Influence of Folate Conjugation on the Cellular Uptake Degree of Poly(allylamine hydrochloride) Microcapsules

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ABSTRACT: The microcapsules in drug delivery systems can prevent degradation of drugs and help to control the release rate. To enhance the targeted delivery effect of the microcapsules to cancer cells, some specific ligands such as folic acid (FA) are necessarily further conjugated. Herein, covalent poly(allylamine hydrochloride) (PAH) multilayers were fabricated on $CaCO_3$ microparticles under the cross-linking of glutaraldehyde, which were further immobilized with different amount of FA molecules via the spacer of diamino terminated poly(ethylene glycol) (PEG). As a comparison study, four types of microcapsules, i.e., the PAH capsules, the PAH capsules grafted with PEG, and the PAH capsules conjugated with two different amount of FA via the PEG spacer were prepared. Their chemical and physical structures were confirmed by infrared spectroscopy, UV–vis spectroscopy and scanning electron microscopy. *In vitro* cell culture found that the cellular uptake of the PAH capsules grafted with PEG was reduced significantly compared with that of the pure PAH capsules. The FA-modified microcapsules could be selectively delivered into HepG2 tumor cells which overexpress FA receptors but not into the endothelial cells. The number of HepG2 cells which ingested the FA-conjugated capsules showed a positive correlation with FA amount. The results indicate that these FA conjugated capsules have a high selectivity to be delivered to tumor cells, endowing them with a larger opportunity functioning as targeted delivery vehicle for anticancer drugs. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 121: 3710–3716, 2011

Key words: cellular uptake; microcapsules; folic acid; specific recognition; targeted delivery

INTRODUCTION

Colloids in drug delivery systems such as lipids, nanoparticles, and microcapsules etc., can prevent the degradation of drugs, help to control their release rate and lower their toxic side effects. It has been reported that the nanocarriers are also ingested naturally by cells.[1–4] More recently, the polyelectrolyte microcapsules have shown effective encapsulation of a hydrophobic photodynamic therapy drug, hypocrellin (HB). The HB could be delivered and taken up by cancer cells and showed high cytotoxicity after irradiation.[5]

Usually, the surface of many types of tumor cells overexpresses some receptors which can associate with specific ligands. Utilizing the receptor as a potential target, the drugs bounded with specific ligand can be actively delivered and targeted to the tumor cells. Among the targeting agents, folic acid (FA) has been widely used for the targeting delivery of drugs into tumor cells. The folate receptor (FR) is overexpressed on the surface of many cancer cells such as ovarian, endometria, breast, lung, and renal cells.[6] Moreover, the FA is very stable with minimal immunogenicity and has good compatibility with organic and inorganic matters. On account of these advantages, the FA becomes the ideal ligand and is a good prospect for the application of tumor targeting therapy.[7–10]

Utilizing the specific recognition between folate and FR, colloidal drug delivery system can be targeted to specific cells or tissues.[11–14] Zhang et al. prepared the bovine serum albumin nanoparticles (BSANPs) conjugated with folate and cocultured them with SKOV3 cancer cells. The association of the folate-conjugated BSANPs to SKOV3 cells could be inhibited by competition with excess amount of free FA in the culture medium, confirming that the binding and/or uptake can be mediated by the FR.[15] Oyewumi et al. prepared gadolinium nanoparticles coated with the folate ligands via poly(ethylene glycol) (PEG) spacers. The *in vivo* studies carried in KB

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tumor-bearing athymic mice showed that the cellular uptake and tumor retention of the folate-coated nanoparticles was significantly enhanced over PEG-coated nanoparticles.[16]

After *in vivo* injection, the surface of the colloidal particles would adsorb proteins and recognized by macrophages. Consequently, they would be cleared out of the blood circulation system.[7] It was reported that the colloidal particles modified with hydrophilic PEG would reduce the protein adsorption and prolong their circulation cycle.[17]

