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A liposomal hydrogel with enzyme triggered release for infected wound

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ABSTRACT

A novel liposomal hydrogel with enzyme triggered release of drug as drug carrier was presented for usage of infected wound. The liposomal hydrogel was prepared by an acombined method of thin-film evaporation and supercritical carbon dioxide technique (TE-scCO_2) with freeze-drying. In the liposomal hydrogel, curcumin as a model drug was embedded in the bilayer of the liposomes, and the liposomes were encapsulated in the gelatin-chitosan hydrogel. Phospholipase A_2 (PLA_2) riched in wound exudate was served tactfully as a trigger to hydrolyze lecithin in liposome and destroy the liposomes to release drug. Hydrolysis of phospholipids in liposomes was monitored by fluorescence assay of the released curcumin, and relationship between the hydrolysis of lecithin and the release of drug was discussed. The microstructure changes of the liposome after treated by PLA_2 were probed by using florescence probeembedded in the liposomes for the first time. Besides, relationship between the microstructure of liposome and hydrolysis of liposome was revealed. This liposomal hydrogel can not only inhibit the degradation of drug in conveying process and enhance the efficiency of drug release to the infected skin, but also the release of drugs can be on demand by PLA_2 biological activity changed with wound exudate in infected tissue.

1. Introduction

Biological hydrogel dressings were designed for modern dressing with an improved mechanical, antibacterial, occlusive, absorbent and adherence properties. It also could provide moist environment for wound healing (Thirumaleshwar* et al., 2012). These new dressings usually consist of polymers such as collagen, hyaluronic acid, chitosan, alginates and elastin (Boateng et al., 2008). Various materials with wound healing properties, such as antimicrobials, vitamins and growth factors, were added into chitosan/gelatin hydrogel to improve functionality of biological hydrogel dressings. However, there still exist some shortages, such as lacking of the stability of active substance, difficult degradation of the residual support materials, cytotoxic effect and so on (Sampathi et al., 2014). As a new drug delivery carrier, liposomes have obvious advantage, including good biocompatibility, strong adhesion ability on the skin and cell surface (Elsayed et al., 2006), and rapid penetration across the cell membrane (Zhai and Zhai, 2014; Hood et al., 2014), therefore they are applied in pharmaceutical technology. Previous research in our lab has also shown that liposomes are excellent carrier under many lipid vesicles circumstances (Li and An, 2016a). The liposomes have closed vesicular structure, hydrophilic pharmaceutical can be loaded in their internal water compartment, and hydrophobic pharmaceuticals can be entrapped in the bilayer of the liposomes. As a result, the liposome has high capacity of drug loading, improved drug utilization, the ability of accurately controlling drug release in targeting area (Koshkaryev et al., 2013). Combining the advantages of liposomes and hydrogel dressings, liposomal hydrogel was prepared by embedding the liposome loaded drug into hydrogel. Using liposomal hydrogel as carrier, the concentration of drug in the target site may be enhanced and the therapeutic efficacy of drug could be improved. However, liposomal hydrogel still has some shortage, for example, drug release is low and uncontrolled. To solve the problem, liposomal hydrogels modified by environmentally sensitive substance have been presented to realize drug release by changing pH, redox potential, unique enzymatic, temperature and light source (Lee and Thompson, 2017). The mechanism of drug release by environmentally responsive liposomes is that external stimuli cause the liposomes membrane destabilization and local defects, eventually leading to release the liposome-entrapped drugs (Torchilin, 2005). A thermos-pH sensitive chitosan-based hydrogel has been designed for wound dressing, which can control the drug release by changing temperature and pH (Qureshi and Khatoon, 2014).

