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Fabrication of pH sensitive microcapsules using soft templates and their application on drug release

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A method based on soft templates for pH sensitive microcapsule fabrication was developed using layer-by-layer assembly technique. Toluene-in-water emulsion droplets were first stabilized by a surfactant sodium dodecyl benzene sulfonate (SDBS). Poly(diallyldimethyl ammonium chloride) modified latex beads were then adsorbed onto the droplet surfaces to make the emulsion more rigid. PSS(poly (sodium 4-styrenesulfonate))/PDDA (poly(diallyldimethyl ammonium chloride)) was assembled alternatively onto the emulsion surface to form the microcapsules. Zeta potential analyzer, scanning electron microscopy (SEM), transmission electron microscopy (TEM), FT-IR, and fluorescence microscopy were used to characterize the samples. The toluene droplet templates were removed by ethanol upon heating. The fluorescein, as the water-soluble model drug, was loaded into the microcapsules. Its release behaviors were investigated as a function of wall thickness and pH. The maximum release percentage 61% was obtained after 36 hours at 37°C at pH 7 with one double layer capsules. The capsule itself is nontoxic, while 5-fluorouracil (5-FU) loaded capsules killed 64.18% SK-RC-2 cells at the concentration of 17 μM at pH 7, which shows the great potential of this type microcapsule in cancer chemotherapy. The olive oil and liquid paraffin were used to replace the toluene for forming soft templates in order to obtain microcapsules which are suitable for loading hydrophobic drugs.Sudan-1 was chosen as a hydrophobic model drug and 25% release was obtained after 36 hours at 37 °C.

Introduction

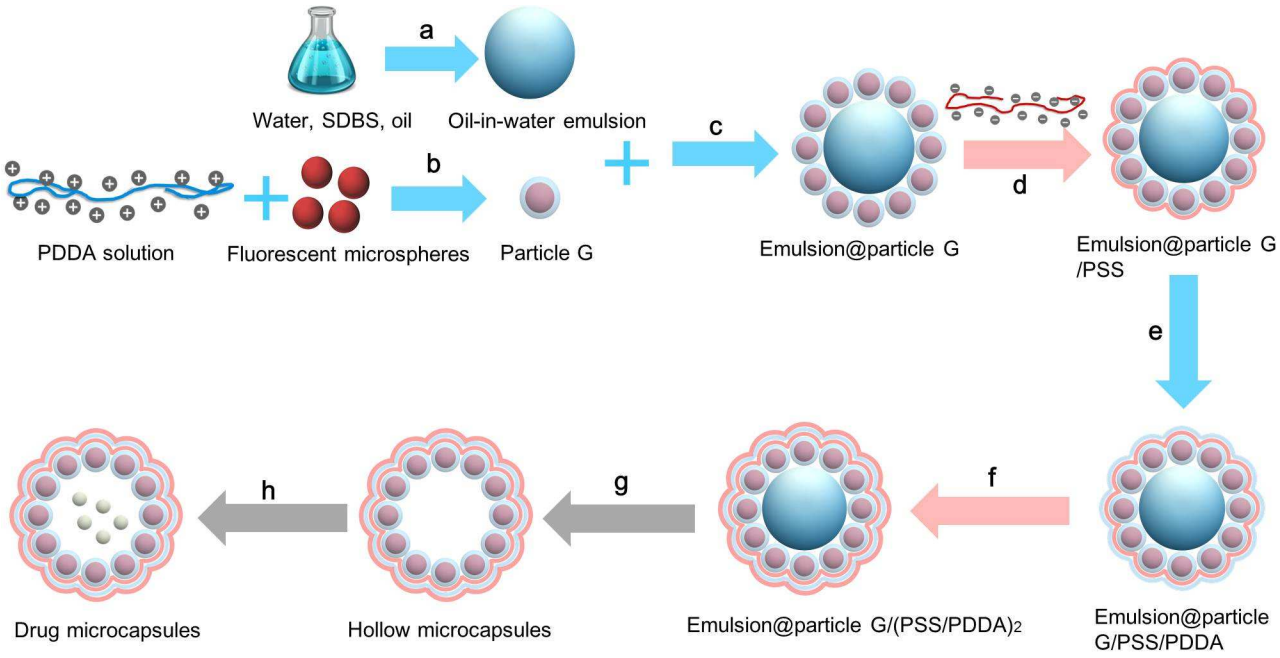
The past ten years have witnessed a dramatic increase of attention in the microcapsule field. There are several methods on making microcapsules including coacervation,[1] in-situ radical polymerization,[2, 3] interfacial condensation polymerization,[4] internal phase separation[5] and layer-by-layer assembly.[6, 7] Microcapsules prepared by layer-by-layer (LBL) method are spontaneously formed when two different charged polymers alternately adsorbed onto the templates.[8, 9] LBL technique allows the deposition of thin films on different kinds of template materials with a high degree of control over the physicochemical and other parameters of the microcapsules.[10] The different assembly methods,[11-15] wall materials[16-20] and templates[21-24] make microcapsules a variety of properties[25] and many great applications in fields of catalysis,[26] drug delivery,[27] and cosmetics [28], etc.

