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Liposomes self-assembled from electrosprayed composite microparticles

Deng-Guang Yu¹, Jun-He Yang, Xia Wang and Feng Tian

School of Materials Science and Engineering, University of Shanghai for Science and Technology, Shanghai 200093, People’s Republic of China

E-mail: ydg017@gmail.com

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Abstract
Composite microparticles, consisting of polyvinylpyrrolidone (PVP), naproxen (NAP) and lecithin (PC), have been successfully prepared using an electrospraying process and exploited as templates to manipulate molecular self-assembly for the synthesis of liposomes in situ. Field emission scanning electron microscope (FESEM) and transmission electron microscope (TEM) observations demonstrate that the microparticles have an average diameter of 960 ± 140 nm and a homogeneous structure. X-ray diffraction (XRD) patterns, differential scanning calorimetry (DSC) and attenuated total reflectance-Fourier transform infrared (ATR-FTIR) results verify that the building blocks NAP and PC are scattered in the polymer matrix in a molecular way owing to the very fast drying of the electrospraying process and the favorable secondary interactions among the components. FESEM, scanning probe microscope (SPM) and TEM observations demonstrate that the liposomes can be achieved through molecular self-assembly in situ when the microparticles contact water thanks to ‘like prefers like’ and by means of the confinement effect of the microparticles. The liposomes have an encapsulation rate of 91.3%, and 80.7% of the drug in the liposomes can be freed into the dissolution medium in a sustained way and by a diffusion mechanism over a period of 24 h. The developed strategy not only provides a new, facile, and effective method to assemble and organize molecules of multiple components into liposomes with electrosprayed microparticles as templates, but also opens a new avenue for nanofabrication in a step-by-step and controllable way.

(Some figures may appear in colour only in the online journal)

1. Introduction

‘How far can we push chemical self-assembly?’ is, without doubt, a most intriguing challenge that waits to be solved in the decades to come [1, 2]. Traditionally, sophisticated methods have often been used to assemble and organize molecules into functional nano-objects. These methods invariably result in mixtures of different products because: (1) we are unsure about how to directly manipulate transfer and contact of building blocks on a molecular scale and (2) building blocks always undergo Brownian movement randomly in the environment and the forces between them are very small. New methodologies, mechanistic insights, and precisely controlled assemblies are just a few of the important topics in this interdisciplinary field of science. In particular, strategies that offer simple routes to synthesis of nano-objects, straight from templates, could be the next big thing in nanofabrication [3–5].

Electrospinning, a ‘top-down’ nanofiber fabrication process, is very popular today because of its ease of implementation, ability to treat a lot of macromolecules, and the potential applications of its products in a wide variety of fields [6–15]. Electrospun nanofibers have not only been exploited as good templates (by means of their configuration as a whole and through post-treatment of nanofiber
mat) in producing functional nano-objects indirectly, such as inorganic nanotubes, fibrous hydrogel materials with encapsulated microbes, and carbon nanofibers [16–19], but also as templates that directly manipulate molecular self-assembly of multiple components to fabricate nano-objects in situ, such as solid lipid nanoparticles and liposomes, by means of the nanometer confinement effect of the nanofibers [20, 21].

Based on the same principles as electrospinning, electrospraying (or sometimes electrohydrodynamic atomization, EHDA) is growing in popularity because of its ability to easily fabricate particles and thin films. Electrospraying and electrospinning produce fibers and particles at the micro- and nanoscales by exploiting electrical forces directly. Electrospraying generates particles with mean diameters that vary between hundreds of micrometers and tens of nanometers in a simple and straightforward process [22–26]. Compared with electrospinning, electrospraying is also a ‘top-down’ fabrication process, which is simple and easy to implement. Correspondingly, its products, particles at micro- or nanoscales, may be good templates in the production of microstructures by means of their configurations and in manipulating molecular self-assembly by means of the confinement effect at the micro-scale. Almost all the polymer excipients can be electrosprayed into micro- or nanoparticles with active ingredients, provided that suitable solvents are employed. Only limited polymers with appropriate molecular weights have electrospinnability, and only often in a narrow window. Thus, electrosprayed particles may provide versatile ways for developing novel nanodrug delivery systems, and lead to a wide variety of new functional materials for nanotechnological applications.