Wound infection has been defined as the product of the growth and metabolic activities of excessive amounts of pathogenic bacteria in tissue (Hasmann et al., 2011). Phospholipase A_2 (PLA_2) is one of the large family of lipolytic enzymes, which is produced by the pathogenic

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bacteria (e.g., *S.aureus* and *P.aeruginosa*) and rich in infected wound exudates (Zhou et al., 2010). Based on the concept that bacteria secrete enzymes (including PLA_2) can damage membrane, a responsive dressing which can release antimicrobials and change colour only relying on the production of infected wounds (Zhou et al., 2010). Phospholipids are the main component of liposomes, which can be hydrolysed by PLA_2 to form haemolytic lecithin and free acid, and to destroy the liposome in the process (al. KJe, 2002). Enzyme triggered release has been concerned in stimuli-responsive systems with the advantage that it doesn't need to add other materials such as inorganic nanoparticles or polymers into liposome bilayer. PLA_2 has been used to lyse the surface-bound vesicles and to release the encapsulated substance (Chifen ‡ et al., 2007). However, rarely literature are reported about the mechanism of the release, change of the microstructure and macro property of the liposomes in the presence of enzymes, which is very important for the basic research and applied research.

In this work, liposomal hydrogel was prepared by a combined method TE-$scCO_2$ with freeze-drying. Curcumin as a model drug was loaded in liposomes, and the liposomes were encapsulated in chitosan/gelatin to form liposomal hydrogel for wound dressing. Enzyme triggered release of curcumin liposome was studied in the presence of PLA_2 which simulate the infected wound exudates environment. The Effect of PLA_2 on the microstructure changes of the liposomes were revealed by using fluorescent probe which was embedded in the liposomes. The relationship between microstructure of liposomes and drug release was explored.

2. Experimental

2.1. Materials

Egg phosphatidylcholine (EPC) (East China Normal University Chemical Reagent Co. Ltd Shanghai, China), curcumin (Sinopharm Chemical Reagent Co. Ltd Shanghai, China), PLA_2 (Sigma-Aldrich Co. LLC), chitosan (Aladdin Industrial Co. Ltd Shanghai, China), gelatin (Sinopharm Chemical Reagent Co. Ltd Shanghai, China). Chloroform, methanol, calcium chloride, glacial acetic acid and other reagents were obtained from Shanghai Lingfeng Chemical Reagent Co. Ltd and used without further purification. The water used in the experience was double distilled, and the other chemicals were of analytical reagent grade.

2.2. Preparation of liposomes and liposomal hydrogel

Curcumin liposomes were prepared by the method of TE-$scCO_2$. Briefly, egg phosphatidylcholine (EPC) and curcumin were dispersed in a binary solution of chloroform and methanol(25% *v/v*), and evaporated to a component of the film formed on the flask walls. Then an appropriate amount of double distilled water was added into disperse the lipid film to acquire liposomes. The resulted liposomal suspension was transferred to a stainless steel autoclave, and incubated under temperature of 40 ℃ and pressure of 18 MPa for an hour in $scCO_2$. The organic solvents were removed after depressurization, and a transparent liposome solution was obtained. The liposomal solution was further stirred overnight to achieve self-assembly and to remove remaining CO_2 in the liposome solution at room temperature and pressure. At last, a clear and stable liposome solution was obtained.

A mass of chitosan powder was added into acetic acid solution to dissolve it completely. Gelatin was dissolved in distilled water at 60℃. After that, the chitosan solution was mixed up with the aqueous gelatin with stirring at 40℃. After fully mixed, curcumin liposome solution was added into the mixture to form a hydrogel. Next, the obtained hydrogel was dried by vacuum freeze dryer for 35 h. At last the liposomal hydrogel sheet was obtained.

2.3. Characterization of liposomes and liposomal hydrogel

The morphology and size of liposomes were obtained by transmission electron microscope (TEM) and dynamic light scattering (DLS) technique, respectively. The encapsulation efficiency of curcumin in the liposomes was measured by centrifuge method. The free curcumin was separated from the liposome solution at a speed of 12,000 r/min. Precipitate loaded with curcumin liposome was dissolved with methanol, and fluorescence intensity of curcumin in liposomes were detected fluorescence spectrophotometer, in which excitation wavelength and emission wavelength were 425 nm and 540 nm, respectively. And the curcumin concentration was determined by the standard curve of fluorescence intensity. 1,6-diphenyl-1,3,5-hexatriene (DPH) as a hydrophobic fluorescence probe was embedded in the bilayer of liposomes to probe the microstructure (Qiu and An, 2013). Zate potential of the liposome was used to evaluate the stability of liposome, and the zate potential of the liposomes for several weeks to even months were determined to assess the long-term stability of liposomes. All experiment was repeated over five times, and significance test and detailed assay condition were shown in ESI.