The templates of microcapsules include solid particles such as silica,[29, 30] calcium carbonate,[31] melamine formaldehyde (MF) particles.[32] However, the hard core of the microcapsules may need to be dissolved using strong chemicals, which may influence capsule integrity, wall properties[6] and their safety for their further applications especially on medicine and food fields. One way to solve the problem is to use soft templates, such as Pickering emulsions.[28, 33-35] Pickering emulsion (PE)[36-38]is an emulsion that stabilized by solid particles. Compared with normal emulsion, PE is much stable[33] and easy to be recycled. PE is also easily functionalized by using particular particles, e.g., magnetic PE can be obtained using iron oxide nanoparticle as stabilizer. Owing to these characteristics, apart from a variety of applications in food science and pharmaceutics, PEs were also used as templates to fabricate Janus particles,[39-41] foams[42] and other functional materials.

The challenges of making well-performing microcapsules suitable for drug release are the biocompatibility and safety of the shell materials, and trigger-ability for drug release by some stimuli, such as pH, temperature, magnetic field, etc. In previous studies, it was found that the cationic polymer PDDA showed an excellent microbicidal and fungicidal effect. PDDA as cationic agent for LBL was chosen in below study and assembled at the outermost layer of the microcapsules. In this way PDDA can better play its effectiveness.[43] The anionic polymer PSS has

a role in inhibiting sperm and HIV cell binding.[44] Due to the microcapsules prepared with PSS shrink or stretch against pH changes,[45, 46]PSS was chosen as anionic polymers to fabricate pH sensitive microcapsules.

In this study, a mild method based on soft templates for microcapsules which can deliver both the water-soluble and fat-soluble drugs is proposed. Under sonication, the surfactant (SDBS) stabilized toluene-in-water emulsions were obtained, which were then used as soft templates for



Scheme 1 Scheme of the preparation of PSS/PDDA hollow microcapsules and drug loading. a) SDBS stabilized emulsion. b) PDDA modification of fluorescent latex beads. c) Particle G assembly onto the emulsion droplets. d) Adsorption of PSS. e) Adsorption of PDDA layer. f) Layer-by-layer assembly of PSS and PDDA. g) Template removing using ethanol upon heating. h) Drug loading by incubation at 50 °C.

the fabrication of microcapsules via LBL technique. Due to the negatively charged surface property of the templates, PDDA stabilized polystyrene particles were adsorbed onto the emulsions. Afterwards PSS/PDDA was alternatively adsorbed onto the surface to fabricate microcapsules. After removing the toluene core, the hollow microcapsules were obtained. The releasing study of fluorescein from the microcapsules was also conducted. Furthermore, the typical chemotherapeutic agent, 5-fluorouracil (5-FU) was loaded into the microcapsules. The cytotoxicity of them to cancer cell was demonstrated. We also replaced toluene with liquid paraffin or olive oil to prepare microcapsules, which is safe and nontoxic for loading oil-soluble drugs without removing the templates. The releasing behavior of Sudan-1(oil-soluble model drug) from liquid paraffin microcapsules was conducted as well.

