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Novel pH-Sensitive Drug Carriers of Carboxymethyl-Hexanoyl Chitosan (Chitosonic Acid®) Modified Liposomes

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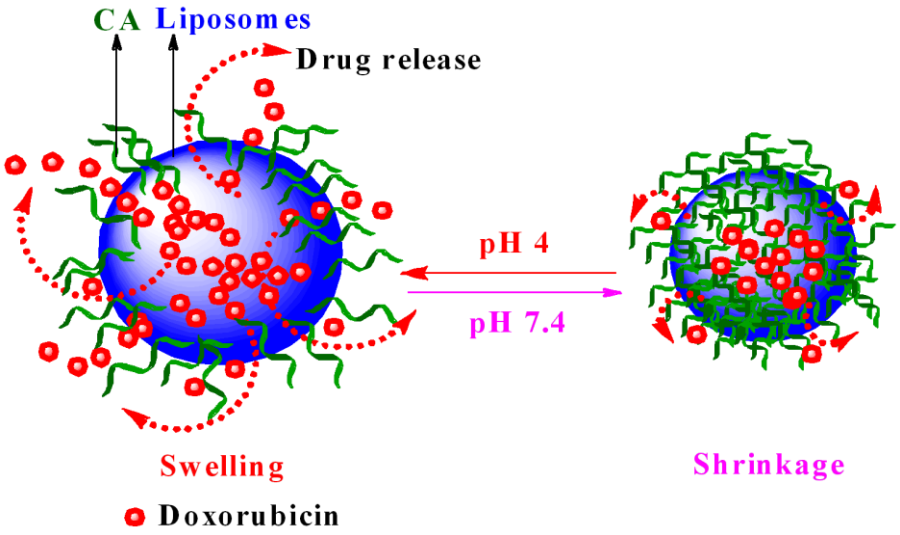


Chitosonic Acid® modified liposomes as a novel drug carrier displayed pH-sensitive, drug controlled released characterizations and a good cellular internalization

Abstract

In this study, novel hybrid nanocarriers composed of carboxymethyl-hexanoyl chitosan (Chitosonic Acid®, CA) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE)-liposomes were developed. CA was immobilized onto the DSPE-liposomes by EDC/NHS reaction using the carboxyl group of CA and amino group of DSPE. The characterizations of the resultant CA-modified liposomes were evaluated by transmission electron microscopy, dynamic light scattering, zeta potential, FTIR spectroscopy, X-ray photoelectron spectroscopy, and contact angle measurement. The results show that the particle size and surface charge of the CA-modified liposomes varied with the concentration of CA, and exhibited pH-sensitive behavior. *In vitro* drug release studies demonstrated the sustained release behavior of the doxorubicin in the CA-modified liposomes, related to the rapid release in the free doxorubicin. Interestingly, the doxorubicin release rate from CA-modified liposomes was lower at higher pH values (pH 7.4) than at lower pH values (pH 4), indicated that the drug carrier displayed pH-sensitive released behaviors. Furthermore, CA-modified liposomes exhibited no cytotoxicity to the fibroblast cells (L-929 cells), suggesting an excellent biocompatibility. Fluorescence and confocal microscopy images showed a good cellular internalization of CA-modified liposomes into the cellular compartment. These results confirm that the novel CA-modified liposomes could respond to pH environment, which is promising to the drug controlled release applications, especially in the field of cancer cells therapy (the lower pH environments).

Keywords: Chitosonic Acid®, liposomes, pH-sensitivity, biocompatibility, drug controlled release

1. Introduction

Development of smart materials responding to the environmental stimuli is gaining importance in the drug delivery system. Remote control drug carrier behavior has been regarded as a function that could enhance the efficacy and efficiency of drug delivery to the target sites.[1-3] Various pharmaceutical nanocarriers such as nanospheres, nanocapsules, micelles, liposomes, and dendrimers are currently being used for delivery of therapeutic or diagnostic agents. The most common and well investigated nanocarriers are liposomes which are artificial phospholipids vesicles with sizes varying from 50 to 1000 nm and greater, which can be loaded with a variety of drugs. Since the discovery of the formation of closed bilayered structures by phospholipids in aqueous media, liposomes have been developed to the point where they are now recognized as a promising material candidate for so called 'smart' delivery systems. Moreover, liposomes exhibit a number of excellences. It is highly biocompatible and biodegradable and can encapsulate both hydrophilic and hydrophobic pharmaceutical agents and protect them from the inactivating effect of external condition. Moreover, liposomes provide a unique characteristic to deliver pharmaceuticals into cells or even inside individual compartments.[3] The growing number of liposomal formulations in the market or currently under clinical evaluation provides proof of their enormous potential.[2]

Recently, trends in liposomal technology include designing 'smart' liposomal carriers for site-specific triggered release of their contents. Strategies used to design stimuli-sensitive liposomes that go through structural changes in response to physicochemical stimuli that allow for more controlled release of encapsulated drug fall in this category. The idea of designing stimuli-sensitive liposomes for programmed drug delivery relies on the fact that certain stimuli intrinsically characteristic of a pathological zone or when applied to this externally, can trigger a

change in properties of the drug-loaded liposomal system to provide an enhanced or controlled drug release and improve cellular drug uptake, or control intracellular drug fate.[2] Researchers have developed novel liposomes to provide a smart treatment in human body which can undergo the releasing of encapsulated contents as the response to the environmental stimuli including pH[4], temperature[5], magnetic field[6, 7], and light[8]. These specific environment-stimuli are used as the driving force for triggered drug release based on the interaction between the stimuli and liposomes. pH-triggered release system is one of the most interesting strategies as the controlled release stimuli from the liposomes system. To achieve the pH-sensitive release of liposome content, liposomes are constructed from pH-sensitive components; after being endocytosed in the intact form, these fuse with the endovacuolar membrane as a result of the lower pH inside the endosome, and release their contents into the cytoplasm. Studies of pH-sensitive liposomes focus on the development of new lipid compositions that confer pH-sensitivity to liposomes; liposome modification with various pH-sensitive polymers; and combining liposomal pH-sensitivity with longevity and ligand-mediated targeting.[3]

Recently, chitosan (CS) has aroused great interest as a coating material for liposomes. A set of advantageous properties achieved by coating of chitosan onto the liposomes including increasing the interaction between liposome and cell membrane, the fusion efficiency[9] and the stability of liposomal structure.[10, 11] Chitosonic Acid® (CA) is a new material derived from CS, wherein the pristine CS was modified by carboxymethylation, to increase the flexibility of chitosan molecular chains in water and followed by hydrophobic modification with hexanoyl groups to create the amphiphilic molecules.[12, 13] CA exhibited a good biocompatibility, biodegradability, non-toxicity, and solubility in aqueous medium in the physiological pH range. In addition, it is expected that CA might exhibit pH-sensitive behavior since it bears both acidic

(-COOH) and basic (-NH_2) functional groups.[14] Representative properties of CA could be exploited as a coating material which present pH-sensitive characteristic.[13-16]

In the present work, novel hybrid nanocarriers composed of carboxymethyl-hexanoyl chitosan (Chitosonic Acid®, CA) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE)-liposomes were developed. The resultant CA-modified liposomes were subject to characterization including transmission electron microscopy, dynamic light scattering, zeta potential, Fourier transform infrared spectroscopy, X-ray photoelectron spectroscopy, and contact angle measurement. *In vitro* drug release studies were evaluated using dialysis method. Cytocompatibility was evaluated with mammalian cells (fibroblast cells). Furthermore, fluorescence and confocal microscopy were conducted to investigate the cellular uptake and internalization of this drug carrier in the mammalian cells.

