spectrometer (Bruker, Germany). Fluorophore-labelled pseudo-peptides were prepared by conjugating 6-aminofluorescein to the pendant carboxylic acid groups by DCC/DMAP coupling. A low level of labeling at 2 mol% was chosen to avoid a significant change to the properties of pseudo-peptides.

2.3. Preparation of virus-mimicking liposomal systems

Firstly, unmodified liposomes were prepared using the lipid film hydration method.²³ Briefly, EPC and cholesterol with required ratios were dissolved in chloroform containing 1% (v/v) ethanol. A lipid film was formed by rotary evaporation overnight to remove the organic solvent. The lipid film was hydrated using a 5 mL of HBS buffer (20 mM HEPES, 150 mM NaCl, titrated to pH 7.4) for 1 h. The size of the liposomes was controlled to be around 100 nm by sonication (Sonicator, 120 watt, Fisher Scientific) using a pulse mode for 20 min. After filtration through a 0.22 μ m filter, the sample was stored at 4 °C. Virus-mimicking liposomes were then prepared by incubation of the unmodified liposomes with an aqueous solution of a specific pseudo-peptide (in HBS buffer at pH 7.4) at a desired concentration for overnight at room temperature. Excess pseudo-peptides were removed through dialysis (Float-A-Lyzer[®], MWCO 300 kDa, Spectrumlabs, USA) against the HBS buffer (pH 7.4) for 7 h. Buffer was frequently changed to ensure the efficiency of dialysis.

To encapsulate DOX into the liposomes, a lipid film was prepared and hydrated with HBS buffer containing DOX at certain concentration. After sonication and filtration, unloaded DOX was removed by dialysis against HBS buffer at pH 7.4 at 4 °C for 3 h. The PP75-coated, DOX-loaded liposomes were prepared following the same protocol as described before.

2.4. Measurement of polymer adsorption

Fluorophore-labelled pseudo-peptidic polymers were used to coat the EPC/cholesterol liposomes following the step described in the Section 2.3. The fluorescence intensity of the fluorescent polymer-modified liposomes was measured by a spectrofluorometer (GloMax[®]-Multi Detection System, Promega, USA) with an excitation wavelength of 490 nm and emission wavelength from 510 to 570 nm. The amount of the adsorbed polymer was calculated using the standard linear curve of fluorescence intensity versus the concentration of fluorescent polymer.

2.5. pH-dependent drug release

The release profiles of the virus-mimicking liposomes mediated by the polymer coating layer at different pHs were investigated using a fluorescence dequenching assay.²⁴ The model drug calcein was encapsulated into the liposomes at a self-quenching concentration (100 mM). The virus-mimicking nanostructures were acidified to the desired pHs with 1 wt% HCl. An aliquot was withdrawn and equilibrated for 1 h at 37 °C. Then the pH was adjusted to 6.5-7.5 in order to minimize the effect of pH on calcein fluorescence. The fluorescence intensity was measured by the spectrofluorometer (GloMax[®]-Multi Detection System, Promega, USA) under excitation at 490 nm and emission at 510-570 nm. The percentage of calcein released was calculated using the following equation:

$$\% \text{ Release} = \frac{F_t - F_0}{F_{\max} - F_0} \times 100\%$$

Where F_t is the fluorescence intensity at time t , F_0 is the initial fluorescence and $F_{\max}$ is the fluorescence upon addition of detergent (triton[®] X-100) to completely lyse liposomal membranes.

For the measurement of pH-triggered calcein release from the diluted liposomal particles containing the PP75 coating, the assembled liposomal particles were diluted for different folds with HBS buffer at pH 7.4 and then acidified to pH 4.5 with 1 wt% HCl. The release percentage was determined following the method described above.

2.6. pH-dependent change of liposomal size

The hydrodynamic sizes of the virus-mimicking liposomes at different pHs were measured by dynamic light scattering (DLS, Zetasizer Nano S, Malvern, UK) at an angle of 137° in 10 mm diameter cells at 25 °C, repeated for 11 times. When needed, liposomal samples were diluted with specific HBS or citrate buffer and equilibrated for 5 min before analysis to yield an appropriate counting rate.

To measure the reversibility of size change in response to pH, the virus-mimicking liposomes at pH 7.4 were titrated to pH 4.5 using 1% HCl and back to pH 7.4 using 0.1 M NaOH, and then repeated for several cycles. The size was measured by DLS after 5 min of each titration. In order to visualize the change of liposomal size, the fluorescent polymer-modified liposomes (as described in the Section 2.4) were imaged by laser scanning confocal microscopy (Zeiss LSM-510 inverted laser scanning confocal microscope, Germany) at the excitation wavelength of 488 nm and the emission wavelength of 535 nm.

2.7. Hemolysis assay

The membrane-disruptive activity of the virus-mimicking liposomes was examined using a hemolysis model.¹⁸ Liposomal samples were prepared at required concentrations in the phosphate or citrate buffer at specific pHs. Defibrinated sheep red blood cells (RBCs) were washed three times with NaCl aqueous solution (150 mM) and resuspended in the liposomal solutions to achieve a final RBC concentration ($1-2 \times 10^8$ RBC mL⁻¹) within the range of a linear relationship between the absorbance of hemoglobin solution and the number of lysed RBCs. The samples were incubated in a shaking water bath at 37 °C for 1 h, and then centrifuged at 4000 rpm for 4 min. Two controls were prepared by resuspending RBCs either in buffer alone (negative control) or in deionized water (positive control). The absorbance of the supernatant from each sample was measured at 541 nm using UV-Visible spectrophotometer (GENESYS™ 10S UV-Vis, Thermo Scientific, USA), and the percentages of relative hemolysis were determined.

2.8. Cell culture

HeLa adherent epithelial cells (human cervical cells) were grown in DMEM supplemented with 10% (v/v) FBS and 100 U mL⁻¹ penicillin unless specified otherwise. The HeLa cells were trypsinized using trypsin-EDTA and maintained in a humidified incubator with 5% CO₂ at 37 °C.

2.9. Laser scanning confocal microscopy

In order to assess the endosomal escape ability of the virus-mimicking liposomes, membrane-impermeable calcein was used as a tracer molecule. 2 mL of HeLa cells (2×10^5 cells mL⁻¹) were cultured for 24 h followed by the treatment with 2 mL of the serum free DMEM containing 0.22 μ M filter-sterilized 3.2 mM calcein and the unloaded virus-mimicking liposomes or bare liposomes. After incubation for 2 h, the cells were washed three times with D-PBS and replenished with the complete medium for 2 h of further incubation. LysoTracker (50 nM) and Hoechst 33342 (1 μ g mL⁻¹) were then employed to stain endosomes/lysosomes and nuclei respectively. The cells were imaged following the same procedure as described above. Calcein was excited at 488 nm and the emission at 535 nm was collected.