Materials and methods

All reagents were used as received without further purification. Toluene andsodium dodecyl benzene sulfonate (SDBS) were purchased from Sinopharm Chemicals (China). Poly(diallyldimethyl ammonium chloride) (PDDA), (*M*w~20,000), poly(sodium 4-styrenesulfonate) (PSS)(*M*w~70000), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), dimethylsulfoxide (DMSO) and fluorescein were purchased from Sigma Aldrich(China). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific. 5-fluorouracil (5-FU) was purchased from Tianjin Jinyao Amino Acid Co., Ltd (China). The SK-RC-2 cells were purchased from Memorial Sloan Kettering Cancer Center. Sodium phosphate monobasic dehydrate, Sodium phosphate dibasic dodecahydrate, Sudan-1 and sodium chloride were purchased from XILONG chemicals (China). Two kinds of carboxylate-modified microspheres with diameter of 100 nm ((yellow-green fluorescent and orange fluorescent) were purchased from Eugene (USA). Ultrapure water was generated using a Millipore Milli-Q plus system. The whole preparation process for microcapsules is illustrated in Scheme 1.

Oil-in-water emulsions were prepared by ultrasonication technique, as illustrated by step a. Briefly, 590 μL (3.3 mg mL^{-1}) SDBS solution, 410 μL deionized water and 70 μL toluene were mixed together, followed by vortex for 30 seconds and ultrasonication for 5 minutes. The bath sonication was used with controlled temperature at 25°C. The surface of emulsion is negatively charged. The oil droplets were washed by centrifuging (5000 rpm, 5 min) and then removing bottom solution with syringe and adding 1 mL deionized water into the vial. This washing procedure was repeated for three times. In step b, 4 μL carboxylate modified green fluorescent latex beads (100 nm in diameter) was added into 1 mL PDDA (2 mg mL^{-1}) solution to get particle G solution. In order to observe the emulsions using fluorescence microscope, particle G was adsorbed onto emulsion surface by adding 100 μL particle G solution into 1 mL microcapsules solution with shaking for 15 minutes, as shown by step c. Afterwards PSS and PDDA were

alternatively adsorbed onto the surface (step d-f). The concentrations of PSS and PDDA were both 2 mg mL^{-1}.[47] The adsorption of polyelectrolytes was carried out in 1mM NaCl solution for 15 minutes. Afterwards, the solution was centrifuged at 5000 rpm for 5 minutes, followed by replacing bottom solution with pH 7 PBS. This washing procedure was repeated three times after each assembly.

When using olive oil to replace the toluene for loading hydrophobic drugs, the microcapsules have been done at this stage. However, to load water soluble drugs, the template needs to be removed. In step g, 100 μL ethanol was added into 2 mL microcapsules solution at 80 °C for removing toluene. The toluene cores were then replaced with water. By the same way, the drugs can be loaded using the drug aqueous solution. We also research the releasing behavior of fat-soluble drug from the microcapsules. The liquid paraffin microcapsules were produced and load fat-soluble drug via incubation. In this study, we use fluorescein as water-soluble model drug, and Sudan-1 as oil-soluble model drug to study their releasing behaviours.

The SK-RC-2 cells (Human kidney cancer) were incubated in RPMI-1640 (GIBCO) medium with 10% fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% CO_2. The antitumor activity of the compounds was tested by MTT experiments. The stock solutions were typically prepared in phosphate-buffered saline (PBS) (pH=7.4) at a concentration of 0.01 mol L^{-1}. Cells were plated at a density of 6×10^3 cells per well, then incubated under the same culture conditions for 24 h. After 24 h, 20 μL of MTT solution at a concentration of 5 mg mL^{-1} dissolved in PBS buffer (pH 7.4) was added to each well. After another 4 h of incubation the medium was removed and 100 μL dimethylsulfoxide (DMSO) was added for each well. The intensity of the absorbance was measured using an automaticmicroplate reader (Infinite M200, Tecan) at a wavelength of 495 nm.