2. Materials and Methods

2.1 Materials

Synthetic lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) (purity>99%) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) (purity>99%) were purchased from Avanti Polar Lipids, Inc. AL, USA. Cholesterol, chloroform, fluorescein isothiocyanate (FITC), 1,10-dioctadecy-3,3,30,30-tetramethylindocarbocyanine perchlorate (Dil) and doxorubicin were purchased from Sigma-Aldrich, St. Louis, MO, USA. Chitosonic Acid® (CA) was purchased from Advanced Delivery Technology, Taiwan. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Acros, USA. Ethanol (95%) was purchased from Shimakyu Pure Chemicals, Japan. All the chemicals were used without further purification. Fibroblasts (L-929) cells were obtained from ATCC CRL-

1503TM. Dulbecco's modified Eagle's medium-high glucose (DMEM), trypsin, dimethylsulfoxide (DMSO), trypan blue, and 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) powder were purchased from Sigma Aldrich, St. Louis, MO, USA. Fetal bovine serum (FBS) was purchased from BD Biosciences, San Jose, CA, USA.

2.2 Preparation of liposomes

Liposomes composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)/ 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE)/cholesterol at molar ratios of 3:1:1 were prepared *via* thin-film hydration technique as method described previously with minor modification.[9, 17] Briefly, DPPC, DSPE, and cholesterol were dissolved in chloroform and ethanol (3:1 *v/v*). Then, the mixture was transferred into a 300-mL round bottom flask and placed to rotary evaporation system (N-1200 series, Eyela®, Tokyo Rikakikai Co., Ltd, Tokyo, Japan) for the elimination of any traces and residual of organic solvents. Further, a thin dry lipid film would form on the wall of the round bottom flask. This thin dry lipid film was kept in rotary evaporation system for 6 h to ensure complete removal of organic solvents. Hydration process of the dry lipid film was accomplished by adding 10 mL phosphate buffer solution (PBS) of pH 7.4 at 80 ^{o}C, which resulted in liposome suspension. This suspension was subjected to a bath-type sonicator (Ultrasonic Cleaner, Kudos, Shanghai, China) for harvesting the liposomes. Afterward, the liposomes were homogenized using an ultrasonicator (Probe-type sonicator, VCX 750, Vibra-Cell™, SONICS®, Sonics and Materials, Inc., Newton, CT, USA) at 24 W for 10 min. The suspension was extruded through a 0.22-µm filter for sterilization and to reduce the size. Furthermore, the resulting suspensions of liposomes were then preserved at 4°C prior to

characterizations. For labeling, Dil was mixed together with the raw material of liposomes and was prepared as the aforementioned method.

2.3 Preparation of Chitosonic Acid® (CA)-modified liposomes

CA-modified liposomes were prepared through the carbodiimide coupling reaction.[12] CA was dissolved in D.I water and stirred at room temperature until reach the homogeneity, and then EDC (1 mg/mL) and NHS (1 mg/mL) were added simultaneously into CA solution and continue with stirring at room temperature. Liposome suspension (4.5 mg/mL, 5 mL) was added drop-wisely to various concentration of CA solution (5 mL) and then were mixed through continuously stirring to minimize the possibility of intra- and inter-molecular reaction of free –NH_2 groups with free –COOH groups of either same or a different chains of CA that resulting in a cross-linked CA coating on the surface of liposomes, CA and liposomes were mixed simultaneously in the stirring condition.[18] Residual EDC, NHS, and unreacted CA were removed through centrifugation and dialysis. The product termed as CA-modified liposomes. In order to investigate the effect of CA concentration on the properties of CA-modified liposomes, CA-modified liposomes with different weight of CA ratios ranging from 0-1 wt% were prepared.

2.4 Characterization

Particle size was measured using a dynamic light scattering (DLS) spectroscopy (Horiba Instrument, Horiba, Kyoto, Japan) with helium-neon laser with wavelength of 633 nm, scattering angle of 90°, and refractive index of 1.33 at 25 °C. The zeta potential was determined through electrophoretic mobility measurement (Horiba Instrument) with the following specifications: a dispersion medium viscosity of 0.894 mPa.s, a refractive index of 1.33, and temperature of 25

°C. The morphology of liposome and CA-modified liposomes was observed using transmission electron microscope (TEM-7650, Hitachi, Chiyoda-ku, Japan) at an acceleration voltage of 75 kV. Phosphotungstic acid (PTA) 1% w/v was used as the staining agent. The infrared spectra were recorded using a FTIR-460 PLUS (Jasco Co.Ltd., Tokyo, Japan) to determine the major characteristic of functional groups. Briefly, the sample was mixed with KBr then the mixtures were pressed into a pellet prior to characterization. Liposomes, CA, and CA-modified liposomes were further investigated using X-ray photoelectron spectroscopy (XPS) (ESCALAB 250, Thermo VG Scientific, West Sussex, U.K.) equipped with Mg K_α at 1253.6 eV at the anode to evaluate their chemical binding energy characteristics. Contact angle measurements were conducted to investigate the hydrophilicity of the drug carrier. Briefly, an aliquot of liposomes suspension was put on glass slide, and dried overnight to form a thin film layer. Afterward, a drop of water was instilled carefully on the surface, and the droplet of image was analyzed using a contact angle machine (Sindatek model 100SB, Taiwan). Degree of coupling between CA and liposomes was obtained by estimating the free amine groups on CA-modified liposomes with and without coupling reaction using EDC/NHS.[18] Free amino groups were calculated using trinitrobenzene sulphonic acid (TNBS) assay. Briefly, CA-modified liposomes (reaction with EDC/NHS) and CA-coated liposomes (reaction without EDC/NHS), about 3.3 mg each were reacted with a mixture of 1 ml of 0.5% picrylsulfonic acid and 1 ml of 4% sodium bicarbonate at 60 ^{o}C for 4 h. 1 ml of this solution was further treated with 3 ml of 6N hydrochloric acid at 40 ^{o}C for 1.5 h. The absorbance of this solution was determined at 334 nm after suitable dilution using UV-Visible spectroscopy (UV-VIS model V-670, Jasco). Degree of coupling was determined as follows:

$$\text{Degree of coupling} = 1 - \frac{\text{Absorbance of CA-modified liposomes}}{\text{Absorbance of CA-coated liposomes}}$$

Colloidal properties of liposomes and 1wt%CA-modified liposomes was determined by investigating their particle size and surface charge after incubation for 24 h at 37 °C in the medium with different pH values (pH 4 to pH 7.4). Fresh samples were prepared for all measurements and were carried out three times.

2.5 *In vitro* drug release studies

Doxorubicin (DOX) was used as a drug model. Briefly, DOX-loaded 1wt%CA-modified liposomes were prepared by introducing of DOX (5 mg) in the hydration process and followed by the aforementioned process of liposomes preparation. The DOX-loaded-1wt%CA modified liposomes was then separated from the aqueous solution by centrifugation at 11,000 *g* for 30 min. DOX concentration in the supernatant was measured using a UV-visible spectroscopy at 480 nm. The amount of DOX in DOX-loaded-1wt%CA modified liposomes could be calculated by the total amount of DOX subtracting the residual DOX in the supernatant. The encapsulation efficiency (EE) was obtained as follows [19, 20]:

$$\text{EE} = \frac{D_o - D_s}{D_o} \text{ x } 100\%$$

where D_o is the total amount of DOX and D_s is the amount of DOX remaining in the supernatant. *In vitro* drug release test was evaluated using the dialysis method in the medium with pH 4 and 7.4 at 37°C. For each release experiment, 1 mL a solution of DOX-loaded 1wt%CA-modified liposomes was loaded to a dialysis bag. Further, the dialysis bag was placed on the bath containing release medium (20 mL). Proper sink conditions were maintained throughout each experiment. For all *in vitro* drug release studies, 1 mL of the release medium was withdrawn at certain time intervals and replaced with the same quantity of fresh medium. The solution was

then analyzed using a UV-visible spectroscopy at 480 nm. The drug release percentage was evaluated as follows:

$$\text{Cumulative release (\%)} = R_t/D \times 100\%$$

where D and R_t represent the initial amount of drug loaded and the cumulative amount of drug released at time *t*, respectively.