The intracellular delivery of DOX to HeLa cancer cells was examined by confocal microscopy. 2 mL of HeLa cells (2×10^5 cells mL⁻¹) were seeded into a 35 mm glass bottom plate (MatTek, USA) and cultured for 24 h. The spent medium was removed and replaced with 2 mL of the serum free DMEM containing 0.22 μ M filter-sterilized, DOX-loaded liposomal samples (e.g. virus-mimicking liposomes or bare liposomes) or free DOX at the fixed DOX concentration of 2.5 μ M. After incubation for 1 h, the cells were washed three times by D-PBS buffer and stained with Hoechst 33342 at 1 μ g mL⁻¹ for 5 min. The cells were then rinsed with D-PBS for three times and imaged using the laser scanning confocal microscope. DOX was excited using a 480 nm laser and the emission at 560 nm was collected.

2.10. Flow cytometry

Flow cytometry was applied to evaluate the cellular uptake. 2 mL of HeLa cells (2.5×10^5 cells mL⁻¹) were cultured for 24 h. The spent medium was replaced with 1 mL of the serum-free DMEM

containing free DOX, DOX-loaded bare liposomes or DOX loaded, PP75 coated liposomes. The amount of DOX for all the samples was fixed at $2.5 \mu\text{M}$. After 1 h of incubation, the cells were washed with D-PBS for three times and then detached via the treatment with trypsin for 1 min. The cell suspensions were centrifuged to remove the supernatants and the cell pellets were then resuspended in serum-free DMEM. The cell suspensions were filtered through $40 \mu\text{m}$ Flowmi™ tip strainers (Bel-Art, USA) to remove cell aggregates before flow cytometry measurements (LSRFortessa cell analyzer, BD, USA) with an excitation wavelength of 488 nm.

2.11. Alamar Blue assay

The cytotoxicity of the virus-mimicking liposomes with or without DOX loading was evaluated by Alamar Blue assay. HeLa cells were seeded into a 96-well plate (Corning, USA) containing the complete DMEM (0.1 mL per well) at a density of 10^4 cells per well and cultured for 24 h. The spent medium was replaced with 0.1 mL of $0.22 \mu\text{m}$ filter-sterilized sample solutions in the serum free DMEM. After 24 h of incubation, the medium was completely removed and the cells were washed with D-PBS for three times. After that, only for the HeLa cells treated with the DOX-loaded liposomal samples or free DOX, was conducted a 24 h of further incubation with the complete DMEM medium. The plate with replenished complete DMEM containing 10% (v/v) alamar blue was then incubated for 4 h according to the manufacturer's instructions and the fluorescence in each well was measured by the spectrofluorometer at the emission wavelength of 580-640 nm and the excitation wavelength of 525 nm. The cytotoxic effect was determined from the fluorescence readings. The concentrations leading to 50% inhibition, IC_{50} , were determined from the concentration-dependent cell viability curves.

2.12. Lactate dehydrogenase (LDH) assay

The effect of the virus-mimicking liposomes on the cell membrane integrity was investigated by LDH assay. HeLa cells were seeded into a 96-well plate (Corning, USA) containing the complete DMEM (0.1 mL per well) at a density of 10^4 cells per well and cultured for 24 h. The spent medium was replaced with 0.1 mL of the serum free DMEM containing 0.22 μm filter-sterilized virus-mimicking liposomes or bare liposomes without drug loading at various lipid concentrations. After incubation for 24 h, the LDH released into the supernatant was quantified using a LDH Cytotoxicity Assay Kit. Briefly, 50 μL of supernatant in each well was transferred to a new plate and mixed with the reaction mixture from the kit. After 30 min of incubation, the reaction was stopped by the stop solution and the absorbance at 490 nm (using 680 nm as reference) was measured using the spectrofluorometer.

2.13. Statistical analysis

All data points were repeated in triplicate ($n = 3$). Reported results and graphical data are mean values with standard deviation encompassing a 95% confidence interval. The Student's t-test was performed to evaluate the statistical significance of difference. A value of $P < 0.05$ was considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. Effect of cholesterol

Cholesterol is an important component to maintain the integrity and fluidity of the phospholipid membrane. The effect of cholesterol on the membrane permeability of the virus-mimicking liposomes was investigated. Figure 1A shows the leakage of the model drug calcein from the liposomes containing a variety of cholesterol proportions during functionalization with PP75 for overnight at pH 7.4. For the liposome in the absence of cholesterol, 98.9 ± 1.9 % of the loaded calcein was lost. The

calcein leakage percentage decreased gradually with the increase in the proportion of cholesterol in the liposomal formulation. The liposome containing 40 mol% cholesterol only showed 9.8 ± 0.3 % calcein leakage. When the cholesterol proportion was increased to 60 mol%, no calcein leakage was observed.

After the post-functionalization removal of the excessive pseudo-peptide, ideally there should be no leakage of drug payload from the virus-mimicking liposomal delivery system at pH 7.4. Figure 1B shows the pH-dependent release profiles of the PP75-coated, virus-mimicking liposomes containing 40 and 60 mol% cholesterol. Although no content leakage was observed in the multifunctional liposome containing 60 mol% cholesterol at pH 7.4, it showed a much less efficient release of the model drug calcein in response to pH than the liposome containing 40 mol% cholesterol. This could be due to the enhanced rigidity of the liposomal membrane as result of an increased amount of cholesterol.²⁵ The virus-mimicking liposome containing 40% cholesterol also had no calcein leakage at pH 7.4, but showed an efficient calcein release facilitated by the adsorbed polymer within the endosomal pH range (5.0 – 6.8), reaching up to 92.5 ± 3.0 % at pH 5.0. Therefore, 40 mol% cholesterol was chosen for the liposomes used in the following experiments to ensure significant pH-responsive behavior.

It is proposed that the addition of cholesterol should change the mechanism of interaction between the pseudo-peptidic polymer and the liposome (Figure 1C), therefore impacting the permeability of the virus-mimicking liposomal system and its pH-responsive drug release profiles. In the absence of cholesterol, the hydrophobic segments of the polymer chain are not stable in the aqueous solution and tend to be embedded into the apolar region of the lipid membrane.²⁶ However, the addition of cholesterol could condense the lipid packing²⁷, change the membrane fluidity to make it more rigid²⁵, and reduce the permeability of liposomes^{28,29}. As a result, cholesterol could hamper the insertion of the hydrophobic parts of the polymer into the liposomal membrane. Furthermore, cholesterol could enhance the hydrophobicity of liposomal surface and therefore the hydrophobic interaction between the polymer and the liposome would mainly take place at the membrane surface instead of translocation

through the membrane.²⁷ This hypothesis was validated by the DLS results in Table S1, showing that after the surface coating with PP75 the hydrodynamic diameter of the liposome containing 40% cholesterol was increased by 6.4 nm, higher than the size increase (2.7 nm) for the liposome without cholesterol. The efficient, pH-triggered release behavior of the PP75-coated liposomes (Fig. 1B) is in agreement with the results of other researchers demonstrating that a deep polymer insertion into the bilayer is not always required for membrane destabilization.³⁰ When increasing the cholesterol proportion in the liposomal formulation, the enhancement of membrane rigidity and elasticity as well as the adsorption of polymer on the liposomal surface lead to a lower leakage at pH 7.4.