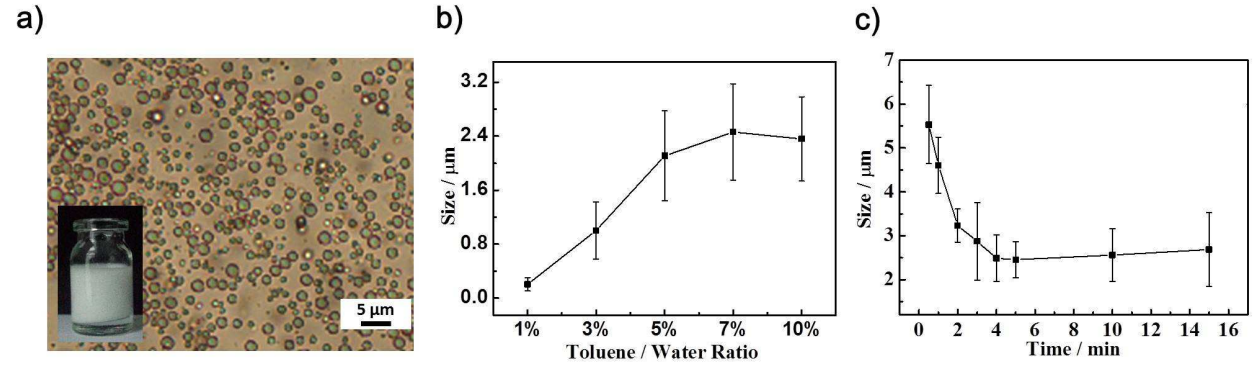


Fig. 1 (a) The optical microscopy image of oil-in-water emulsions. Inset is the photo of corresponding sample. (b) Emulsion size as a function of toluene/water percentage at fixed sonication time of 5 min. (c) Emulsion size as a function of sonication time at oil concentration of 7%.

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Fluorescence microscope (Nikon 80i, Japan) was used to image the optical and fluorescent microcapsules. The Zetasizer Nano ZSP (Malwern, UK) was used to measure the size and charge property of emulsions. The Fourier transform infrared (FT-IR) measurement was characterized by a Thermo Scientific Nicolet iS10 FT-IR Spectrometer. Transmission electron microscopy (TEM) measurements were carried out on H-7650 transmission electron microscope (Hitachi, Japan). The TEM samples were prepared by dropping sample solution onto the grids and dried in the air.

The calibration curves for fluorescein and Sudan-1 were obtained from the standard solutions. The fluorescein and Sudan-1 loaded microcapsules were washed to remove the excess dyes outside the microcapsules and dispersed into 2 mL PBS (pH=7 or 4). One sample was used to obtain the total encapsulated dye amount in the microcapsules by destroying them by chloroform to release all the dyes out. The other identical sample was put into dialysis bag, which were in turn placed into 40 mL PBS buffer (pH=7 or 4) at 37 °C under magnetic stirring. In each desired time intervals, 2 mL sample was taken out to analyze the drug concentration and replaced with the same volume of fresh buffer solution. The amount of fluorescein and Sudan-1 released was examined by fluorophotometer at 519 nm[48] and 580 nm respectively. The release at each time interval was obtained by the amount of dye released at that time over the total amount of dye inside the microcapsules. To study the influence of the thickness of the microcapsule wall on the drug releasing behavior, the microcapsules with one double layer and two double layers were prepared.

Results and discussion

Since the LBL technique is used for microcapsule fabrication, the emulsions should be charged. After carefully screening, SDBS was chosen at the concentration of 2 mg mL^{-1}. It gives negatively charged surface of the emulsion, which is favourable for particle G adsorption. The volume concentration of oil in water of 1%, 3%, 5%, 7%, and 10% were investigated for emulsion formation. As shown in Fig. 1b, with the increasing amount of toluene, the size of oil-in-water emulsion also increases from 1 to 5%, and levels off from 5 to 10%. 7% was chosen to produce emulsions. At this concentration, the influence of sonication time on emulsion size was investigated and plotted as shown in Fig. 1c. The size decreases gradually and reaches the lowest value at 5 minutes. The sonication time was chosen to be 5 min, since it gives more even distribution observed under microscope.