2.6 Cell culture

Mouse fibroblast (L-929 cells) were cultured using DMEM containing 10 vol.% FBS and 1 vol.% antibiotic antimycotic solution. The cultures were incubated under saturated humid conditions at 37 °C with 5% CO_2. The medium was changed every day until reaching approximately 70 to 80% confluency.

2.7 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Cell growth and cytotoxicity were determined using MTT assay. In 24-well plates, 5,000 L-929 cells were cultured in each well. These plates were divided into several groups and incubated under saturated humid conditions at 37°C and 5% CO_2. After 24 h of incubation, the medium was replenished. Among these plates, some plates were added with liposomes-contained medium (final concentration of liposomes was 0.45 mg/mL) and CA-modified liposomes-contained medium (final concentration of CA-modified liposomes was 0.45 mg/mL). Prior to the experiments, the samples were extruded through 0.22-μm filter for sterilization. In addition, the negative control contained only medium, while the positive control was medium containing 5% DMSO. After culturing for 24 h, 0.1 mL of MTT solution and 0.9 mL of medium were added to each well. After incubating for another 3 h at 37 °C, the medium was withdrawn, and replaced

with 500 µL DMSO and allowed to stand about 15 min for complete reaction. Furthermore, the plates were shaken, and the readings were taken at 570 nm using an ELISA reader (Sunrise, Tecan, Männedorf, Switzerland). Proliferation of the cells was calculated as follows[9]:

$$\text{Proliferation (\%)} = A_c/A_o \times 100\%$$

where A_c is the absorbance of each system in day one and A_o is the absorbance of the control on day 0.

2.8 Fluorescence microscope

Fibroblasts (L929) cells (~15,000 cells) were seeded on the well (Lab-Tek II Chamber Slide w/cover RS Glass 2 Slide sterile, Nunc, Denmark) with DMEM containing 10 vol.% FBS and 1 vol.% antibiotic antimycotic solution and incubated for 24 h to allow cell attachment. Following the incubation, either Dil-liposomes (0.45 mg/mL) or Dil-CA-modified liposomes (0.45 mg/mL) was added to each well and incubated for another 24 h. After 24 h of incubation, the cells were washed twice with PBS. The fluorescence images were obtained with a fluorescence microscope (Olympus, Tokyo, Japan). Image J was used to analyze the images from fluorescence microscopy to determine the red area. The fusion ratio (F) was calculated as follows[9]:

$$F = A_r/A_t \times 100\%$$

where A_r is the luminous red area with liposomes and CA-modified liposomes in the fluorescence image and A_t is the total area in the fluorescence image.

2.9 Confocal Microscopy

Cellular internalization of 1wt%CA-modified liposomes into the cell compartment of L-929 cells was investigated using confocal laser scanning microscopy (CLSM) (TCS SP5, Spectral Confocal and Multi Photon System, Leica Microsystems, Wetzlar, Germany). Briefly, L-929 cells (15,000 cells) was seeded in each well of a 4 well-plate (Lab-Tek II Chamber Slide w/cover RS Glass 2 Slide sterile, Nunc, Denmark), and incubated at 37 °C in a 5 % CO_2 incubator for 24 h. Following the incubation, Dil-1wt%CA-modified liposome suspension (0.45 mg/mL) was added into the well. After 24 h of incubation, cells were washed with PBS, and mounted on a slide with for confocal microscope observation. The fluorescence signal was observed by confocal microscopy using the detection at wavelength of 570 nm.[9]

3. Results and Discussion

3.1 Characterizations of CA-modified liposomes

Liposomes were prepared through thin film hydration method and CA was incorporated into the liposomes via chemical-conjugated reaction using EDC/NHS. A covalent conjugation strategy was chosen because it is more stable than electrostatic interactions. Moreover, it is generally accepted that this cross-linked method leads only to the formation of amide linkage between carboxyl-contained compound (CA) and amino-contained compounds (DSPE-liposomes) (Fig. 1a).[21] As a reference, DPPC/cholesterol exhibits a slightly negative zeta potential of -9.1 ± 0.7 mV. Addition of DSPE into the DPPC/cholesterol increased the zeta potential to 0.6 ± 0.3 mV this probably because DSPE contains amino groups with positive charges. This is in accordance with the research previously reported which revealed that PE-conjugation was found to increase zeta potential value of liposomes system.[22]

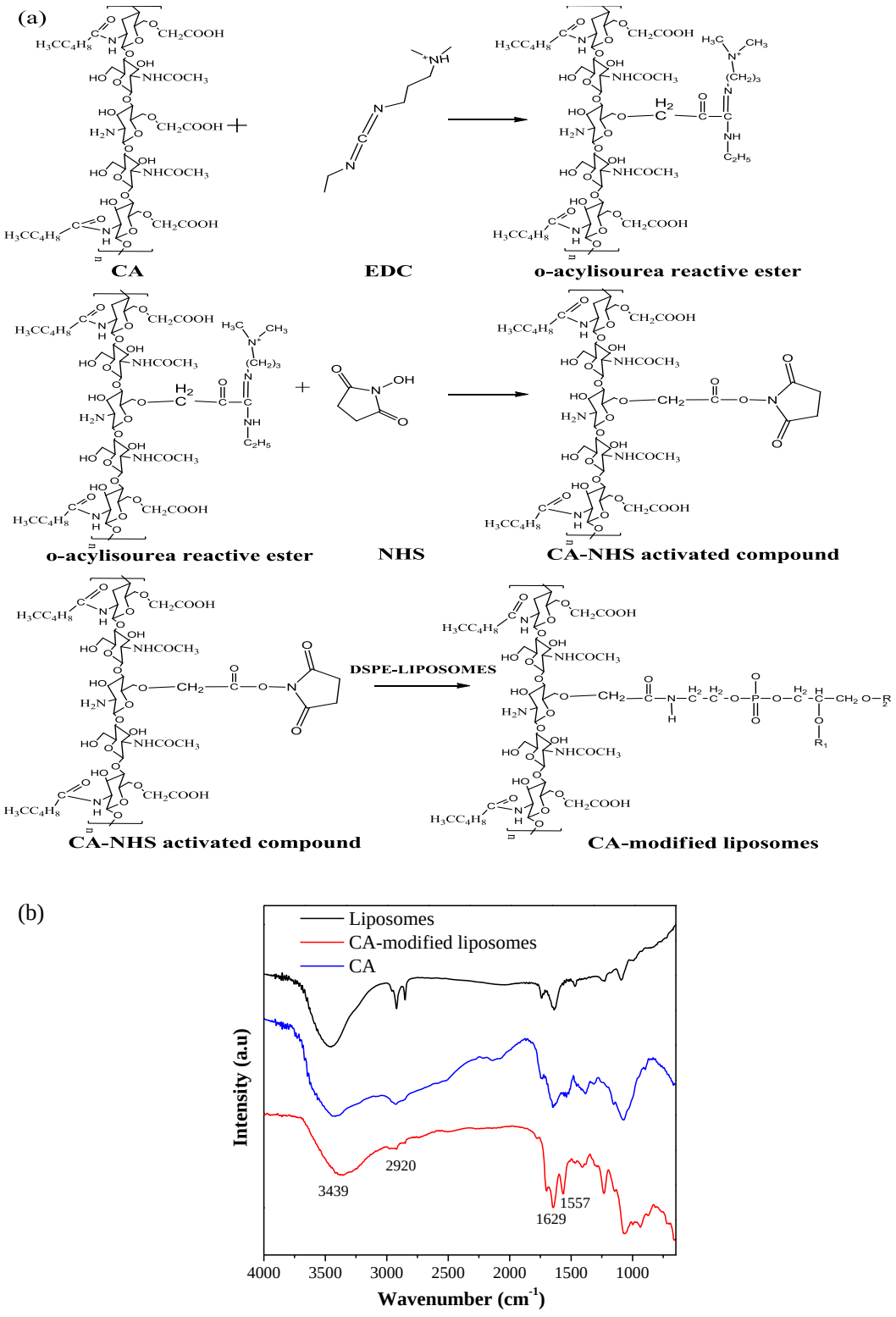
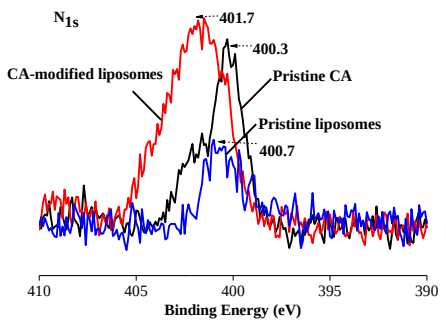