Swelling behaver of the liposomal hydrogel samples were explored by immersing the hydrogel in distilled water (pH = 6.0) at 25℃. Porous performance of hydrogel sheets was observed by polarized microscope.

2.4. Hydrolysis of lecithin and liposomes by PLA_2

Phospholipids can be hydrolysed by PLA_2, and product fatty acid and lysolecithin. The hydrolysis process can be estimated by measuring the concentration of fatty acids. The concentration of fatty was determined at 559 nm of wavelength by on-line measuring of UV–vis spectroscopy with phenol red as an acid-base indicator (Morgado et al., 1995).

The effect of the PLA_2 hydrolysis on the size distribution of the curcumin liposomes solution was explored by DLS and the effect on morphology of liposomes was studied by TEM. The transmittance changes of liposome solution was determined by UV–vis spectrophotometer, which indicates the stability has changed with the the hydrolysis of liposomes. Liposome solution without PLA_2 were used as control group in the experiment.

2.5. Drug release from liposomes and liposomal hydrogel

Liposomes was added into the simulate wound exudate solution contained with PLA_2, and phospholipids in the liposomes, which reacted with PLA_2 at 37℃ for 3 h. Then the solution was centrifuged for 20 min at 12,000 r/min and the fluorescence intensity of curcumin released from liposomal hydrogel was measured in the supernatant by fluorescence spectrophotometer. The excitation wavelength and emission wavelength were 425 nm and 540 nm, respectively. The curcumin concentration was determined by fluorescence intensity - concentration standard curve, which was obtained by the relationship of fluorescence intensity and concentration of curcumin methanol solution. The intensity - concentration standard curve has been shown in ESI. Liposome solution without PLA_2 was used as control group in the experiment.

In order to explore effect of PLA_2 on curcumin release of liposome hydrogel, the curcumin fluorescence intensity with time in solution with PLA_2 and without PLA_2 was measured. The fluorescence intensity of curcumin was transferred to the concentration of curcumin by using the intensity-concentration standard curve. The intensity - concentration standard curve has been shown in ESI. The curcumin release was determined by following equation.

$$Curcumin\ Release\ (\%) = m/m_0 \times 100$$

Where m_0 is the weight of total curcumin encapsulated in liposome solution, m is the weight of released drug from liposome solution.

Liposomal hydrogel in PLA_2 solution was submerged in sodium

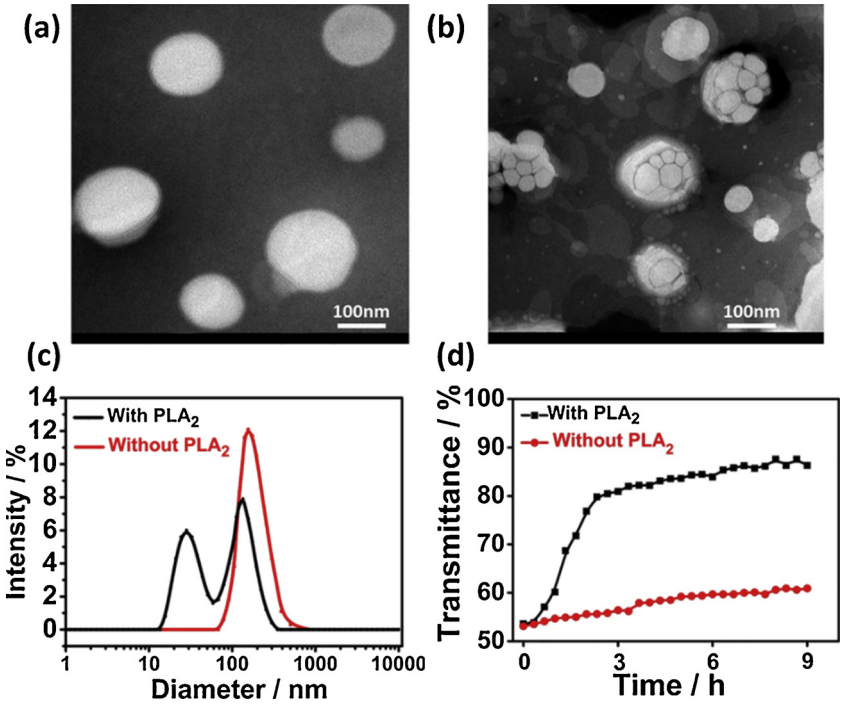

Fig. 1. (a) TEM images of curcumin liposomes in water solution; (b) TEM images of curcumin liposomes incubated with PLA_2 for 3 h. (c) Particle size distribution of curcumin liposomes in water solution and in PLA_2 water solution for 3 h. (d)Transmittance change of curcumin liposomes with time in PLA_2 solution.