When we used the emulsion droplets as templates to directly assembly polyelectrolyte, the majority of microcapsules were damaged during the centrifugation and washing process. In order to solve the problem, we used particle G to stabilize the emulsions before LBL assembling. Another function of particle G is to enable the droplets to be observed under fluorescent microscope, since the latex beads are fluorescence labelled. Fig. 2a shows the particle G stabilized emulsions, which have even

red fluorescence circles. The corresponding fluorescence profile along the line in Fig. 2a is shown in Fig. 2b. Then the layer-by-layer assembly was carried out by adsorbing PSS and PDDA in turn to form two double-layered microcapsules. Between each coating, centrifugation-accelerated creaming and washing were carried out to remove unbound polyelectrolytes. The zeta-potential measurements were conducted to follow multilayer growth, as shown in Fig. 2c. The uncoated emulsion is negatively charged with potential of –32.93 mV. After adsorbing particle G layer, it becomes +30.21 mV. The first PSS/PDDAlayers give –21.79mV and +58.62 mV respectively; while the second PSS/PDDA layers present –49.28 mV and +59.19 mV respectively. With each additional layer was successfully assembled onto the microcapsules, the bigger surface area may cause the zeta-potential to be more negative or positive. The alternative charge variation gives a typical zigzag-shape plot, which suggests the successful coating with oppositely charged polyelectrolytes during each step. FT-IR spectrum also confirmed this. FT-IR spectrum (Fig. 2d) shows that the asymmetric stretching frequency of the S=O band of the sulfonic group in PSS locates at 1224 cm^{-1}, and the C-N stretching vibration band of the ammonium group in PDDA roughly appears at 1632 cm^{-1}.[49] Fig. 2e shows the fluorescence image of the microcapsule sprepared with two PSS/PDDA double layers. From the line profile across a microcapsule (Fig. 2f), the shell intensity became weaker due to the thicker wall. Considering the use of microcapsules for aqueous soluble drug delivery, the toluene should be removed. Due to the strong volatile feature of the template, the ethanol was used to remove toluene core under heating condition. Fig. 2g shows a typical TEM image of the microcapsule after removing the toluene core, which preserved its structural integrity and showed typical folds and creases in its dried state.