Fig. 1 Proposed conjugation mechanism of CA and liposomes via EDC/NHS reaction (a); and FTIR spectra (b) of liposomes, CA and CA-modified liposomes.

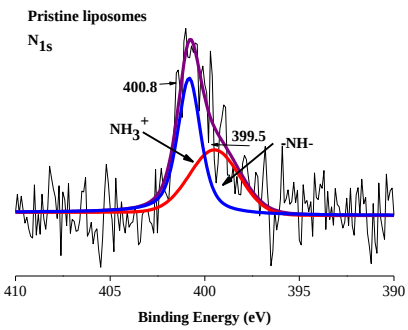
FTIR spectra of liposomes and CA-modified liposomes (Fig. 1b) showed peaks at 3,439 and 2,920 cm^{-1} for hydroxyl groups and C-H stretching, respectively. Furthermore, the interaction between CA and liposomes in CA-modified liposomes was observed through formation of amide I (CO) and amide II (NH) bond at 1,629 and 1,557 cm^{-1}, respectively. This result revealed the conjugation reaction of carboxyl groups of CA with amino groups of DSPE-contained liposomes.[18, 23] Degree of coupling between CA and liposomes was measured through analytical method of TNBS assay.[18] Based on this method, the degree of coupling between CA and liposomes in 1wt%CA-modified liposomes was 36.22 ± 2.58 %.

Figure 2 shows the X-ray photoelectron spectroscopy (XPS) spectra of N_{1s}, O_{1s}, and C_{1s} spectra of liposomes, CA, and CA-modified liposomes which were deconvoluted into several main peaks. Generally, shifting peaks were identified for the spectra of N_{1s} (Fig. 2a), O_{1s} (Fig. 2e), and C_{1s} (Fig. 2i) for CA-modified liposomes suggesting the molecular interaction between liposomes and CA. In the N_{1s} spectra of liposomes (Fig. 2b), the peak at 399.5 eV was assigned to chemical bindings of -NH, while the peak at 400.8 eV was assigned to amino groups in the ammonium form ($-NH_3^+$).[24] The presence of NH_3^+ relatively higher than content of –NH, probably because the presence of DSPE molecules on the surface of liposomes (DSPE carries-NH_3^+). CA exhibited N_{1s} spectra consist of N-C=O and NH_2 chemical bindings (-NH) at 400.2 eV and the spectra of NH_3^+ chemical bindings at 402.2 eV (Fig. 2c). The presence of –NH chemical binding was relatively higher than NH_3^+. This is probably because CA carriers hexanoyl moieties that contributed to the increase of –NH sites. CA-modified liposomes exhibited two peaks of N-C=O and NH_2 (-NH) and also $-NH_3^+$ chemical bindings at 401.2 and 403.1 eV, respectively (Fig. 2d). Regarding the O1s spectra of liposomes, two peaks were identified (Fig. 2f). The peaks at 530.2 and 532.1 eV were assigned to C=O and C-OH chemical

bindings, respectively. In the O_{1s} spectra of CA, three peaks were identified (Fig. 2g). The peak at 531.7 eV was assigned to N-C=O chemical bindings in *N*-acetylated-glucosamine units. The peaks at 533.1 and 533.8 eV were assigned to C-OH and O-C-O chemical bindings, respectively. In the O_{1s} spectrum of CA-modified liposomes, three peaks were identified (Fig. 2h). The peaks at 532.4, 533.8, and 534.8 eV were assigned to N-C=O in *N*-acetylated-glucosamine units, C-OH and O-C-O chemical bindings, respectively. The resolved C_{1s} spectrum regarding liposomes revealed two main peaks (Fig. 2j). The C_{1s} peak at 285.5 eV was mainly assigned to the CH_2 and C-NH_2 chemical bindings. 24 The peak at 287.4 eV was assigned to C=O chemical bindings. The spectrum of C_{1s} of pristine CA showed three main peaks (Fig. 2k). The C_{1s} peak at 285.3 eV was mainly assigned to the CH_2 and C-NH_2. The peaks at 286.9 eV was attributed to the C-O, C-OH and C-N-C=O chemical bindings meanwhile the peak at 288.4 eV was assigned to O-C-O and C=O chemical bindings.[24] The spectra of C_{1s} from CA-modified liposomes also presented three main peaks (Fig. 2l). The C_{1s} peak 285.4 eV was attributed to CH_2 and C-NH_2 chemical bindings. The peak at 286.8 eV was attributed to C-O, C-OH, and C-N-C=O chemical bindings and the peak at 288.5 eV was correspond to the peak of C=O chemical bindings. Furthermore, the presence of –NH_2 and specific peak found correspond to O-C-O chemical bindings groups on the CA-modified liposomes spectra and chemical shift in each component indicated the immobilization process of CA on the surface of liposomes *via* amide bonding formation.



(a)



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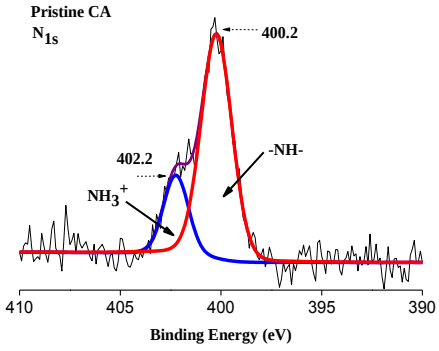

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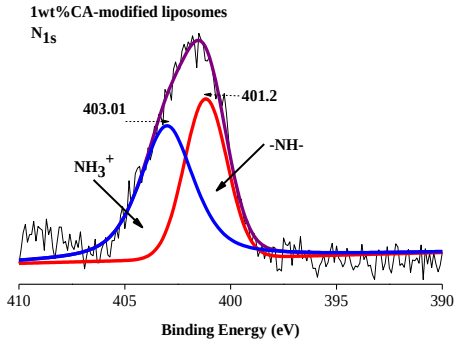

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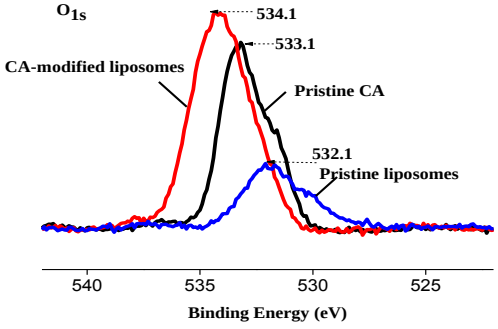