dodecyl sulfonate (SDS) solution (5% *w/w*) which was used as a release acceptor with the presence of PLA_2 at 37°C for several hours, and the released curcumin in the SDS solution was identified by fluorescence spectrophotometer. Liposomal hydrogel in SDS solution without PLA_2 were used as control group in the experiment.

2.6. Microstructure of the liposomes

Fluorescence probes of pyrene and diphenylhexatriene were used to explore the membrance fluidities and micropolarity of liposomes, respectively. In order to probe the loading position of curcumin as drug in the liposomes, diphenylhexatriene (DPH) as a fluorescence probe was encapsulated in the lipid bilayers for both systems of blank liposomes and curcumin liposomes. Fluorescent probe pyrene enveloped in the bilayer of the liposomes was used to explore the change of micropolarity of membrance under the action of PLA_2. The pyrene monomer fluorescence spectrum exhibits significant fine structure in the form of five predominant peaks at excitation wavelength of 335 nm. Pyrene monomer (I_m) and excimer (I_e) present fluorescent intensity of 398 nm and 475 nm respectively, the fluorescence intensity ratio of pyrene (I_e/I_m) was usually used to probe the change of free volume of the membrane (Ioffe and Gorbenko, 2005). Peak I (I_1, at 374 nm) shows significant intensity enhancement when the environment is more polar, whereas peak III (I_3, at 384 nm) shows minimal intensity variation with polarity. Thus, the intensity ratio of I_1/I_3 is an empiric measure of polarity(Zhang and Wang, 2016).

3. Results and discussion

3.1. Characterization of liposomes and liposomal hydrogel

Liposomes with favourable properties are essential for the preparation of liposomal hydrogel. Curcumin liposomes were synthesized by TE-$scCO_2$ method. Size of the liposome obtained by DLS method which was 188 ± 12 nm. The encapsulation efficiency of curcumin in the liposome was about 89%, which is higher than that prepared by thin-film evaporationmethod (Zhang and Wang, 2016). Zeta potential of liposomes was used to estimate the stability, and the value (about −35 mV) remained almost unchanged for 3 months, it means that the designed liposomes can stable for at least 3 months.

The liposomes were efficiently packaged in polyporous gelatin/chitosan hydrogels with three-dimensional cross structure by freeze-drying method as shown in ESI, which was beneficial to the water vapour permeation. Furthermore, the liposomal hydrogels can absorb 17–18 times water of their own weight. The water absorbing capacity of this liposomal hydrogel was much higher than previously published work (Fan et al., 2016). This amazing water absorption capacity was beneficial to absorb wound exudates.

3.2. Lecithin hydrolysis and liposomes destroy by PLA_2

Lecithin can be hydrolysed by phospholipase A_2 to produce free fatty acids and hemolytic lecithin. Therefore, the hydrolysis of lecithin can be estimated by producing free fatty acids concentration. In the hydrolysis process, phenol red was used as an acid-base indicator to determine the free fatty acids concentration by using ultraviolet and visible spectrophotometer. (Morgado et al., 1995). The lecithin that make up the liposome were hydrolysed by PLA_2 in the simulate wound environment (temperature of 37°C, pH of 6 and calcium concentration of 2.5 mM) (Hansen et al., 2015; Fluhr et al., 2004). It was found that PLA_2 showed higher hydrolytic activity under the above conditions, and the results were showed in the ESI. It has been shown that liposomes and PLA_2 are stable respectively in simulate wound environment (Qiu and An, 2013; Li and An, 2016a; Saetern et al., 2005; Lahdesmaki et al., 2012; Morgado et al., 1996), and the reaction after their mixing can be considered as the hydrolysis of PLA_2 on liposome. It means that the hydrolysis of lecithin by PLA_2 in the simulate wound environment can demonstrate the effectiveness of PLA_2 as a liposomes release drug switch.