Liposomes are unique in their ability to accommodate many types of active ingredients, such as drugs, genes, diagnostic agents, nutrients, and cosmetic ingredients. In the medical field, a variety of possible applications are currently under investigation worldwide, including targeting cancer cells, vaccine and protein delivery, gene therapy, combating multi-drug resistance, and developing long-circulation drug delivery systems [27–29]. New facile and straightforward processes that yield high-quality products, particularly with enhanced stability, are therefore much sought after.

Based on current knowledge mentioned above, we investigate here the use of electrosprayed microparticles, which are exploited as templates, to manipulate molecular self-assembly to produce liposomes in situ. The electrospraying process provides an opportunity for us to seal the building blocks into a confined micro-region in molecular composites. The outcome of a molecular self-assembly process is not only determined by the building blocks and the possible connections between them, but also by the means through which the building blocks are brought into contact with each other. However, the difficulty of agitating a viscous solution on a small scale (beyond changes in solution temperature and viscosity) means that there is little control over the transport and the contact of building blocks on a molecular scale [30, 31]. Pre-positioning the building blocks in a confined micro-region can give us the ability to manipulate molecular transfer and contact on a micro-scale.

2. Experimental details

2.1. Materials

Polyvinylpyrrolidone K17 (PVP K17, \(M_w = 8500\)) was purchased from Shanghai Yunhong Pharmaceutical Aids and Technology Co., Ltd (Shanghai, China). Naproxen (NAP) was obtained from Shanghai Greentech Industries Co., Ltd (Shanghai, China). Lecithin (PC, extracted from soy bean) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China), as was analytical grade chloroform. Water was double distilled before use.

2.2. The electrospraying process

A co-dissolving solution of PVP K17, PC, and NAP in chloroform, with a ratio of 25%:4.5%:0.5% (w/v), was prepared as the electrospraying solution.

The co-dissolving solution was fed to a metal capillary with an inner diameter of 0.5 mm. The flow rate of the electrospraying solution was controlled using a syringe pump (KDS100, Cole-Parmer®, Vernon Hills, IL, USA) and maintained at 1.0 cm\(^{-1}\) h\(^{-1}\). The microparticles were collected on a plate wrapped using aluminum foil, which was kept at a fixed distance of 25 cm from the needle tip of the electrospraying head. A 12 kV voltage was applied using a high-voltage power supply (Shanghai Sute Electrical Co., Ltd, Shanghai, China). The electrospraying process was recorded using a digital video recorder (Canon PowerShot A640, Tokyo, Japan).

2.3. Characterization of the electrosprayed microparticles

2.3.1. Morphology. Morphologies of the microparticles were assessed using an S-4800 field emission scanning electron microscope (FESEM, Hitachi, Tokyo, Japan). Average particle size was determined by measuring the diameters of more than 100 microparticles in FESEM images using the Image J software (National Institutes of Health, Bethesda, MD, USA). Samples were carbon coated prior to observation.

Transmission electron microscope (TEM) images of the samples were recorded on a JEM 2100F field emission transmission electron microscope (JEOL, Tokyo, Japan). TEM samples of microparticles were collected by fixing a lacey carbon-coated copper grid on the collector.