Figure 1. The effect of cholesterol addition into the PP75-coated, virus-mimicking liposomes. (A) Calcein leakage from the liposomes containing different proportions of cholesterol during functionalization with PP75 for overnight at pH 7.4. (B) pH-responsive calcein release from the PP75-

coated liposomes (5 mg mL⁻¹ lipids, 58.2 μ M PP75) containing 40 (■) and 60 mol% (●) cholesterol. Mean $\pm$ S.D. (n=3). (C) Schematic of the interactions between the polymer and the liposome in the absence (left) and presence of cholesterol (right).

3.2. Effect of polymer amphiphilicity

In general, interactions between polymers and lipid membranes include hydrophobic interaction, hydrogen bonding, columbic association and other possible effects.³¹ The hydrophobicity-hydrophilicity balance of the amphiphilic pseudo-peptides and their hydrophobic interaction with the neutral EPC/cholesterol membrane can be well controlled by side-chain modification. Herein, a variety of pseudo-peptidic polymers (e.g. PP25, PP50, PP75) were synthesized by grafting different amounts of hydrophobic amino acid Phe onto the PLP polymer backbone, and the effect of polymer amphiphilicity on the pH-dependent release profiles of the virus-mimicking liposomes was assessed.

Figure 2. pH-dependent calcein release from the bare liposomes (◆), and the virus-mimicking liposomes coated with PLP (▼), PP25 (▲), PP50 (●), and PP75 (■) respectively. Mean $\pm$ S.D. (n=3).

As shown in Figure 2, no calcein leakage was observed at pH 7.4 for all the virus-mimicking liposomal systems coated with different pseudo-peptidic polymers, indicating that these liposomal nanostructures were stable at pH 7.4. At pH below 7.0, a dramatic calcein release was observed in the liposomes coated with PP50 and PP75, whilst the liposomes coated with PP25 and PLP only showed a marginal increase in the calcein release compared to the bare liposomes without polymer coating. Increasing the amount of pendant hydrophobic amino acids on the polymer backbone could significantly enhance the hydrophobicity of the polymer.¹⁶ The calcein release percentages of the pH-responsive liposomes in response to acidification were ranked in the order of PLP-coated liposomes $\approx$ PP25-coated liposomes < PP50-coated liposomes < PP75-coated liposomes, which well correlated to the order of the increase in the degree of substitution with Phe and in the level of polymer hydrophobicity.

For non-covalent polymer-liposome complex, hydrophobic interactions were shown to be crucial in affecting the polymer's affinity for phospholipid vesicles.³² The higher polymer hydrophobicity, the tighter membrane association can be obtained. This is consistent with our recent report that the main driving force for the association of PP75 with the 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) monolayer model membrane is the hydrophobic interaction.³³ Side-chain modification with Phe, the most hydrophobic amino acid in nature with an aromatic residue, could facilitate the anchoring of the polymer into the liposomal membrane. This is in agreement with the report that aromatic residues in peptide structures can cause a dramatic increase in their interaction with the lipid membrane.³⁴

3.3. Effect of polymer adsorption

Figure 3A shows the pH-responsive calcein release profiles of the virus-mimicking liposomes (with a fixed lipid concentration at 3.0 mM) coated with different amounts of PP75, i.e. different polymer-to-lipid molar ratios. For the liposomes coated with 58.2 μ M PP75, up to 92.5% $\pm$ 3.0 % calcein was

released upon acidification to pH 5.0, which approaches the lower end of endosomal pH range. It was considerably higher than the calcein release percentages of the liposomes coated with 33.3 and 3.6 μM PP75 (both less than 20% at pH 5.0). This demonstrated that the pH-responsive membrane-destabilizing pseudo-peptidic polymer well retained its membrane activity upon acidification.¹⁸ A higher concentration of the polymer adsorbed would interconnect different vesicles and destabilize the membrane more efficiently.^{35,36}

Figure 3. The effect of the amount of PP75 adsorbed on the liposomal membrane. (A) pH-dependent calcein release from the virus-mimicking liposomes (containing a fixed lipid concentration at 3.0 mM) coated with PP75 at 3.6 μM (▲), 33.3 μM (●) and 58.2 μM (■) respectively. (B) pH-triggered calcein release from the diluted liposomal particles containing the PP75 coating. The assembled liposomal particles were diluted at pH 7.4 for 10 and 100 folds (i.e. decrease of the concentration of adsorbed polymer from 58, to 5.8 and 0.58 μM respectively but the polymer-to-lipid molar ratio was fixed at 1.9:100) and then acidified to pH 4.5. Mean $\pm$ S.D. (n=3).

The assembled liposomal particles with PP75 coating were diluted with HBS buffer at pH 7.4 for 10 and 100 folds. Consequently the concentration of adsorbed polymer was decreased from 58 to 5.8 and

0.58 μM respectively, but the polymer-to-lipid molar ratio was fixed at 1.9:100. Figure 3B shows the drug release behavior of the diluted liposomal particles upon acidification to pH 4.5. The difference in calcein release percentage was insignificant, indicative of the dilution-independent, pH-responsive drug release behavior of this virus-mimicking liposomal system. This characteristic is of crucial importance for a drug delivery system as the concentration of delivery formulations would be dramatically decreased due to the dilution during circulation in the bloodstream.²⁶

It is interesting to note that the 100-fold diluted pH-sensitive liposome (concentration of the adsorbed PP75 at 0.58 μM , polymer-to-lipid molar ratio at 1.9:100) retained a high calcein release rate of $91.8 \pm 28.7\%$ at pH 4.5 (Figure 3B). As comparison, the PP75-coated liposome with over six times more adsorbed polymer (3.6 μM) but with a lower polymer-to-lipid molar ratio (0.12:100) released only $25.6 \pm 3.4\%$ calcein at pH 4.5 (Figure 3A). This indicates that the polymer-to-lipid molar ratio played a more important role than the concentration of adsorbed polymer in destabilization of the liposomal membrane and controlled drug release.