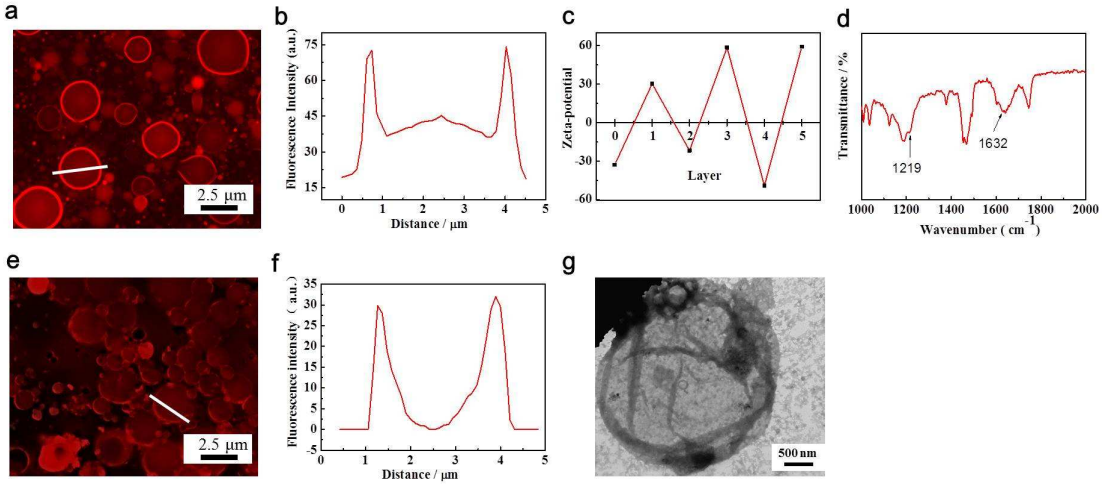

Fig. 2(a) The fluorescence image of the microcapsules stabilized by particle G. (b) Corresponding fluorescence intensity profile along the line indicated in (a). (c) Zeta potential measurements after each step of PSS/PDDA adsorption. (d) FT-IR spectrum of the microcapsule assembled with two PSS/PDDA layers. (e) The fluorescence image of the two PSS/PDDA layers modified microcapsules. (f) Corresponding fluorescence intensity profile along the line indicated in (e). (g)TEM image of the microcapsule assembled with two PSS/PDDA layers after removing oil core.

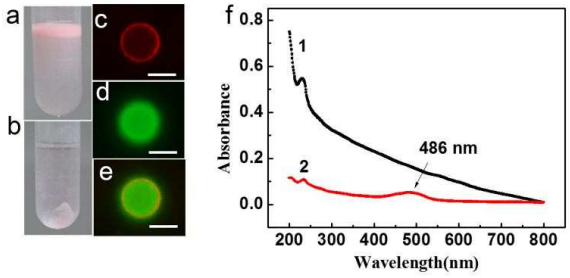

Fig. 3 a) The microcapsules with toluene droplets as templates after centrifugation. b) The toluene droplet removed microcapsules after centrifugation. Fluorescence microscopy images of orange particle G stabilized microcapsules containing fluorescein inside using red filter (c) and green filter (d), respectively. e) Merged image of c and d. All scale bars are 5 μm. (f) UV-vis of spectra of the microcapsules with water (black curve) and fluorescein (red curve) inside.

The model drug (fluorescein) was loaded into the microcapsules by incubation template removed microcapsules in 0.2 mg mL^{-1} fluorescein solution at 50 °C for 24 hours. The fluorescein outside microcapsules was removed by centrifuging. Since the fluorescein is in green color, the green latex beads in particle G were replaced with red latex beads to fabricate the microcapsules. Before removing the toluene template, the emulsions sit on the top layer in the tube after centrifugation (Fig. 3a), because the density of the microcapsules is less than that of water. But after removing the template, they sink to the bottom after centrifugation (Fig. 3b). This is an evidence of success elimination of toluene. After loading fluorescein inside the microcapsules, the fluorescence images were taken with red filter (Fig. 3c) and green filter (Fig. 3d), respectively. Fig. 3e is the merge image of Fig. 3c and d. The outer red ring and green core are from red particle G and fluorescein respectively. Those images clearly show the successful loading of fluorescein inside the microcapsules. In order to further confirm the loading of fluorescein, UV-Vis spectra of the microcapsules before (curve 1 in Fig. 3f) and after loading fluorescein (curve 2 in Fig. 3f) were recorded. The peak at 486 nm[48] indicates the loading of fluorescein.

The releasing behavior of water-soluble fluorescein from microcapsules were investigated at 37 °C, as shown in Fig. 4a. It is noted that the thicker wall microcapsule released drug slower and with lower final releasing percentage than the thinner ones.

For the microcapsules with the same wall thickness, the release at pH 7 is faster and with higher final release percentage than that at pH 4, because the PSS inside the wall of microcapsules shrank at lower pH,[45, 46] consequently slowing down the drug release. The maximum release percentage is 61%. In order to confirm the possibility of these capsules in chemotherapy, the cytotoxicities of free microcapsules and the 5-FU loaded microcapsules were determined by MTT assays at pH 7 (Fig. 5). The free microcapsules did not show any inhibition of SK-RC-2 cell growth, which indicated the biocompatibility of microcapsules made by our method. While the 5-FU loaded microcapsule inhibited the cancer cell growth, since the cell viability is 35.82% at the concentration of 5-FU in the microcapsule of 17 μM, which is similar to the positive control. This shows the great potential of these drug loaded microcapsules in chemotherapy.