2.3.2. Physical status of components in the microparticles and their interactions. Differential scanning calorimetry (DSC) analyses were carried out using an MDSC 2910 differential scanning calorimeter (TA Instruments Co., USA). Sealed samples were heated at 10°C min\(^{-1}\) from 20 to 250°C under a flow of nitrogen gas (40 ml min\(^{-1}\)). X-ray diffraction patterns (XRD) were obtained on a D/Max-BR diffractometer (RigaKu, Tokyo, Japan), with Cu Kα radiation over a 2θ range 5°–60° at 40 mV and 30 mA. Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) analysis was carried out on a Nicolet-Nexus 670 FTIR spectrometer (Nicolet Instrument Corporation, Madison, USA) over a 500–4000 cm\(^{-1}\) range and at a resolution of 2 cm\(^{-1}\).
2.4. Characterization of the self-assembled liposomes

2.4.1. Morphology and diameter. A drop of water from a micro-injector was placed on some microparticles collected on a glass slide, which was allowed to dry naturally, to observe the self-assembled liposomes using FESEM and a scanning probe microscope (SPM; DI-NSIV Digital Instruments, New York, USA).

The average hydrodynamic diameter and size distribution of the self-assembled nanoparticles were determined using BI-200SM static and dynamic light scattering (SDLC) instruments (Brookhaven Instruments Corporation, Austin, TX, USA). To prepare the samples, 0.2 g of the composite microparticles that had been peeled away from the collector was placed in 100 ml of water to self-assemble into liposome suspensions.

One drop of the self-assembled suspensions was then spread onto a carbon-coated copper grid for TEM observation of the self-assembled liposomes.

2.4.2. Drug entrapment efficiency. Drug content in the self-assembled liposomes was determined by calculating the difference between the total drug concentrations in the liposome suspension and the amount of free drug in its supernatant ($w_f$). The supernatant was separated from the liposomes by ultra-centrifugation. The amount of free drug present in the supernatant was determined using a UV–vis spectrophotometer (Unico Instrument Co., Ltd, Shanghai, China) at 331 nm. A standard calibration curve of concentration versus absorbance was plotted for this purpose. The amount of drug in the supernatant was subtracted from the total amount of drug in the electrosprayed microparticles ($W_t$) to calculate the amount of drug entrapped in the self-assembled nanoparticles. Percentage drug entrapment was calculated using the formula: 

$$\text{Entrapment} = \frac{(W_t - w_f)}{W_t} \times 100\%.$$ 

2.4.3. In vitro drug release tests. One milliliter of the self-assembled liposomes was placed in a dialysis tube (MWCO = 3500 Da). The dialysis sacs were then dialyzed in 10 ml of phosphate buffer solution (PBS; pH 6.8, 0.1 M) at 37 °C, with a stirring rate of 100 rpm. At predetermined times, the PBS was removed and replaced with the same volume of fresh solution. The amount of drug released was determined by UV–vis spectrometry, as described above. Released studies were conducted six times, and mean values were plotted against time.

3. Results and discussion

3.1. Electrospraying process

As shown in figure 1(a), akin to an electrospinning system, a common electrospaying system comprises four major components, namely a high-voltage power supply, an earthed collector, a fluid-driving device-syringe pump, and a capillary for introducing the sprayed fluid.

Figure 1(b) shows a typical electrospraying process under an applied voltage of 12 kV, a flow rate of 1 ml h$^{-1}$, and a collected distance of 25 cm. The co-dissolving solution was fed to a metal capillary with an inner diameter of 0.5 mm, at the end of which a droplet forms. When the droplet is exposed to an electric field, a charge is induced on its surface. Provided the liquid has sufficient electrical conductivity, there is a range of combinations of liquid flow rate and applied voltage for which the drop assumes a conical shape (the Taylor cone). At the apex of this cone, a narrow straight jet is formed (cone-jet mode), which subsequently breaks up into fine droplets.

Electrospraying first generates near-monodisperse droplets, whose size can vary between a few micrometers to hundreds of micrometers, which provide the possibility of faster drying than a bulk drying process, such as casting film. Later, the droplets shrink rapidly because of the fast evaporation of solvents as a result of atomization. The huge surface areas of the micro-droplets provide the possibility of exhaustion of the solvents and the solidification of products. Facile interactions of electrons with the fluid solvents accelerate their evaporation [32].