3.4. pH-triggered change of liposomal size

The interaction between the adsorbed pseudo-peptidic polymers and the liposomes was also investigated via monitoring the change of liposomal size with pH. Figure 4A depicts the pH-responsive size change of the bare liposomes and the virus-mimicking liposomes. The particle sizes at pH 7.4 were similar between modified ($96.5 \pm 2.5\text{ nm}$) and unmodified liposomes ($90.1 \pm 1.4\text{ nm}$) (Figure S3), demonstrating that the surface coating with the pseudo-peptides did not considerably change the particle diameter. The size of the bare liposomes without polymer coating was stable within pH range tested. For the PLP- and PP25-coated liposomes, only a marginal size increase was observed at pH 4.0. As comparison, the particle size of the PP50- and PP75-coated liposomes increased remarkably at pH below 5.0. This trend well correlates to the above results showing that the PP50- and PP75-coated

liposomes exhibited a significantly enhanced content release at acidic pHs compared to the PLP- and PP25-coated liposomes (Figure 2). This could be because the increased polymer hydrophobicity introduced the stronger interaction between the adsorbed polymers and the liposomes, leading to the enhanced intervesicular interaction and the subsequent aggregation or fusion of the liposomes.³⁷

In order to further investigate whether the size increase at acidic pH was due to membrane fusion or particle aggregation, the reversibility of the size change of the PP75-coated liposomes was assessed by titration between pH 7.4 and 4.5. As shown in Figure 4B, upon titration from pH 4.5 to 7.4, the particle size decreased to the original size within 5 min. Once pH was decreased back to 4.5, the particle size again immediately increased significantly. The reversible size change was validated by confocal microscopy using the pH-sensitive liposomes coated with the 6-aminofluorescein-labelled PP75. At pH 7.4, green fluorescence was homogeneously distributed in the solution. Immediately after pH decrease to 4.5, aggregation of the fluorescent liposomes was observed. Upon titration back to pH 7.4, the aggregation disappeared quickly and the homogeneous green fluorescence distribution was visible again. This suggests that the size increase at acidic pH should not be due to liposomal fusion.³⁸ This was further confirmed by the lipid mixing assay through the detection of the NBD–rhodamine fluorescence resonance energy transfer (FRET) (Figure S4). As shown in Figure 4S, the bare liposomes and the PP75-coated liposomes exhibited negligible lipid mixing at pHs 7.4 and 4.5, indicating that the polymer coating prevented membrane fusion at pH 7.4 and the size increase at pH 4.5 (Figure 4) was due to aggregation instead of fusion between the liposomes.

Figure 4. pH-dependent change of the liposomal size. (A) The mean hydrodynamic size of the bare liposomes (Bare-LP), PLP-coated liposomes (PLP-LP), PP25-coated liposomes (PP25-LP), PP50-coated liposomes (PP50-LP) and PP75-coated liposomes (PP75-LP) at different pHs. (B) Reversible size change of the PP75-coated liposomes between pH 7.4 and 4.5, as measured by DLS and imaged by confocal microscopy. Mean $\pm$ S.D. (n=3).

Scheme 2 depicts the bridging mechanism which could be used to explain the pH-triggered, reversible size change of the PP75-coated liposomes. It has been previously reported that the pseudo-peptidic polymer displays a coil-to-globule conformational change upon reduction of pH and this process is reversible.¹⁶ When PP75, which bears a significant amount of hydrophobic pendant groups, was coated on the liposomal surface, some hydrophobes on the polymer could be associated with the liposomes, whilst others could facilitate the intermolecular interaction, leading to formation of the PP75-bridged liposomal network. At pH 7.4, the PP75 polymer exhibited an expanded structure due to the electrostatic repulsion between the negatively charged carboxylic groups outweighing the hydrophobic interaction.^{16,39} Therefore the virus-mimicking liposomes would be uniformly dispersed in the loose polymer-bridged networks, displaying the similar particle size with the bare liposomes. However, with decreasing pH, the carboxyl groups were protonated and the hydrophobic interaction

becomes predominant, leading to the transition of polymer conformation to the collapsed globular structure.⁴⁰ The size of the virus-mimicking liposomal system would increase tremendously as result of the significantly enhanced intermolecular interaction between polymer coating layers. When pH was increased back to 7.4, the expansion of the polymer structure would lead to a considerable reduction to the original size of the virus-mimicking liposomes. The proposed mechanism is in agreement with the report that tethering hydrophobic groups onto the backbone of biopolymers (e.g. chitosan and hydroxyethylcellulose) could enhance the intermolecular interaction and the subsequent formation of networks of surfactant vesicles and micelles.^{41,42} Other researchers reported the similar aggregation and disaggregation of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) giant unilamellar vesicles (GUVs).⁴³

Scheme 2. Schematic representation of the mechanism of the reversible size change of the PP75-coated, virus-mimicking liposomes between pH 7.4 and 4.5.

It has been reported that the bridging effect involving phospholipid vesicles and cationic (e.g. polylysine) or anionic polymers (e.g. polyacrylic acid) would usually lead to membrane fusion between vesicles or formation of polymer-lipid micelles.^{35,44} However, in the case of the virus-mimicking

liposomes coated with the anionic pseudo-peptidic polymers in this study, the liposomal membrane was not lysed or fused during the formation liposomal aggregates as the size change was reversible for several pH cycles (Figure 4B). The aggregates of the PP75-coated liposomes, which were kept at pH 4.5 for 7 days, could still be readily disaggregated to the original liposomal size with increasing pH to 7.4. The lipid mixing assay (Figure S4) further confirmed the size increase at pH 4.5 was due to aggregation instead of membrane fusion. Comparing the pH-triggered content release (Figure 2) and pH-triggered liposomal size change (Figure 4A), the release of the encapsulated model drug calcein from this virus-mimicking liposomal system did not seem to be necessarily dependent on the size change. At pH above 5.0 (e.g. 5.5-7.0), the PP75-coated liposomes already showed a significant calcein release, reaching 60.9 ± 0.5 % at pH 5.5, whilst no obvious size change was observed. The content release could be dependent on the transformation of PP75 on the liposomal surface to a membrane-destabilizing state upon acidification, instead of arising from the change of colloid stability.

3.5. Cell membrane destabilization

As shown above, the virus-mimicking, pH-sensitive liposomes showed efficient and controllable release of the encapsulated model drug calcein at endosomal pH through the liposomal membrane destabilization by the adsorbed pseudo-peptides. Herein, the hemolysis assay was performed using the RBC membrane as a model of the endosomal membrane¹⁷ to assess the endosomal escape ability of the pH-sensitive liposomes and their potential for further enhancement of the cytoplasmic delivery. The relative hemolysis of the PP75-coated liposomes, with a fixed polymer-to-lipid molar ratio at 1.9:100 but varying concentrations of the adsorbed polymer, were compared between physiological pH 7.4 and a typical early endosomal pH 6.5 (Figure 5A). With decreasing the PP75 concentration, the hemolytic activity of the virus-mimicking liposomes was gradually reduced at both pHs. Ideally, an effective cytoplasmic delivery system should have no or low hemolysis at physiological pH, but can efficiently

destabilize the endosomal membrane upon acidification in endosomal compartments. In Figure 5A, the liposome coated with 0.5 μM PP75 showed the largest difference between the hemolysis at pH 7.4 ($27.7 \pm 1.6 \%$) and pH 6.5 ($77.6 \pm 1.8 \%$).