This method can be used to prepare any other oil droplet templated microcapsules by simply replacing toluene with other oils. We took liquid paraffin (Fig. 6a) and olive oil (Fig. 6b) as examples. The microcapsules prepared with liquid paraffin and olive oil are very stable since they are less volatile. Fig. 6a and 6b shows liquid paraffin microcapsules and olive oil microcapsules respectively. Like Fig. 2e, two or more double layers assembly makes the fluorescence of particle G in Fig. 6a and Fig. 6b weaker and not very inhomogeneous. The releasing behavior of oil-soluble Sudan-1 from the liquid paraffin core microcapsules was investigated at 37 °C, as shown in Fig. 4b. Similarly, the thicker wall microcapsule released drug slower and with lower final releasing percentage than the thinner ones. The release of Sudan-1 from oil-core microcapsules are all less than the water-soluble ones, which may be attributed to its less solubility in water. Due to the nature of the oil phase itself,[50] the microcapsules are non-toxic and much stable. The hydrophobic drug can be loaded into these microcapsules. During the research, we found that the microcapsules for both hydrophilic and hydrophobic drug loading are stable up to at least two months.

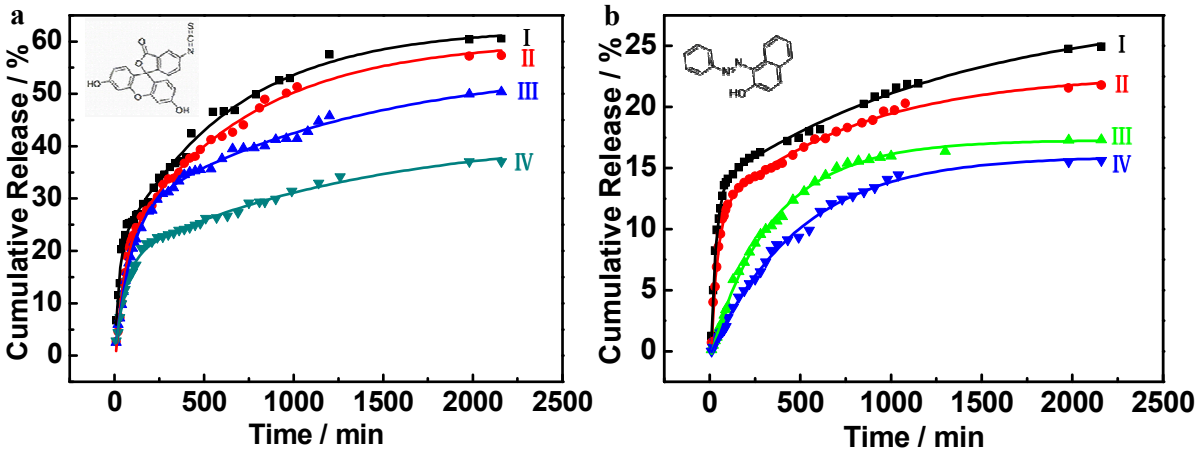

Fig. 4 Cumulative releasing curves of fluorescein (a) and Sudan-1 (b) from the microcapsules with a double layer at pH 7 (I), two double layers at pH 7 (II), one double layer at pH 4 (III), two double layers at pH 4 (IV), respectively. Releasing temperature is 37 °C. Insets at top left corner of the figures are the chemical structures of fluorescein and Sudan-1 respectively.

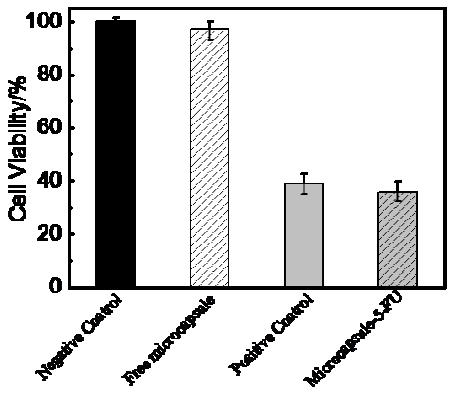

Fig. 5 The viability of SK-RC-2 cells after 24 h treatment with free microcapsules and 5-FU loaded microcapsules .