Solvent removal from the droplets is a key in determining the format of the final products. If the solvent in the droplets does not evaporate effectively, the droplets connect together to form thin films on the collector. If the solvent evaporates...
well, the droplets shrink and solid particles are formed finally. Exhausting all the solvents is important in the preparation of electrosprayed particles. Different methods have been employed for this, such as using an auxiliary apparatus to raise and keep the sprayed fluid at a high temperature, using a cross flow of nitrogen, air, or a liquid bath to extract sufficient solvent to coagulate the particles [22, 23, 33].

Here, the process is easier and more practical to implement than reports in the literature [22], in which a heated sleeve is employed to maintain the electrospraying solution at an elevated temperature of 50 °C. To avoid the formation of spindle and filament structures and to achieve an instant drying effect, a PVP with a relatively lower molecular weight is better because it allows a higher concentration of the spraying solutions. The volatile solvent chloroform (61.7 °C) was selected for the electrospraying process. A high concentration of 25% (w/v) PVP and the use of chloroform can facilitate the exhaustion of solvents and the solidification of the microparticles synergistically.

3.2. Morphologies of microparticles

FESEM images of the electrosprayed microparticles are shown in figure 2(a). The microparticles have uniform structures, with an average diameter of 960 ± 140 nm. Microparticle formation, using electrospraying, involves a series of complex electrical, fluid, and mechanical stages that are governed by many factors. Among these, a steady drying process, with a drying rate that matches the traveling rate of the droplets, is a key to achieving monodisperse particles. All the microparticles here are solid and round spheres, different from those in the literature [22], which have a lot of dents on their surface resulting from un-matched evaporation of solvent.

Figure 2(b) shows clearly that all the microparticles exhibit almost the same level of contrast, reflecting a homogeneous inner structure without phase separation. In electrospraying, a liquid jet is ejected and accelerates toward the collector and, as the jet travels toward the target, the solvents evaporate rapidly. This results in the formation of microparticles in a very short time period, often several tens of milliseconds. As a consequence, and because of the compatibility between the polymer matrix and the carried components, PC and NAP are distributed uniformly throughout the solid polymer matrix, with the physical state of the multiple components in the liquid spraying solution propagated into the composite microparticles.

3.3. Physical status of the components and their interactions

To investigate the physical status of the components in the composite microparticles, DSC and XRD were conducted. As shown in figure 3, because of dehydration PVP K25 displays a broad endotherm, lying between 80 and 120 °C, with a peak at 85.4 °C. PVP is a polymer and exists in a glassy or rubber-like state. The change from one state to another can be detected as a glass transition from 170 to 180 °C [34].

PC exhibits three clearly different endothermal peaks. The first endothermal peak at 136 °C is probably caused by hot movements of the polar part of the PC molecule. The second and third endothermal peaks are probably a result of phase transitions from a gel-like state to a liquid crystal state, and of the carbon chain in the PC undergoing melting, isomeric, or crystal changes [35, 36].

DSC thermograms of the composite microparticles do not show any phase transitions corresponding to either NAP or...
PC, except for the temperature of the broad endotherm which shifts slightly, a result of PVP dehydration. The disappearance of signature peaks corresponding to NAP and PC suggests that NAP and PC lost their original structure and a PVP–PC–NAP complex was formed because of secondary interactions.

The XRD pattern of PVP (figure 4) displays a diffuse background with two diffraction halos, which demonstrates that the polymer is amorphous. PC also exhibits an amorphous pattern, which lacks any diffraction peaks except for a single broad hump. When NAP and PC are included in the PVP matrix particles, the left halo disappears. This indicates changes in the orientation, the conformation, and the organization of PVP chains in the amorphous phase because of the inclusion of NAP and PC [37]. All XRD results concur with those from DSC and TEM observations, which demonstrate that a molecular mixture or a complex of PVP–PC–NAP is formed with the present ratio of components in the composite microparticles.