The liposome composed of lecithin was destroyed in the simulate wound environment due to hydrolysis of lecithin. The change of liposomes morphology can explain this disruption process which was observed by TEM as shown in Fig. 1(a) and (b). The curcumin liposomes exhibited a spherical vesicular structure with particle size of

150˜200 nm in Fig.1(a). After treated by PLA_2 for 3 h, liposomes present a multicapsular structure with smaller liposomes of 100˜150 nm and micellar structure about 20˜30 nm shown in Fig.1(b). It could be due to the lecithin, which make up the liposomes, was hydrolysed by PLA_2 to haemolytic lecithin and fatty acid, and the haemolytic lecithin can't form stable liposomes (Staneva et al., 2004). The size distribution of the curcumin liposomes after adding PLA_2 for 3 h as shown in Fig.1(c). As shown in Fig. 1(c), the average diameter of liposomes in water solution was 175 ± 21 nm, but the size distribution of liposomes in PLA_2 water solution showed two peaks (134 ± 14 nm and 32 ± 2 nm), which was consistent with the results from TEM. It indicated that the lecithin that forms the liposome was hydrolysed gradually by PLA_2, causing the liposomes to be destroyed. Furthermore, the influence of PLA_2 hydrolysis on liposomes stability was inspected by changes in the transmittance of liposome solution from the perspective of particle size change. As shown in Fig.1(d), the transmittance was changed from 54.6% to 84.8% in 3 h, it means that the particle size became trail off. This result may be because lecithin is hydrolysed and resulted in the liposome to be split.

3.3. Microstructure of curcumin liposomes

Anisotropy values of fluorescence probe (DPH) encapsulated in the liposome bilayer was applied to evaluate the membrane structure and the changes of liposome. The fluorescence anisotropy values of DPH in blank liposome and curcumin liposome were 0.15 ± 0.01 and 0.08 ± 0.01(n = 4), respectively. Anisotropy value (0.08) for the curcumin liposomes was lower than that (0.15) for blank liposome. This means that the arrangement of the curcumin liposome membrane was looser than the blank one and the membrance fluidity of the curcumin liposomes is higher than that of the blank liposomes (Dhanikula and Panchagnula, 2008; Sugikawa et al., 2016; Dhanikula and Panchagnula, 2008; Sugikawa et al., 2016), because the original structure in the bilayer was altered due to insertion of curcumin. A similar phenomenon has been reported when hydrophobic nanoparticles was inserted in bilayer of liposomes (Yan and An, 2013). The above results also evidence that curcumin was encased in the bilayer of the liposome from the perspective of microstructure.

The pyrene monomer fluorescence spectrum exhibits significant fine structure in the form of five predominant peaks. Peak *I* (I_1, at 374 nm) shows significant intensity enhancement when the environment is more polar, whereas peak *III* (I_3, at 384 nm) shows minimal intensity variation with polarity. Thus, the intensity ratio of I_1/I_3 is an empiric measure of polarity (Li and An, 2016b). Fluorescent probe pyrene was embedded in the bilayer of liposomes, and the micropolarity of liposomes was estimated from measurement of the pyrene polarity index (I_1/I_3) (Zhang and Wang, 2016). The values of pyrene polarity index (I_1/I_3) of pyrene encapsulated in curcumin liposomes in water solution and in PLA_2 solution were shown in Fig. 2(a). The value was gradually increased with time in the presence of PLA_2, this observation revealed that liposomes tend to have a higher micropolarity than before. This may be caused by the lecithin in the membrane lose one of non-polar hydrophobic chain which made it easier for polar water molecules to enter the non-polar area of the membrane, which increased the polarity of membrane.