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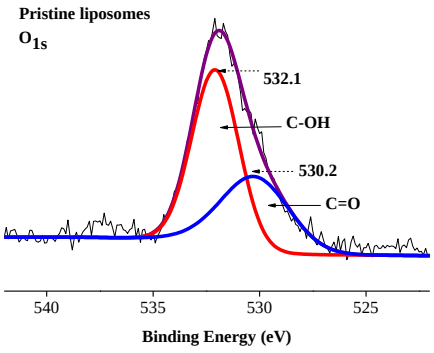

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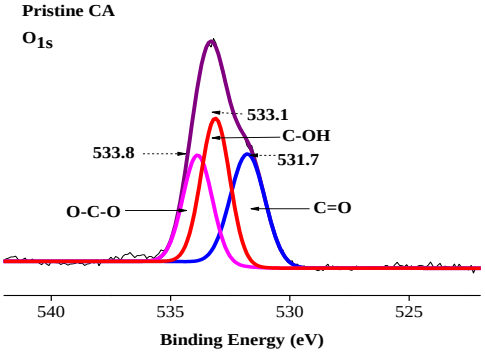
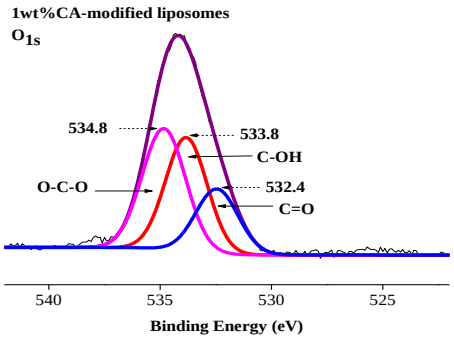


(g)



(h)

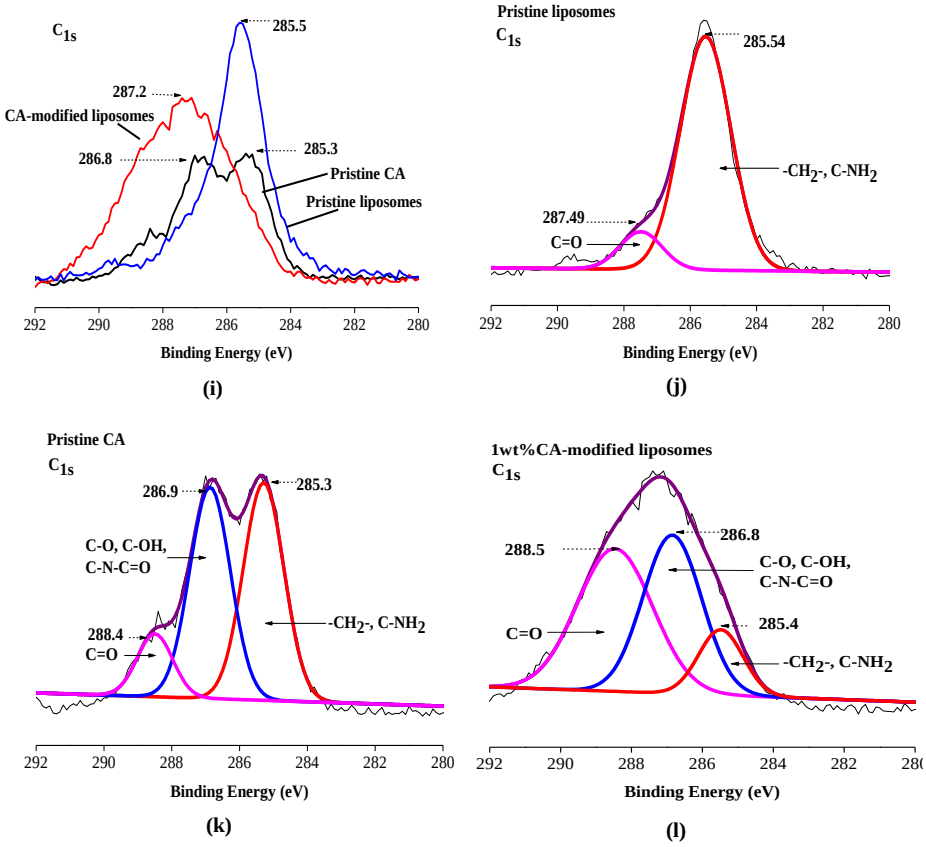


Fig. 2 XPS spectra of N_{1s} (a-d), O_{1s} (e-h), and C_{1s} (i-l) in the CA, liposomes, and 1wt% CA-modified liposomes

Figure 3 shows the TEM images of liposomes (Fig.3a) and CA-modified liposomes with various CA concentrations (Fig. 3b-3f). Based on TEM observation, conventional phospholipid vesicles are supposed to be self-closed structure, whereas liposomes composed of DPPC/DSPE /cholesterol are spherical morphology with diameter approximately 88 nm. Spherical structures also found in CA-modified liposomes (Fig. 3b-3f). Figure 3g shows the particle size and zeta potential of liposomes and CA-modified liposomes. The particle size and zeta potential of CA-modified liposomes increased with increasing CA concentration, suggesting the formation of immobilized CA layer on the surface of the liposomes.[25] Interaction of negatively charged

carboxyl groups of CA with the amino groups of DSPE resulting in a net positive charge from surface exposed amino groups of CA molecular chains. Nanocarriers with the cationic surface charge are known to enter cells relatively easily because of adsorptive interactions with the cell membrane. Therefore, cationic nanocarriers are often employed to enhance cellular delivery of payloads that are unable to enter the cells (e.g., nucleic acids) or poorly retained in the cells (e.g., antineoplastic drugs in multi-drug-resistant cells).[26-28]