Figure 5B shows the relative hemolysis of the liposomes with and without polymer coating at pH ranging from 7.4 to 4.5, mimicking the pH range of the endosomal and lysosomal environments. For the liposomes with 0.5 μM adsorbed PP75, $77.6 \pm 1.8 \%$ relative hemolysis was reached at a typical early endosomal pH 6.5, whilst the hemolysis at pH 7.4 was relatively low. As comparison, the conventional liposomes without polymer coating showed no hemolysis within the pH range tested, indicating that the membrane-destabilizing activity of the virus-mimicking liposomes resulted from the polymer layer instead of the liposomes. This hemolysis result well correlated to the membrane-destabilizing activity of the PP75 polymer¹⁸, suggesting that the polymer in the coating layer well retained its membrane activity. With increasing the concentration of the adsorbed PP75 to 5.8 μM , the virus-mimicking liposomes displayed a high level of relative hemolysis at $71.9 \pm 3.1 \%$ at pH 7.4. This is consistent with the report that a sufficiently high concentration of poly(α -ethylacrylic acid) may lead to complete membrane destabilization⁴⁵. With acidification to pH ≤ 5.0 , the PP75-coated, virus-mimicking liposomes lost their hemolytic activity as a result of the formation of liposomal aggregates (Figure 4A).

Figure 5. Relative hemolysis of the PP75-coated, virus-mimicking liposomes with a fixed polymer-to-lipid molar ratio at 1.9:100. (A) Relative hemolysis of sheep RBCs by the liposomes with different concentrations of the adsorbed PP75 polymer at pH 6.5 (close columns) and pH 7.4 (open columns); (B) pH-dependent relative hemolysis of the bare liposomes (■) and the virus-mimicking liposomes with the concentrations of the adsorbed PP75 at 5.8 μM (●) and 0.5 μM (▲). Mean $\pm$ S.D. (n=3).

3.6. Intracellular drug delivery

Membrane-impermeable calcein was used as a tracer molecule to demonstrate the ability of the co-administered virus-mimicking liposomal carrier to release endocytosed calcein from intracellular vesicles into the cell cytoplasm. As seen in Figure 6A, when HeLa cells were co-treated with free calcein and the bare liposomes, calcein appeared as green punctate spots co-localized with red lysotracker, suggesting that calcein was restricted within intracellular vesicles after endocytosis. When HeLa cells were co-treated with free calcein and the PP75-coated, virus-mimicking liposomes, strong diffuse green fluorescence throughout the cell interior was observed (Figure 6B), indicative of the efficient release of endocytosed materials into the cytoplasm. This further confirmed that the polymer PP75 present in the coating layer of the virus-mimicking liposomes well retained its efficient pH-responsive membrane-destabilizing activity as shown in Figure 5.

Figure 6. Confocal microscopy images of HeLa cells showing the escape of the endocytosed free calcein (3.2 mM) from intracellular vesicles into the cytoplasm mediated by (A) the bare liposomes and (B) the PP75-coated, virus-mimicking liposomes. The polymer-to-lipid molar ratio was kept at 1.9:100 for the liposomes with polymer coating. Images were acquired after 2 h of uptake and 2 h of further incubated (scale bar: 20 μ m).

The synergy of efficient liposomal drug-release behavior and efficient endosomal-escape ability of the novel PP75-coated, virus-mimicking liposomal system has prompted an evaluation of its use for intracellular delivery of encapsulated DOX, a widely used anticancer drug, to the immortalized HeLa human cervix adenocarcinoma cells. The mean diameter of the DOX-loaded liposomes was 94.2 ± 2.6 nm, the encapsulation efficiency was 53.1 ± 0.9 %, and the weigh percentage of drugs over the carrier was 15.2 ± 2.2 %. Figure 7A revealed that the virus-mimicking, PP75-coated liposomal system was highly efficient at delivering DOX into cancer cells, where strong DOX fluorescence was observed inside the cells after only 1 h of incubation. Free DOX entered and accumulated inside cells by diffusion⁴⁶, while the multifunctional liposomes were internalized mainly via endocytosis⁸. After cellular uptake of the DOX-loaded, PP75-coated liposomes, the acidic environment of

endosomes/lysosomes could efficiently trigger not only the DOX release from the liposomes (Figure 2) but also endosomal escape into the cytoplasm (Figures 5 and 6), leading to the consequent nuclear localization to exert its toxicity. As comparison, HeLa cells treated with DOX-loaded bare liposomes showed the substantially lower intracellular DOX fluorescence (Figure 7B) due to the absence of the endosomolytic pseudo-peptide PP75. The significantly enhanced cytoplasmic delivery of calcein that was encapsulated into this virus-mimicking, endosomolytic liposomal system was also demonstrated (Figure S5), further consolidating that the novel system is favorable for intracellular delivery of various drugs.

Figure 7. (A) Confocal microscopy images of HeLa cells showing the subcellular distribution of the anticancer drug DOX. The cells were treated with free DOX, DOX-loaded bare liposomes (DOX-LP), and DOX-loaded, PP75-coated liposomes (PP75-DOX-LP, polymer-to-lipid molar ratio=1.9:100) at the fixed equivalent DOX concentration of 2.5 μ M. Images were acquired after 1 h incubation (scale bar:

20 μm). Cellular uptake of DOX was further analyzed by flow cytometry and presented as (B) relative mean fluorescence intensity (MFI) and (C) representative histogram plots. All the flow cytometry samples were analyzed after 1 h of treatment with a fixed DOX dosage of 2.5 μM .