To distinguish the interactions between PVP, NAP, and PC in the nanofibers, ATR-FTIR analyses were carried out. Spectra are given in figure 5. In the PC spectrum, bands corresponding to the hydrophobic tail regions of PC occur at 2853, 2923, and 1468 cm$^{-1}$, representing symmetric CH$_2$ ($\nu_s$CH$_2$), antisymmetric CH$_2$($\nu_{as}$CH$_2$) stretching, and CH$_2$ scissoring, respectively. The N$^+$CH$_3$ stretching vibration is located at 972 cm$^{-1}$. Peaks at 1233 and 1088 cm$^{-1}$ arise from the antisymmetric ($\nu_{as}$) and the symmetric ($\nu_{s}$) PO$_2^-$ stretching vibrations [38].

Secondary interactions between PC and PVP molecules can be distinguished from the ATR-FTIR spectra of the composite microparticles. Hydrophobic interactions between PVP and PC molecules can be inferred from the shifts to lower wavenumbers of peaks assigned to the $\nu_s$CH$_2$, the $\nu_{as}$CH$_2$ stretching, and the CH$_2$ scissoring motions of PC. Attractive electrostatic interactions should also take place between the negatively charged PC head group and the nitrogen atom on the pyrrolidone ring of PVP [39], and also between the negatively charged PVP oxygen (N$^+$ = C–O$^-$) and the positively charged PC head group (figure 5) [40].

NAP can form hydrogen bonds with PVP between the PVP carbonyl group and the hydroxyl group of the NAP molecule. This is evidenced by the shifts to lower wavenumbers of peaks assigned to the C=O stretching vibrations of PVP (from 1661 to 1657 cm$^{-1}$) and in the fingerprint region, where almost all of the peaks of the functional groups decrease in intensity or disappear from the spectrum of the microparticles. The secondary interactions play a fundamental role in promoting the structural homogeneity of the PVP–NAP–PC composite microparticles.

3.4. Characterization of the self-assembled liposomes

To initiate the self-assembly process, a drop of water from a micro-injector was placed on a sample of electrospayed microparticles and put on a glass slide fixed on the collector for several minutes. The sample was dried naturally in air. Figure 6(a) depicts the FESEM observations of the self-assembled liposomes. The liposomes are surrounded by light ‘haloes’ at the bottom, giving an impression that the liposomes grow from the ‘haloes’. An SPM image of the self-assembled liposomes is in excellent agreement with the FESEM results in figure 6(b). These results demonstrate that (1) the molecular self-assembly processes can happen easily on contact with water and (2) the confinement of composite microparticles plays an important role in allowing the liposomes to ‘grow’ from them. ‘Like prefers like’ is one of the most important fundamental roles in non-covalent bonding [2]. When the composite microparticles make contact with water, the hydrophilic PVP matrix dissolves quickly and...
forms semitransparent ‘haloes’, whereas the water-insoluble PC and NAP co-aggregate the liposomes and stand out of the PVP matrix. TEM images of the self-assembled liposomes are exhibited in figure 6(c). NAP molecules are aggregated into nanoparticles, with an estimated size of 8 nm, and are scattered in the liposomes, mainly in an annular way, with the periphery and the center having a small amount of NAP. A typical SDLC result is shown in figure 7. The liposomes have an average diameter of 197 ± 16 nm and a polydispersity index (PDI) of 0.274 ± 0.018. This suggests the production of vesicles with a narrower size distribution.

During the dissolution of PVP from the microparticles, some NAP was also freed into the dissolution medium. The amount of free NAP in the supernatant from the liposome suspensions was 8.7%, which means that 91.3% of the drug was encapsulated into the liposomes. After 24 h in vitro dissolution, 82.4% of the drug in the original microparticles was freed into the dissolution medium. Therefore, 80.7% \(\left[=\frac{(82.4\%-8.7\%)}{91.3\%}\right]\) of the drug in the liposomes was released during that time period (figure 8).