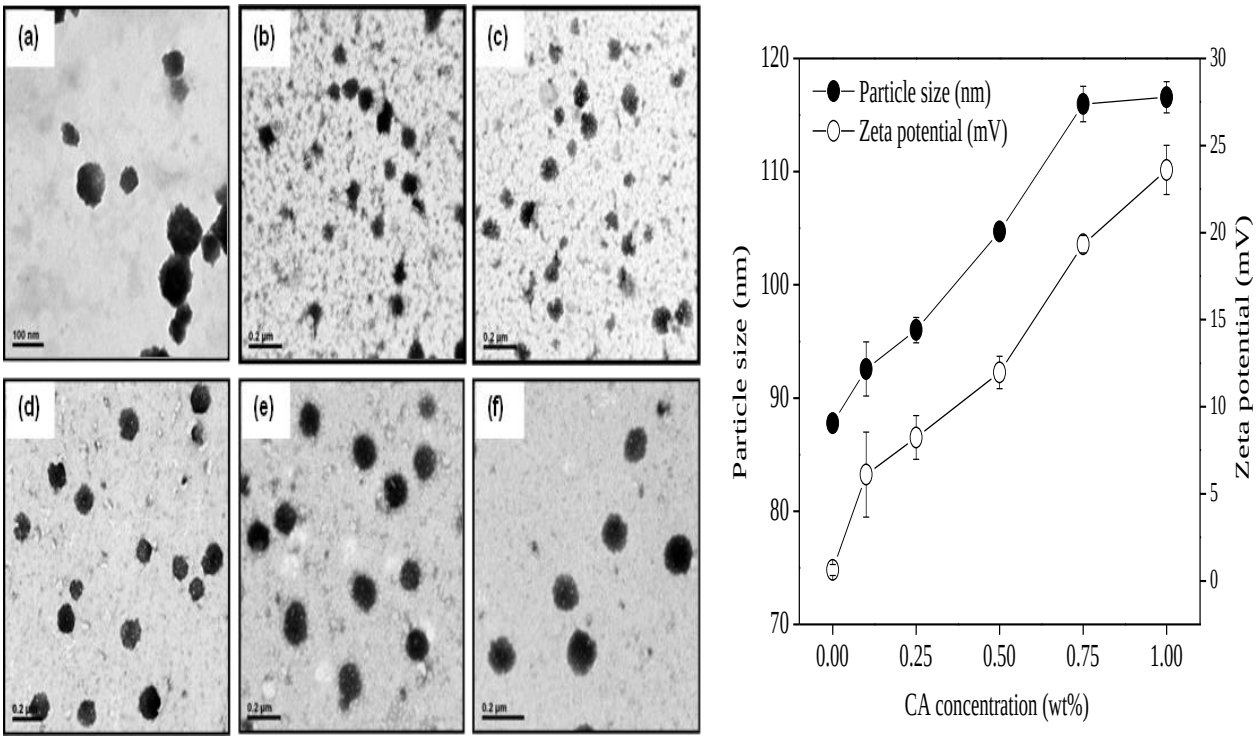


Fig. 3 TEM images of (a) liposomes and CA-modified liposomes with concentration of CA: (b) 0.1wt%, (c)0.25wt%, (d)0.5wt%, (e)0.75wt%, (f)1wt% and effect of CA concentration in the particle diameters and the zeta potential values of liposomes and CA-modified liposomes with various concentration (g)

Contact angle measurement was performed to evaluate the hydrophilicity of CA-modified liposomes. Contact angle of liposomes-DOX was found to be 37.4 ± 4.6^{o}, while the water contact angle of DOX-loaded-1wt% CA-modified liposomes was 14.5 ± 0.3^{o} (Fig. 4). The reduction of contact angle value on the DOX-loaded-1wt% CA-modified liposomes was about ~61.2%, which was statistically significant ($p<0.05$). These phenomena might be explained as DOX-loaded-1wt% CA-modified liposomes being more hydrophilic than liposomes-DOX was due to the presence of CA. The hydrophilicity increased the wettability of the surface, making the fluid spread over a large area on the surface. Previous investigation by Lee et al. explained that CA is more hydrophilic due to the presence of carboxymethyl and hexanoyl groups.[13] These results shows a clear evidence of conjugation of CA on the surface of liposomes.

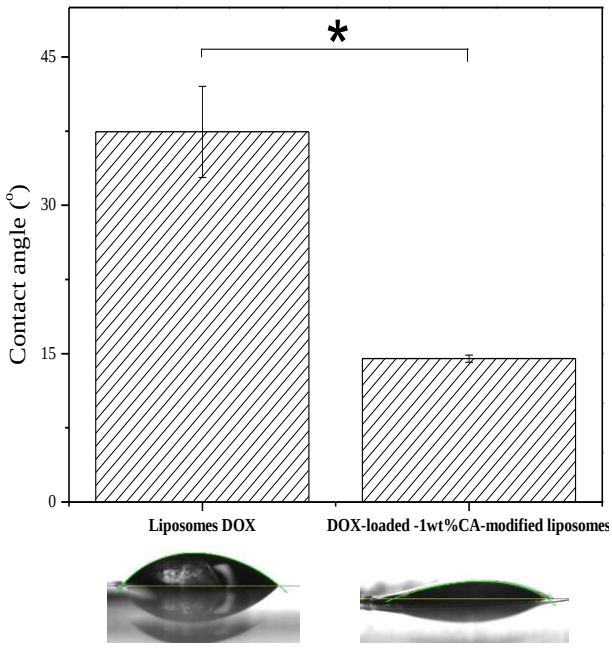


Fig. 4 Contact angle value of doxorubicin (DOX)-loaded liposomes (Liposomes DOX) and DOX-loaded-1wt%CA-modified liposomes

3.2 pH-sensitive behaviors and *in vitro* drug release studies

Figure 5 shows the particle size and zeta potential of 1 wt%CA-modified liposomes at various pH values. The particle size of CA-modified liposomes varied with the pH. This suggests that the pH-sensitivity was closely related to the attachment of CA on the surface of liposomes. On the other hand, the zeta potential of CA-modified liposomes decreased with increasing pH because the amino groups are dissociated at higher pH values. These results suggest that CA-modified liposomes exhibited a unique pH-dependent behavior, which could be exploited as a pH sensitive system. Previous investigation reported that hexanoyl groups on the carboxymethyl-hexanoyl chitosan group altered the state of water in carboxymethyl-hexanoyl chitosan by inhibiting intermolecular hydrogen bonding which makes the pH-sensitivity is more pronounced.[14]

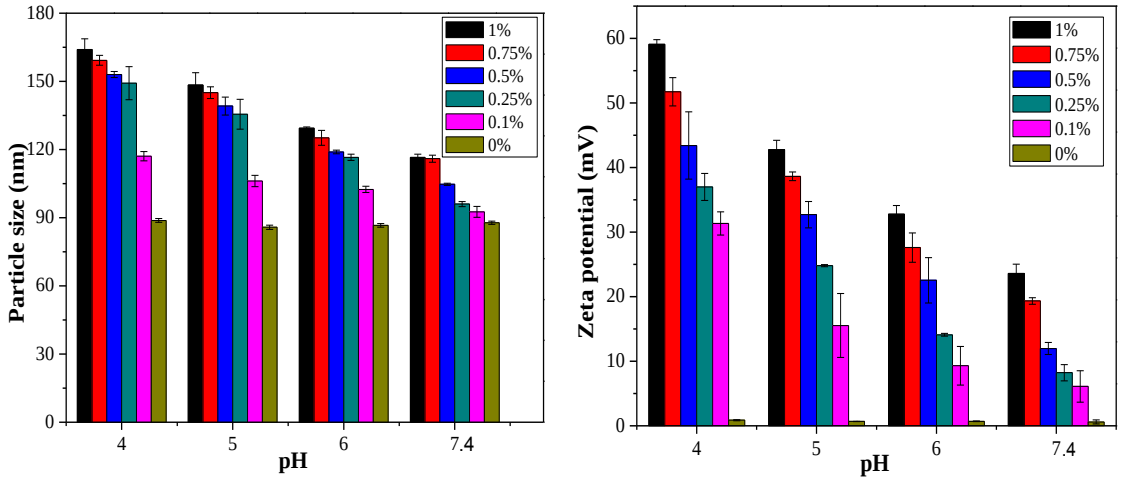


Fig. 5 Particle size and zeta potential of liposomes and CA-modified liposomes as a function of pH and concentration of CA

Encapsulation efficiency of DOX in the DOX-loaded-1wt%CA-modified liposomes was 47.5%. Figure 6a shows the in vitro drug release profile of free DOX, pristine liposomes, and DOX-loaded-1wt% CA-modified liposomes. It was observed that free DOX had a faster

diffusion rate such that completed release in less than 10 h. It was consistent with the expected rate of diffusion for low molecular weight molecules across the dialysis membrane. This is probably because the dialysis membrane affects the-rate determining step. This characteristic is in accordance with previous reported investigations.[18, 29, 30] Pristine liposomes display lower release behaviors and no significant pH-sensitivity. Significant retardation of drug release was observed for DOX in DOX-loaded-1wt% CA-modified liposomes comparing to the release of free DOX. Interestingly, DOX in DOX-loaded-1wt% CA-modified liposomes show two-step release profile. Firstly, DOX rapidly released due to the diffusion of DOX on the surface of liposomes out of the dialysis membrane then followed by a relatively slow release, which might be dominated by DOX entrapped in the aqueous compartment of liposomes. It was also found that the pH of medium could affect the release rate of DOX in CA-modified liposomes. The drug release rate reduced with increasing pH values. This could be attributed to the solubility of DOX (pKa ~8.2) at pH 4 is higher than that at pH 7.4. This characteristic is in accordance with the previous reported investigations.[31-33] Moreover, the increasing drug release rate in the lower pH could also be attribute to the changing of the structure of CA-modified liposomes as a pH-sensitive drug carrier. It displays the swelling structure caused by the stronger protonation of NH_2 into NH_3^+, which will increase with the pH value decreasing (pH 4), whereas the shrinkage structure caused by the de-protonation of NH_3^+ into NH_2 and increase with the pH value arising (pH 7.4) (Fig. 6b). Furthermore, pH-sensitive behaviors of the CA-modified liposomes contribute significantly to the drug release system as the structural changes confirmed by dynamic light scattering and zeta potential measurements.