The efficient delivery of DOX by the PP75-coated liposomes was further confirmed by quantitative analysis using flow cytometry. As shown in Figures 7B and 7C, the HeLa cells treated with the DOX-loaded, PP75-coated liposomes showed almost an identical mean fluorescence intensity (MFI) to those treated with free DOX, but over twice of those treated with the DOX-loaded bare liposomes. This is in agreement with the confocal microscopy results shown in Figure 7A. The enhanced intracellular DOX fluorescence in the cells treated with PP75-coated liposomes could be due to the enhanced release of DOX into the cytoplasm. Furthermore, Surface functionalization of liposomes with the amphiphilic PP75 polymer containing hydrophobic amino acid grafts could enhance the surface hydrophobicity of liposomes, leading to the enhanced interaction between the polymer and the cell membrane.^{32,39} Scavenger receptors, a family of glycoproteins overexpressed on the surface of various cells including HeLa used herein⁴⁷, could play a role in the enhancement of cellular uptake of PP75-coated liposomes. Similar observations have been reported by other researchers for the liposomes coated with different anionic polymers.^{37,48}

3.7. *In-vitro* cytotoxicity

The effect of the virus-mimicking liposomes (without drug loading), at a fixed polymer-to-lipid molar ratio of 1.9:100 but varying lipid concentrations, towards the metabolic activity of HeLa cells was evaluated by Alamar Blue assay. As shown in Figure 8A, the bare liposomes without polymer coating had no cytotoxic effect within the lipid concentration range of 7.5-300.0 μM . HeLa cells also well tolerated the virus-mimicking liposomes, displaying no cytotoxic effect at the lipid concentration below 60 μM . The relative viability of HeLa cells decreased to $75.9 \pm 19.2 \%$ with increasing the

concentration of lipid present in the PP75-coated liposomes to 300.0 μM , suggesting that at this concentration, the presence of PP75 (5.7 μM) led to a reduction of cell metabolic activity.

Figure 8. Concentration-dependent relative viabilities of HeLa cells treated with the liposomes with (close columns) or without (open columns) PP75 for 24 h as determined by (A) Alamar Blue assay and (B) LDH assay. The polymer-to-lipid molar ratio of the PP75-coated, virus-mimicking liposomes was kept constant at 1.9:100. Mean $\pm$ S.D. (n=3).

Plasma membrane damage is a good indicator of serious injury prior to cell death and can be assayed by measuring the lactate dehydrogenase (LDH, a stable cytosolic enzyme) activity in the extracellular medium. The effect of the virus-mimicking liposomes (without drug loading) on the cellular membrane integrity of HeLa cells was investigated using a standard LDH assay. As shown in Figure 8B, HeLa cells well tolerated all the liposomes with or without PP75 coating within the lipid concentration tested, indicating that the liposomes did not cause significant cell membrane damage.

Figure 9. Potency of free DOX (open columns), DOX-loaded bare liposomes (grey columns), and DOX-loaded, PP75-coated liposomes (dark columns) against HeLa cells after 24 h of treatment and 24 h of further incubation. The drug to lipid molar ratio was kept at 20.2:100 and polymer-to-lipid molar ratio was 1.9:100 for the PP75-coated liposomes. Mean $\pm$ S.D. (n=3). * $P < 0.05$, ** $P < 0.01$.

The anticancer drug DOX was then applied as a payload to assess the potency of the PP75-coated, virus-mimicking liposomal system towards HeLa cancer cells. As a small-molecule drug, DOX can readily traverse across the phospholipid membrane into the cytoplasm and the nuclei by simple diffusion. However, due to its non-specific uptake and thus systemic side effects, it needs to be delivered by nano-carriers such as liposomes, which, compared to free DOX, are usually internalized into cells via endocytosis. The change in mode of uptake can lead to a considerable reduction of the potency of DOX delivered by nano-carriers.⁴⁹ This is validated by the results shown in Figure 9 that the DOX-loaded bare liposomes exhibited a substantially lower cell-killing activity than free DOX at all concentrations tested (0.01-10.0 μ M). However, it is interesting to note that the DOX-loaded, PP75-coated liposomes displayed the significantly enhanced potency against HeLa cells ($P < 0.05$) over the DOX-loaded bare liposomes, reaching the same or even higher potency level than free DOX within the concentration range tested (Figure 9). The IC_{50} of DOX-loaded, PP75-coated liposomes was $0.86 \pm$

0.21 μM , almost the same with the IC_{50} of free DOX ($0.83 \pm 0.18 \mu\text{M}$) while 2.5 times lower than the IC_{50} of DOX-loaded bare liposomes ($2.17 \pm 0.10 \mu\text{M}$). The enhanced potency of the DOX-loaded, PP75-coated liposomes was due to the efficient, synergistic liposomal release of DOX and endosomal escape. This suggests the promising applications of this virus-mimicking liposomal delivery system for efficient, pH-triggered intracellular drug delivery.

4. CONCLUSIONS

A novel, virus-mimicking, pH-sensitive liposomal delivery platform was successfully developed by surface-functionalization of the self-assembled EPC/cholesterol liposomes with the influenza viral peptide-mimicking, anionic pseudo-peptides. The pH-responsive release resulted from the liposomal membrane destabilization by the pseudo-peptidic polymers and was well controlled by varying the cholesterol proportion and the amount and amphiphilicity of the adsorbed polymers. The PP75-coated liposomes were optimal, showing no leakage at pH 7.4 but a complete content release at endosomal pH of 5.0-6.8. This pH-sensitive release profile was dilution-resistant, which is of crucial importance for a drug delivery formulation that would be dramatically diluted upon administration into the bloodstream. Liposomal particle aggregation was observed at pH below 5.0 and the size change was reversible, probably due to the bridging effect. The adsorbed polymer well retained its pH-responsive membrane-destabilizing activity, and as a result, the PP75-coated liposomes efficiently destabilized the erythrocyte membrane at early endosomal pH, whilst necessarily exhibited a low hemolysis at physiological pH. The pH-sensitive liposomal carriers were well tolerated by HeLa cells. The high efficiency of the novel PP75-coated, virus-mimicking liposomal system in endosomal escape into the cell cytoplasm, which together with the efficient pH-responsive liposomal release of DOX, led to the considerably enhanced

potency towards HeLa cells over the DOX-loaded bare liposomes. This suggests its great potential applications in intracellular drug delivery.

SUPPORTING INFORMATION

Mean hydrodynamic diameters of the liposomes before and after polymer coating, FT-IR and ^1H -NMR spectra of PLP, PP25, PP50 and PP75, size distribution of the bare and PP75-coated liposomes, lipid mixing assay, and the confocal images showing the subcellular localization of calcein as a model drug loaded inside the liposomes.

AUTHOR INFORMATION

Corresponding Author

* Tel: +44 (0)20 75942070. Fax: +44 (0)20 75945638. Email: rongjun.chen@imperial.ac.uk (R.C.)

Author Contributions

R.C. designed and supervised the research. S.C. performed all of the research. S.C. and R.C. analyzed data; S.C. and R.C. wrote the paper. All authors have read, commented on, and approved the final version of the paper.

Notes

The authors declare no completing financial interest.

ACKNOWLEDGEMENTS

The authors would like to thank the Department of Chemical Engineering at Imperial College London for funding this project and for providing to S.C. a fully-funded PhD Scholarship. The authors would like to acknowledge Professor Sakis Mantalaris for access to spectrofluorometer and Dr João Cabral for access to dynamic light scattering.