The fluorescence intensity ratio of pyrene (I_e/I_m) was usually used to probe the change of free volume of the membrane (Ioffe and Gorbenko, 2005). In order to further illustrate the role of the PLA_2 on the lecithin hydrolysis and the liposome microstructure change, fluorescent probe pyrene was encapsulated in the bilayer of liposomes, and the microstructure change information of the liposomes was obtained by measuring variation of pyrene fluorescence intensity in the PLA_2 solution using fluorescence spectrum. The values of pyrene (I_e/I_m) for both systems of curcumin liposomes with and without PLA_2 were shown in Fig. 2(b), the ratio increased in the presence of PLA_2. It means that enzyme reaction disrupted the originally ordered structure and increased the free volume of the membrane. This may be caused by the lecithin in the membrane lost one of the alkyl chain which product space defects in the membrane. Both analyses of free volume and micropolarity mean that the action of PLA_2 leaded a more incompact structure of liposomes bilayer.

3.4. Drug release from liposomes and liposomal hydrogel with the presence of PLA_2

As mentioned above, the microstructure of liposomes can be destroyed by PLA_2 hydrolysis, and this phenomenon can be used for liposomes drug release. In this research, PLA_2 is a drug release switch in the liposome, and the chitosan-gelatin hydrogel only acts as carrier of liposome. In order to reveal enzyme triggered the release of drug specific function. The relationship of drug release and hydrolysis of lecithin by the PLA_2 were studied. The curcumin released by PLA_2 triggered was evaluated by centrifuge method. The concentration of curcumin remained in liposomes was detected by fluorescence spectra (Aili et al., 2011), and curcumin liposomes without PLA_2 were also tested for comparison. The curcumin release from liposomes in PLA_2 solution changed with time as shown in Fig.3(a). It can be seen that 50% of curcumin was released from liposomes at 10 h in PLA_2 solution, which is much higher than 7% of curcumin release in water solution. It indicated that PLA_2 had an obvious enzyme stimulus-sensitive effect on liposomes, and it can effectively improve the drug release of liposomes.

In addition, to evaluate the PLA_2-sensitive release of drug in liposomal hydrogel, this liposomal hydrogel was submerged in SDS solution in the presence of PLA_2, and the concentration of curcumin in the external fluid was detected by fluorescence spectra, free curcumin hydrogel was used for comparison. Relationship of curcumin release from liposomal hydrogel and time was shown in Fig.3(b). The curcumin release rates of two samples were similar in the initial 6 h. But after 6 h, the release rate of curcumin in the free curcumin hydrogels was lower than that in the liposome hydrogel. Finally, 76% of curcumin in curcumin liposomal hydrogel can be released in 72 h, but only 45% of curcumin was released from free curcumin hydrogel. There are several reasons can explain the higher drug release in liposomal hydrogel.

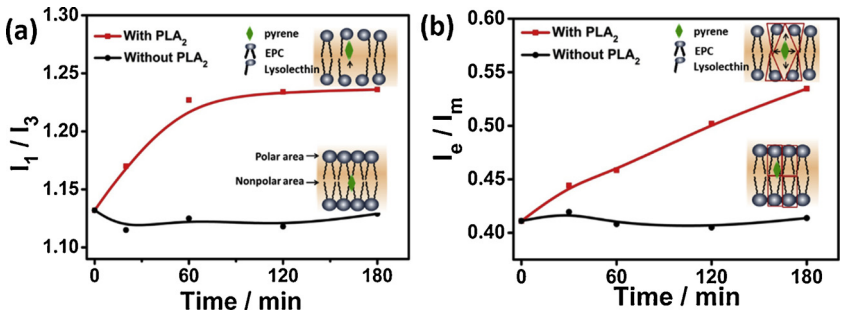

Fig. 2. a) Pyrene polarity index (I_1/I_3) changed with time for curcumin liposomes in water solution and PLA_2 solution. (b) The fluorescence intensity ratio (I_e/I_m) of pyrene changed with time for both systems of curcumin liposomes in water solution and PLA_2 solution.

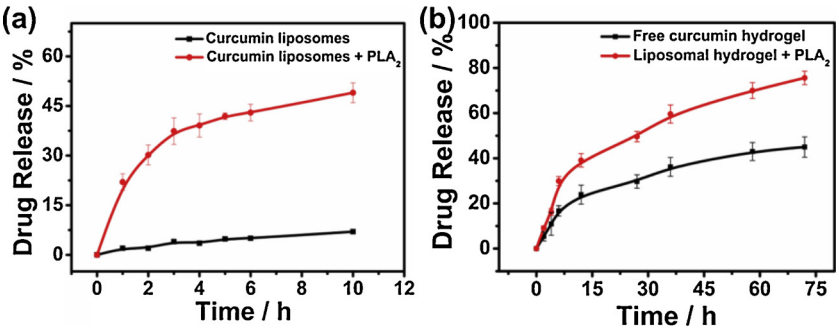

Fig. 3. (a) Relationship of curcumin release from liposomes and time. (b) Relationship of curcumin release from liposomal hydrogel and time.