In this study, covalent poly(allylamine hydrochloride) (PAH) microcapsules are prepared by stepwise assembly on dextran sulfate (DS) doped $CaCO_3$ microparticles under the cross-linking of gluteraldehyde (GA).[18] They are further modified with FA via the PEG spacer. Quantitative analysis is performed by flow cytometry (FCM) to evaluate the selective delivery to HepG2 tumor cells by comparison with the control capsules and endothelial cells which do not overexpress FA receptors. The covalent PAH capsules contains negatively charged DS, and have a stronger elasticity and stability as well as a larger number of free amino groups. These features make the capsules easily operated against various treatments and low enough cytotoxicity. To our best knowledge, the cellular uptake of such a type of microcapsules has not been investigated so far.

MATERIALS AND METHODS

Materials

DS (MW 500kDa), poly(allylamine hydrochloride) (PAH, M_w 70 kDa), 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDAC), *N*-hydroxy succinimide (NHS), 4′,6-diamidino-2-phenylindole (DAPI), fluorescein isothiocyanate (FITC) isomer, and trypsin were purchased from Sigma-Aldrich. Poly(ethylene glycol) bis(amine) (PEG-bisNH_2, MW 4 kDa) was purchased from Aladdin. Human HepG2 cells were purchased from the Chinese type culture collection, and human endothelial cells were provided by the First Affiliated Hospital of College of Medicine, Zhejiang University. Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Hangzhou Sijiqing Biological Engineering Materials Co.. The water used in all experiments was purified by a Millipore water system. Other chemicals were all analytical reagents and used as received.

Preparation of porous $CaCO_3$(DS) microparticles

The $CaCO_3$ microparticles doped with DS were synthesized by mineralization of $Ca(NO_3)_2$ and Na_2CO_3 solutions in the existence of DS. Briefly, 400 mL $Ca(NO_3)_2$ solution (0.05 mol l^{-1}) was mixed with 100 mL DS solution (8 mg/mL), into which 400 mL Na_2CO_3 solution (0.05 mol l^{-1}) was rapidly poured under ultrasonication. After 10–30 min the $CaCO_3$ microparticles were filtered off, washed with water and ethanol for three times, and, then dried in an oven at 45°C.

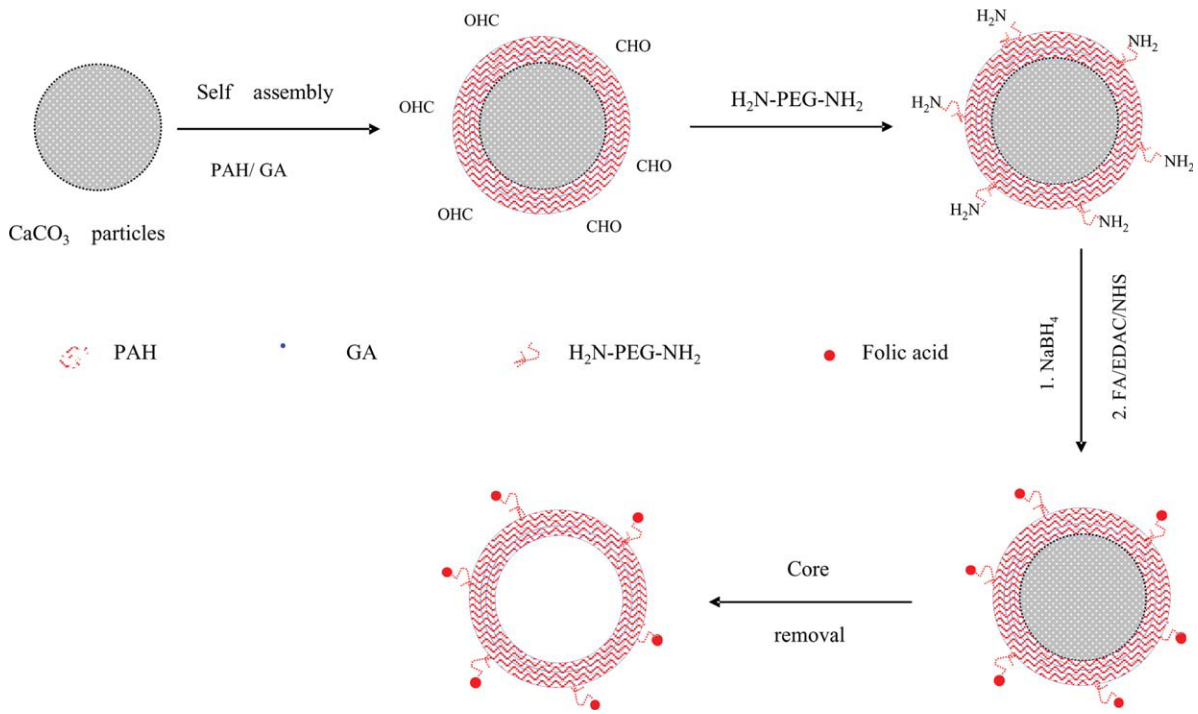
Preparation and modification of PAH microcapsules