The mechanism of release of NAP from the self-assembled liposomes in PBS can be characterized by analyzing the dissolution data using the Peppas equation [41], \(Q = kt^n\), where \(Q\) is the percentage of drug released at time \(t\), \(k\) is a kinetic constant, and \(n\) is the diffusional exponent indicative of the release mechanism. Drug released from the self-assembled liposomes could be fitted with the equation \(Q = 36.52t^{0.12} \) \((R^2 = 0.9923)\). The value of the diffusion index \(n\) is 0.12, indicating that NAP released from
the self-assembled liposomes was controlled by a Fickian diffusion mechanism.

3.5. Self-assembly mechanism

Based on the observations above, the ‘dissolution’ of the microparticles in water is essentially an in situ molecular self-assembly process. The microparticles act as templates, and the secondary interactions among the water, PVP, PC, and NAP molecules play fundamental roles, both in the formation of polymer-based composites and in the self-assembly process as well.

A mechanism for the self-assembly process, which is consistent with the experimental data, is proposed in figure 9. This can be summarized as follows. (1) After the composite microparticles come into contact with water, strong interactions resulting from hydrogen bonding between the $\ce{C=O}$ group of PVP and the water molecules cause the water to liberate the PC and the NAP molecules ‘anchored’ onto the PVP chains, and, concomitantly, the PC molecules are hydrated. This results in the swelling of the nanofibers and gives the NAP and the hydrated PC molecules mobility. (2) The PVP matrix further absorbs water, swells, and is gradually disentangled. The NAP and the hydrated PC molecules are concentrated within the framework of the swelling microparticles because of hydrophobic repulsion from the solvent. Water molecules bridge the phosphate head groups between neighboring PC units [42]. Meanwhile, the NAP molecules co-aggregate and connect with the hydrocarbon tails of PC through hydrophobic interactions. (3) Finally, the hydrated PC molecules co-aggregate into vesicles, with NAP contained within them, and are freed into the environmental medium when the microparticles collapse completely and the PVP molecules disentangle and dissolve. The assembly process takes place spontaneously because of the highly hygroscopic and hydrophilic properties of PVP and the high surface area of the microparticles. In particular, the usage of PVP K17, which has a very small molecular weight and which can dissolve in water faster than any PVP with higher molecular weights, helps to ensure that the self-assembly process occurs instantly.

4. Conclusions and prospects

Composite microparticles, consisting of multiple components prepared using an electrospaying process, have been successfully exploited as templates to manipulate molecular self-assembly for the synthesis of liposomes in situ. A high concentration of the matrix polymer with a relatively lower molecular weight (25% PVP K17) and a suitable selection of high volatile solvent for preparing co-dissolving solutions facilitate the electrospaying process and the solidification of microparticles. By means of the effect of confinement on the microparticles, their components can self-assemble into liposomes when they come into contact with water based on the non-covalent bonding rule of ‘like prefers like’. The liposomes have an encapsulation rate of 91.3%, and 80.7% of the drug in the liposomes is freed into the dissolution medium in a sustained way and by a diffusion mechanism over a period of 24 h.

The present study demonstrates a novel strategy for preparing liposomes in situ from electrospayed composite microparticles consisting of multiple components. The process has significant advantages over traditional routes in the preparation of liposomes. The preparation process is free of problematic heating, cooling, agitation, sonication, or extrusion steps, and does not require sterilization. The final products have significantly enhanced properties. Liposome precursors can be stored ‘frozen’ in the solid state microparticles, giving them high stability. However, they can be converted easily to liquid suspensions on demand.

By combining electrospaying and molecular self-assembly, a strategy is developed in which the first object at a micro-scale is produced using ‘top-down’ electrospaying, and the later objects at a nanoscale are produced through a ‘bottom-up’ molecular self-assembly. This strategy not
only provides a new, facile, and effective methodology to assemble and organize molecules of multiple components into liposomes through electrosprayed templates, but also opens a new avenue for nanofabrication in a step-by-step and controllable way. The method acts in the same manner as traditional mechanical engineering, in which a new part is achieved from a blank through cutting and profiling. Here, the composite microparticles act as blanks. ‘Cutting and profiling’ are achieved using the environmental solvent, resulting in the desired nano-object.

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