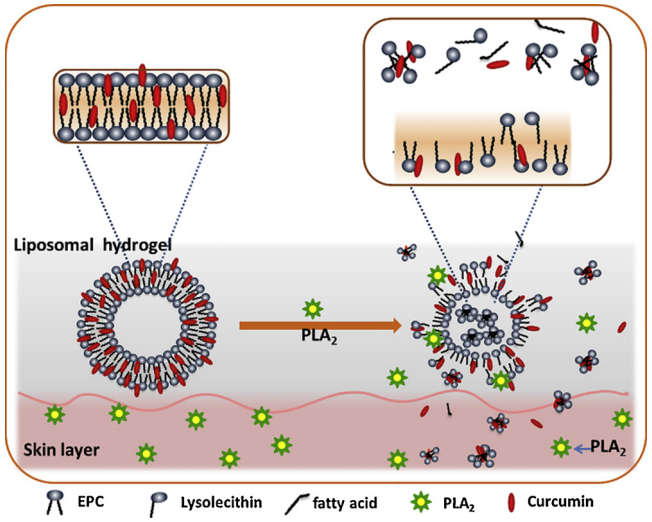

Fig. 4. Schematic representation of curcumin liposomal hydrogel effect at infected skin.

Firstly, the curcumin was protected by liposomes to avoid degradation. More importantly, lecithin in the liposomes was hydrolyzed by PLA_2 and formed to lysolecithin, which can improve the solubility of curcumin in hydrogel and accelerate the diffusion of curcumin and increase the release rate.

Based on the above results from PLA_2 stimulus sensitive drug release and the effect of PLA_2 on the microstructure of liposomes, mechanism of curcumin liposomal hydrogel effect on infected skin could be inferred as follows. As showed in Fig.4, liposomal hydrogel loaded with curcumin is applied to infected skin, and infected wound exudate enriched in PLA_2 produced by the pathogenic bacteria was absorbed by the liposomal hydrogel. Then the phospholipids of liposomal was hydrolysed by the PLA_2, which caused the liposome bilayer became thinner and looser. Finally, the drug was released from the destroyed liposomes. Some of free phospholipids in the outer layers can carried curcumin to be reassembled into micelles, and the phospholipids of the inner layer are reassembled into multicapsular micelles due to surface tension. Finally the micelles carried curcumin was entered the target site through the skin layer.

4. Conclusion

A liposomal hydrogel loaded with curcumin was prepared by a combined method TE-$scCO_2$ with freeze-drying. Phospholipase A_2 (PLA_2) in the infected wound exudate was served tactfully as a release switch trigger to hydrolyze lecithin in liposome and destroy the liposomes to release drug. Effect of PLA_2 hydrolysis on the morphology and size of liposomes was studied by using TEM and DLS method, and it was found that the liposome size before the hydrolysis was 175 ± 21 nm, while the liposome size after the hydrolysis was 134 ± 14 nm and 32 ± 2 nm. Effect of PLA_2 hydrolysis on the microstructure of liposomes was explored by using florescence probe embedded in the liposomes, and both analyses of free volume and micropolarity demonstrated that the action of PLA_2 leaded a more incompact structure of liposomes bilayer. The relationship between the microstructure of liposomes and curcumin release was probed. The results indicated that the phospholipids in the liposomal was hydrolysed by the PLA_2, which caused the liposome bilayer became thinner and looser, and the drug was released from the destroyed liposomes. This liposomal hydrogel can not only inhibit the degradation of drug in conveying process and enhance the efficiency of drug release to the infected skin, but also control release drugs on demand by PLA_2 biological activity changed with wound infected. This phospholipase triggered liposomal hydrogel has high potential for broad application foreground in curing infected tissue.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chemphyslip.2019.104783.

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