REFERENCES

- (1) Hubbell, J. A.; Chilkoti, A. Chemistry. Nanomaterials for Drug Delivery. *Science* **2012**, 337 (6092), 303–305.
- (2) Chen, R. Polymers in Drug Delivery: Concepts, Developments and Potential. In *Drug Delivery Systems: Advanced Technologies Potentially Applicable in Personalised Treatment*; Coelho, J., Ed.; Advances in Predictive, Preventive and Personalised Medicine; Springer Netherlands: Dordrecht, 2013; Vol. 4.
- (3) Chen, R.; Folarin, N.; Ho, V. H. B.; McNally, D.; Darling, D.; Farzaneh, F.; Slater, N. K. H. Affinity Recovery of Lentivirus by Diaminopelargonic Acid Mediated Desthiobiotin Labelling. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* **2010**, 878 (22), 1939–1945.
- (4) Torchilin, V. P. Multifunctional, Stimuli-Sensitive Nanoparticulate Systems for Drug Delivery. *Nat. Rev. Drug Discov.* **2014**, 13 (11), 813–827.
- (5) Andrade, B.; Song, Z.; Li, J.; Zimmerman, S. C.; Cheng, J.; Moore, J. S.; Harris, K.; Katz, J. S. New Frontiers for Encapsulation in the Chemical Industry. *ACS Appl. Mater. Interfaces* **2015**, 7 (12), 6359–6368.
- (6) Farokhzad, O. C.; Langer, R. Impact of Nanotechnology on Drug Delivery. *ACS Nano* **2009**, 3 (1), 16–20.
- (7) Barenholz, Y. Doxil[®] – the First FDA Approved Nano-Drug: Lessons Learned. *J. Control. Release* **2012**, 160 (2), 117–134.
- (8) Torchilin, V. P. Recent Advances with Liposomes as Pharmaceutical Carriers. *Nat. Rev. Drug Discov.* **2005**, 4 (2), 145–160.
- (9) Skehel, J. J.; Wiley, D. C. Receptor Binding and Membrane Fusion in Virus Entry: The Influenza Hemagglutinin. *Annu. Rev. Biochem.* **2000**, 69, 531–569.
- (10) Mura, S.; Nicolas, J.; Couvreur, P. Stimuli-Responsive Nanocarriers for Drug Delivery. *Nat. Mater.* **2013**, 12 (11), 991–1003.
- (11) McTaggart, S.; Al-Rubeai, M. Retroviral Vectors for Human Gene Delivery. *Biotechnol. Adv.* **2002**, 20 (1), 1–31.
- (12) Plank, C.; Zauner, W.; Wagner, E. Application of Membrane-Active Peptides for Drug and Gene Delivery across Cellular Membranes. *Adv. Drug Deliv. Rev.* **1998**, 34 (1), 21–35.
- (13) Eccleston, M. E.; Slater, N. K. H.; Tighe, B. J. Synthetic Routes to Responsive Polymers; Co-Polycondensation of Tri-Functional Amino Acids with Diacylchlorides. *React. Funct. Polym.* **1999**, 42 (2), 147–161.
- (14) Eccleston, M. E.; Kuiper, M.; Gilchrist, F. M.; Slater, N. K. H. pH-Responsive Pseudo-Peptides for Cell Membrane Disruption. *J. Control. Release* **2000**, 69 (2), 297–307.
- (15) Chen, R.; Khormaei, S.; Eccleston, M. E.; Slater, N. K. H. Effect of L-Leucine Graft Content on Aqueous Solution Behavior and Membrane-Lytic Activity of a pH-Responsive Pseudo-peptide. *Biomacromolecules* **2009**, 10 (9), 2601–2608.

- (16) Chen, R.; Eccleston, M. E.; Yue, Z.; Slater, N. K. H. Synthesis and pH-Responsive Properties of Pseudo-Peptides Containing Hydrophobic Amino Acid Grafts. *J. Mater. Chem.* **2009**, *19* (24), 4217–4224.
- (17) Chen, R.; Yue, Z.; Eccleston, M. E.; Williams, S.; Slater, N. K. H. Modulation of Cell Membrane Disruption by pH-Responsive Pseudo-Peptides through Grafting with Hydrophilic Side Chains. *J. Control. Release* **2005**, *108* (1), 63–72.
- (18) Chen, R.; Khormaei, S.; Eccleston, M. E.; Slater, N. K. H. The Role of Hydrophobic Amino Acid Grafts in the Enhancement of Membrane Disruptive Activity of pH-Responsive Pseudo-Peptides. *Biomaterials* **2009**, *30* (10), 1954–1961.
- (19) Ho, V. H. B.; Slater, N. K. H.; Chen, R. pH-Responsive Endosomolytic Pseudo-Peptides for Drug Delivery to Multicellular Spheroids Tumour Models. *Biomaterials* **2011**, *32* (11), 2953–2958.
- (20) Eccleston, M. E.; Williams, S. L.; Yue, Z.; Chen, R.; Lee, C. K.; Anikina, E.; Pawlyn, C.; Barrand, M. A.; Slater, N. K. H. Design and In-Vitro Testing of Effective Poly(L-Lysine Iso-Phthalamide) Based Drug Targeting Systems for Solid Tumours. *Food Bioprod. Process.* **2005**, *83* (2), 141–146.
- (21) Liechty, W. B.; Chen, R.; Farzaneh, F.; Tavassoli, M.; Slater, N. K. H. Synthetic pH-Responsive Polymers for Protein Transduction. *Adv. Mater.* **2009**, *21* (38-39), 3910–3914.
- (22) Khormaei, S.; Choi, Y.; Shen, M. J.; Xu, B.; Wu, H.; Griffiths, G. L.; Chen, R.; Slater, N. K. H.; Park, J. K. Endosomolytic Anionic Polymer for the Cytoplasmic Delivery of siRNAs in Localized in Vivo Applications. *Adv. Funct. Mater.* **2013**, *23* (5), 565–574.
- (23) Guo, F.; Yu, M.; Wang, J.; Tan, F.; Li, N. Smart IR780 Theranostic Nanocarrier for Tumor-Specific Therapy: Hyperthermia-Mediated Bubble-Generating and Folate-Targeted Liposomes. *ACS Appl. Mater. Interfaces* **2015**, *7* (37), 20556–20567.
- (24) Guo, W.; Gosselin, M. A.; Lee, R. J. Characterization of a Novel Diolefin-Based LPDII Vector for Gene Delivery. *J. Control. Release* **2002**, *83* (1), 121–132.
- (25) Liu, D. Z.; Chen, W. Y.; Tasi, L. M.; Yang, S. P. Microcalorimetric and Shear Studies on the Effects of Cholesterol on the Physical Stability of Lipid Vesicles. *Colloids Surfaces A Physicochem. Eng. Asp.* **2000**, *172* (1-3), 57–67.
- (26) Tribet, C.; Vial, F. Flexible Macromolecules Attached to Lipid Bilayers: Impact on Fluidity, Curvature, Permeability and Stability of the Membranes. *Soft Matter* **2008**, *4* (1), 68–81.
- (27) Gutiérrez de Rubalcava, C.; Rodriguez, J. L.; Duro, R.; Alvarez-Lorenzo, C.; Concheiro, A.; Seijo, B. Interactions between Liposomes and Hydroxypropylmethylcellulose. *Int. J. Pharm.* **2000**, *203* (1–2), 99–108.
- (28) Briuglia, M. L.; Rotella, C.; McFarlane, A.; Lamprou, D. A. Influence of Cholesterol on Liposome Stability and on in Vitro Drug Release. *Drug Deliv. Transl. Res.* **2015**, 231–242.
- (29) Wu, G.; Lee, K. Y. C. Effects of Poloxamer 188 on Phospholipid Monolayer Morphology: An Atomic Force Microscopy Study. *Langmuir* **2009**, *25* (4), 2133–2139.
- (30) Walrant, A.; Vogel, A.; Correia, I.; Lequin, O.; Olausson, B. E. S.; Desbat, B.; Sagan, S.; Alves, I. D. Membrane Interactions of Two Arginine-Rich Peptides with Different Cell Internalization