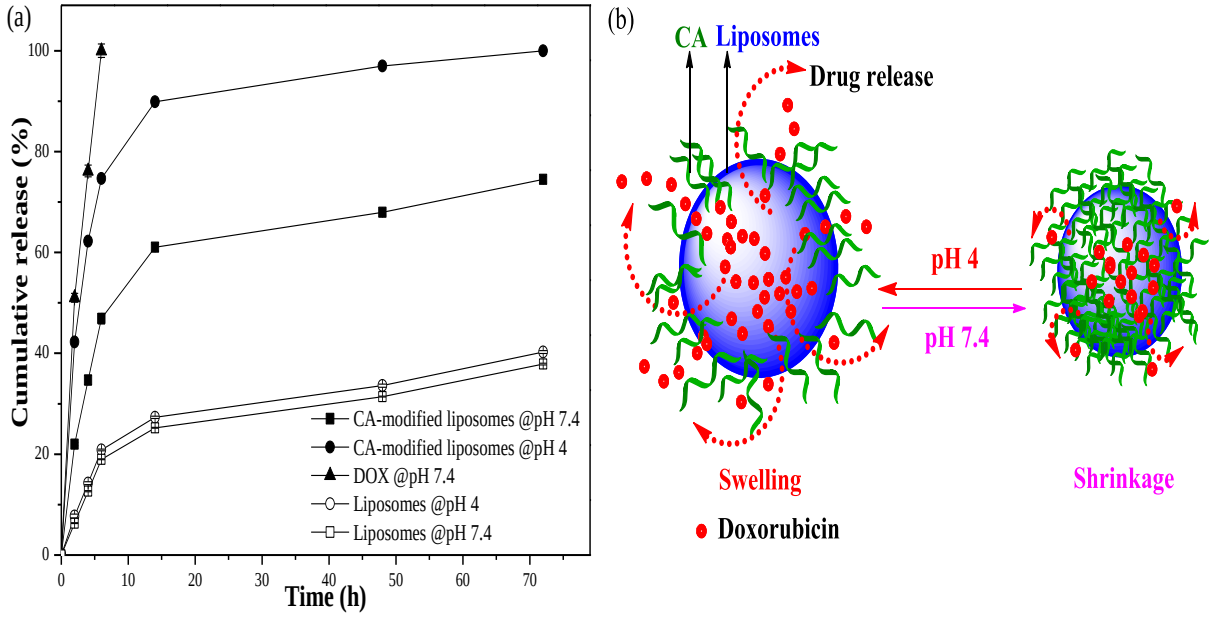


Fig.6 Drug release studies of liposomes and 1wt%CA-modified liposomes as a function of pH (a) and Schematic representation of pH-triggered release of CA-modified liposomes (b)

3.3 Cytotoxicity study

Figure 7 shows the results of the cytotoxicity assay of pristine liposomes (Fig. 7c) and CA-modified liposomes (Fig. 7d-7h) using L-929 cells as the mammalian cell model. The cells proliferated when cultured with all the samples, thus CA-modified liposomes exhibited no cytotoxicity. This might be due to the hydrophobic interaction between the hexanoyl groups and cell membranes favored the formation of initial cell contact.[14] Krajewska et al. proposed that chitosan exhibited less cytotoxic than the DPPC membrane which is similar to mammalian cells.[34] Therefore, the CA-modified liposomes could be used as a platform for biomedical applications.

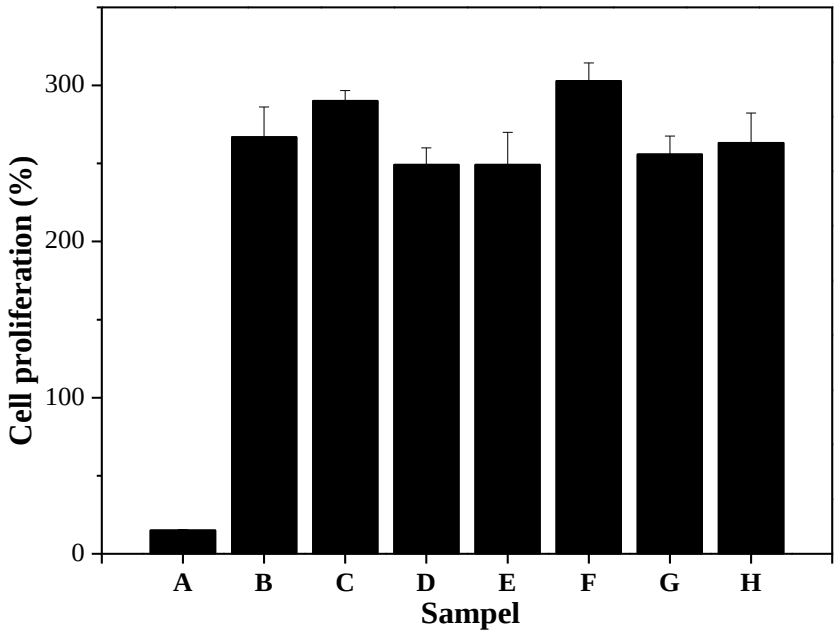
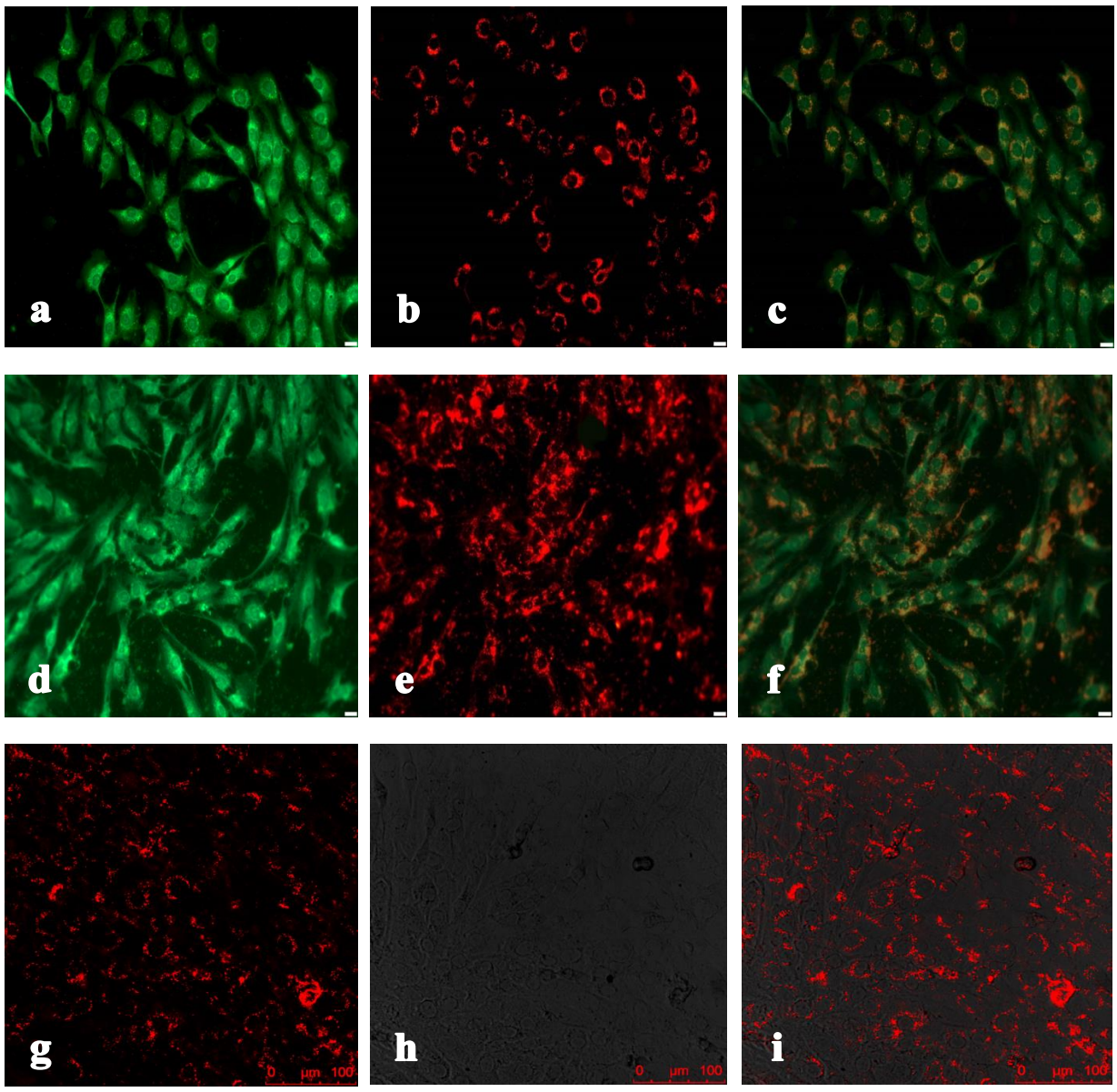