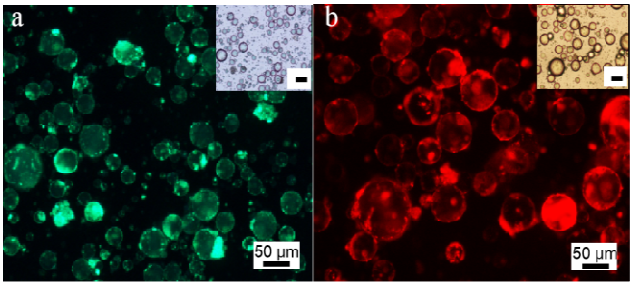

Fig. 6 The fluorescence images of microcapsules using liquid paraffin (a) and olive oil (b) as the oil phase. The insets are corresponding optical microscope images. All scale bars are 50 μm. The particle G in a and b are the green and orange fluorescent latex beads respectively.

Conclusions

Oil-in-water templates were used to fabricate microcapsules together with layer-by-layer technique. The olive oil, liquid paraffin and toluene were used for making oil-in-water droplets. When using volatile organic solvent as template, the template can be removed by ethanol at heating condition. The water soluble drug can be loaded into them by incubation at high temperature. In this study, fluorescein as a model drug was loaded into the microcapsules. Its release behavior was investigated and 61% release was obtained after 36 hours at 37°C. MTT experiments confirmed the biocompatibility of our microcapsules and their treatment potential after loading chemotherapeutic drugs. When using non-toxic oil, such as olive oil, make the system suitable for hydrophobic drug loading. The release behavior of Sudan-1 from liquid paraffin core microcapsules was also investigated and 25% release was obtained after 36 hours at 37°C. The method proposed in this paper has a certain degree of universality, which make it a great potential on drug-sustainable-release field.

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Notes and references

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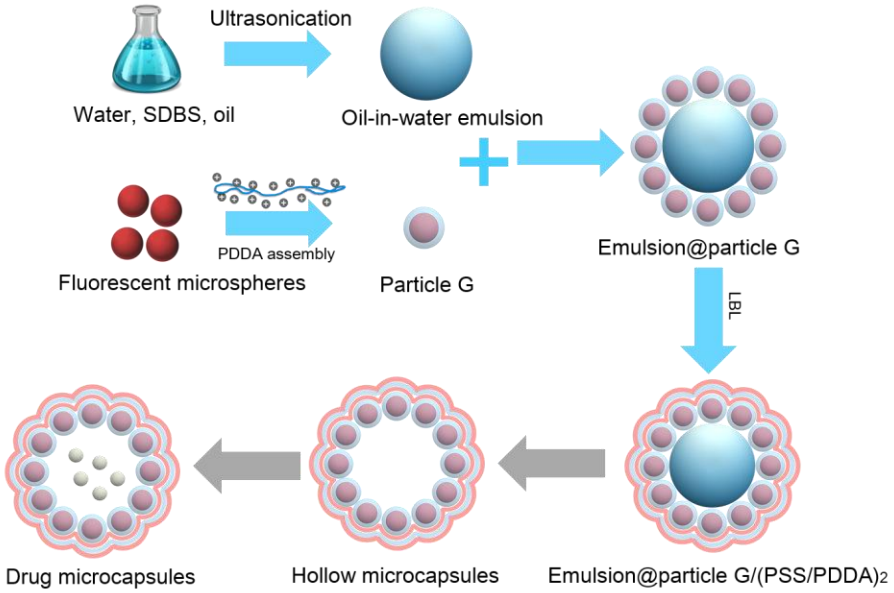

The schematic depiction of the process preparing hollow microcapsules and drug loading *via* layer-by-layer assembly technique.