Capacities. *Biochim. Biophys. Acta* **2012**, 1818 (7), 1755–1763.

- (31) Marie, E.; Sagan, S.; Cribier, S.; Tribet, C. Amphiphilic Macromolecules on Cell Membranes: From Protective Layers to Controlled Permeabilization. *J. Membr. Biol.* **2014**, 247 (9-10), 861–881.
- (32) Schulz, M.; Olubummo, A.; Binder, W. H. Beyond the Lipid-Bilayer: Interaction of Polymers and Nanoparticles with Membranes. *Soft Matter* **2012**, 8 (18), 4849–4864.
- (33) Zhang, S.; Nelson, A.; Coldrick, Z.; Chen, R. The Effects of Substituent Grafting on the Interaction of pH-Responsive Polymers with Phospholipid Monolayers. *Langmuir* **2011**, 27 (13), 8530–8539.
- (34) Plank, C.; Oberhauser, B.; Mechtler, K.; Koch, C.; Wagner, E. The Influence of Endosome-Disruptive Peptides on Gene Transfer Using Synthetic Virus-like Gene Transfer Systems. *J. Biol. Chem.* **1994**, 269 (17), 12918–12924.
- (35) Chieng, Y. Y.; Chen, S. B. Interaction between Poly (Acrylic Acid) and Phospholipid Vesicles: Effect of pH, Concentration, and Molecular Weight. *J. Phys. Chem. B* **2010**, 114 (14), 4828–4835.
- (36) Chieng, Y. Y.; Chen, S. B. Complexation of Cationic Polyelectrolyte with Anionic Phospholipid Vesicles: Concentration, Molecular Weight and Salt Effects. *J. Colloid Interface Sci.* **2011**, 354 (1), 226–233.
- (37) Yuba, E.; Harada, A.; Sakanishi, Y.; Kono, K. Carboxylated Hyperbranched Poly(glycidol)s for Preparation of pH-Sensitive Liposomes. *J. Control. Release* **2011**, 149 (1), 72–80.
- (38) Jahn, R.; Südhof, T. C. Membrane Fusion and Exocytosis. *Annu. Rev. Biochem.* **2003**, 68, 863–911.
- (39) Yessine, M. A.; Leroux, J. C. Membrane-Destabilizing Polyanions: Interaction with Lipid Bilayers and Endosomal Escape of Biomacromolecules. *Adv. Drug Deliv. Rev.* **2004**, 56 (7), 999–1021.
- (40) Wang, H. C.; Zhang, Y.; Possanza, C. M.; Zimmerman, S. C.; Cheng, J.; Moore, J. S.; Harris, K.; Katz, J. S. Trigger Chemistries for Better Industrial Formulations. *ACS Appl. Mater. Interfaces* **2015**, 7 (12), 6369–6382.
- (41) Lee, J. H.; Gustin, J. P.; Chen, T.; Payne, G. F.; Raghavan, S. R. Vesicle-Biopolymer Gels: Networks of Surfactant Vesicles Connected by Associating Biopolymers. *Langmuir* **2005**, 21 (1), 26–33.
- (42) Panmai, S.; Prud'homme, R. K.; Peiffer, D. G. Rheology of Hydrophobically Modified Polymers with Spherical and Rod-like Surfactant Micelles. *Colloids Surfaces A Physicochem. Eng. Asp.* **1999**, 147 (1-2), 3–15.
- (43) Quemeneur, F.; Rinaudo, M.; Maret, G.; Pépin Donat, B. Decoration of Lipid Vesicles by Polyelectrolytes: Mechanism and Structure. *Soft Matter* **2010**, 6 (18), 4471–4481.
- (44) Yaroslavov, A. A.; Sybachin, A. V; Kesselman, E.; Schmidt, J.; Talmon, Y.; Rizvi, S. A. A.; Menger, F. M. Liposome Fusion Rates Depend upon the Conformation of Polycation Catalysts. *J. Am. Chem. Soc.* **2011**, 133 (9), 2881–2883.
- (45) Thomas, J. L.; Barton, S. W.; Tirrell, D. A. Membrane Solubilization by a Hydrophobic

Polyelectrolyte: Surface Activity and Membrane Binding. *Biophys. J.* **1994**, *67* (3), 1101–1106.

- (46) Cui, C.; Xue, Y. N.; Wu, M.; Zhang, Y.; Yu, P.; Liu, L.; Zhuo, R. X.; Huang, S. W. Cellular Uptake, Intracellular Trafficking, and Antitumor Efficacy of Doxorubicin-Loaded Reduction-Sensitive Micelles. *Biomaterials* **2013**, *34* (15), 3858–3869.
- (47) Yang, X. P.; Amar, M. J.; Vaisman, B.; Bocharov, A. V.; Vishnyakova, T. G.; Freeman, L. a; Kurlander, R. J.; Patterson, A. P.; Becker, L. C.; Remaley, A. T. Scavenger Receptor-BI Is a Receptor for Lipoprotein(a). *J. Lipid Res.* **2013**, *54* (9), 2450–2457.
- (48) Copolovici, D. M.; Langel, K.; Eriste, E.; Langel, Ü. Cell-Penetrating Peptides: Design, Synthesis, and Applications. *ACS Nano* **2014**, *8* (3), 1972–1994.
- (49) Koren, E.; Apte, A.; Jani, A.; Torchilin, V. P. Multifunctional PEGylated 2C5-Immunoliposomes Containing pH-Sensitive Bonds and TAT Peptide for Enhanced Tumor Cell Internalization and Cytotoxicity. *J. Control. Release* **2012**, *160* (2), 264–273.

TABLE OF CONTENTS GRAPHIC