Fig. 7 Cell proliferation of L-929 cells treated with varied CA-modified liposomes after 24hr incubation. Each value is defined as the mean ± standard of deviation (n=6). Blank (+) (A) treated with 5% DMSO and Blank (-) (B) treated with medium (DMEM) only. C (Only liposomes), CA-modified liposomes with concentration of CA: (D) 0.1wt%, (E)0.25wt%, (F) 0.5wt%, (G)0.75wt%, (H) 1wt%

3.4 Cellular uptake

The fusion of liposomes with the cell membrane was examined using fluorescence microscopy by observing the dispersion of the Dil inserted in the liposome wall.[9] Fluorescence images showed that the intensity of Dil in cells contacting CA-modified liposomes (Fig.8e) is higher than the cells contacting pristine liposomes (Fig. 8b). The facilitated endocytosis in the case of liposomes might be attributed to the ability of liposomes to fuse with the cell membrane and facilitating the endocytosis mechanism.[18] On the other hand, the coating of CA on the

surface of the liposomes would improve the fusion efficiency of liposomes (Fig. 8g). Highly positive surface charge of CA-modified liposomes would improve the attraction and uptake between the negatively charged cell membrane and the liposomes, hence increasing the fusion efficiency (Fig.8j).[9, 11, 26, 28] The presence of CA-modified liposomes could be detected both in the nuclei of the cells and cells cytoplasm (Fig.8f) comparing to the cells with liposomes (Fig. 8c). In addition, after internalization of large amount of the liposomes (Fig.8a) and CA-modified liposomes (Fig. 8d) for 24 h, the cells showed insignificant change in cell geometry, i.e. size and morphology, suggesting a negligible detrimental effect of the hybrid liposomes.[12] This efficient cellular internalization and outstanding cytocompatibility are in accordance with that previously reported using hybrid of carboxymethyl hexanoyl chitosan against human breast adenocarcinoma (MCF-7)[23], bovine corneal endothelial cell (BCE)[23] and human retinal pigmented epithelium cells[12]. Furthermore, confocal laser scanning microscopy (CLSM) observation (Fig. 8g) also confirmed an excellent cellular internalization of CA-modified liposomes into the cells compartment which confirmed by red fluorescence indicated of CA-modified liposomes in the cellular compartment (Fig. 8i). Therefore, the combination of biocompatibility, cellular uptake, and internalization of the CA-modified liposomes made these hybrid liposomes a potential candidate for biomedical applications.



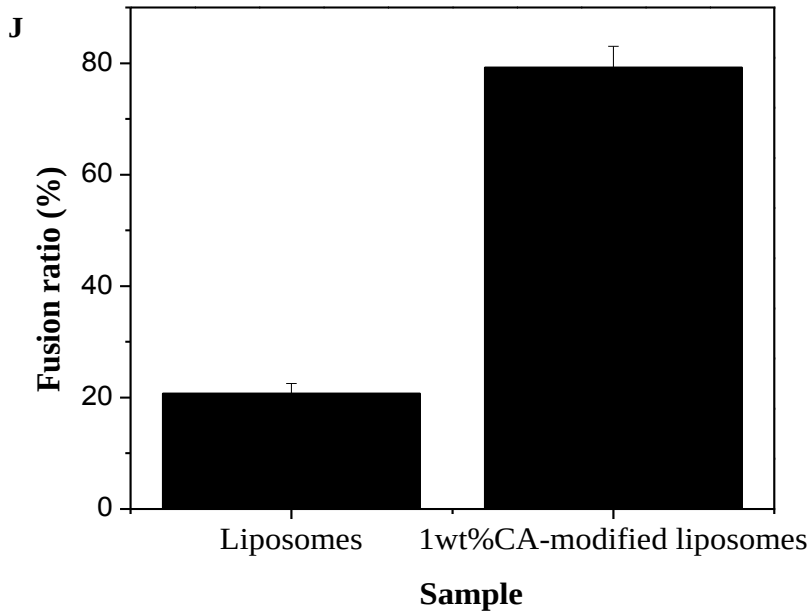


Fig. 8 Fluorescence images after L-929 cells incubated with (a) liposomes, (b) Dil-liposomes, (c) merged picture of (a and b) (d) 1wt%CA-modified liposomes, (e) Dil-1wt%CA-modified liposomes, and (f) merged picture of (d and e); Confocal imaging of Dil-modified 1wt%CA-modified liposomes (g), bright field (h) and merge (i) images; (j) Fusion ratio of liposomes and 1wt%CA-modified liposomes

4. Conclusion

We developed a novel pH-sensitive drug carriers composing of carboxymethyl-hexanoyl chitosan (Chitosonic Acid®, CA) modified liposomes (DSPE-CA) via covalent bonding (amide bonding) between liposomes-DSPE and CA-COOH as confirmed by Fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy measurements. Transmission electron microscopy observation confirmed that the particle size of CA-modified liposomes were in the range of between 88 and 117 nm. DLS and zeta potential measurement revealed that the particle

size and surface charge of CA-modified liposomes increased with increasing CA concentration, suggesting the successful formation of CA layers on the surface of the liposomes. In addition, CA-modified liposomes exhibited pH sensitivity which was confirmed by changes of particle size and surface charge in different pH values. *In vitro* drug release study revealed the sustained and controlled release profile of DOX from CA-modified liposomes. The releasing rate is very fast at lower pH value (pH 4), but it is slow at higher pH value (pH 7.4). Therefore, DOX could be rapidly released at lower pH value in the CA-modified liposomes to achieve the controlled release purpose. From a cellular point of view, CA-modified liposomes exhibit non-cytotoxicity to the L-929 cells, suggesting biocompatibility, and simultaneously increase the fusion and uptake into the cells compartment. Therefore, these novel drug carriers of CA-modified liposomes displayed pH-response behaviors, which will be applicable in the intelligent controlled release for tumor therapy.

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