Fabrication of the FA decorated microcapsules was accomplished following the procedures shown in Scheme 1. Briefly, PAH (2 mg/mL, 0.5*M* NaCl, pH 10) and 0.5% GA solutions were prepared and used for the covalent assembly of PAH multilayers on the $CaCO_3$ particles through a centrifugation protocol.[18] After the $CaCO_3$ (DS) microparticles had been washed with 0.5*M* NaCl solution (pH 10) for three times, the microparticles (300 mg) were incubated in 10 mL PAH solution under shaking for 15 min. The suspension was centrifuged at 3000 rpm for 1 min and the supernatant was carefully discarded. Three washings in water were then conducted. The PAH adsorbing particles were subsequently incubated in 10 mL GA solution under shaking for 15 min to cross-link the adsorbed PAH. After similar washings the particles were further incubated in PAH solution to obtain three bilayers of PAH/GA. A portion of the $CaCO_3(PAH/GA)_3$ core-shell particles were incubated in 2% PEG-bisNH_2 for 24 h to obtain the PEG modified microparticles, $CaCO_3(PAH/GA)_3$-PEG. After centrifugation and washing with water for three times, the $CaCO_3(PAH/GA)_3$ and $CaCO_3(PAH/GA)_3$-PEG particles were reduced with 0.1% $NaBH_4$ solution for 4 h, followed by three washings.

The $CaCO_3(PAH/GA)_3$-PEG particles were further incubated in FA (2.5 mM) solution containing EDAC (20 mg/mL) and NHS (10 mg/mL) for 4 h and 54 h to obtain the $CaCO_3(PAH/GA)_3$-PEG-FA 4 h and $CaCO_3(PAH/GA)_3$-PEG-FA 54 h particles of different conjugation ratios of FA, respectively. Finally, all the core-shell particles were treated with 0.1*M* EDTA to dissolve the $CaCO_3$ template and to obtain the hollow microcapsules of $(PAH/GA)_3$, $(PAH/GA)_3$-PEG, $(PAH/GA)_3$-PEG-FA 4 h and $(PAH/GA)_3$-PEG-FA 54 h, respectively. The microcapsules were further labeled with FITC (10 μg/mL) for 24 h, followed by purification with centrifugation and washing until no fluorescence could be detected in the supernatant.

Cell culture with microcapsules

To testify the cellular uptake performance of the microcapsules, HepG2 cells which overexpress FA receptors were cultured with the microcapsules *in*



Scheme 1 Schematic illustration to show the GA mediated covalent assembly of PAH, PEG grafting, folic acid immobilization, and the final obtained microcapsules. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

vitro, using the endothelial cells of scarce FA receptors as the control. Both types of cells were cultured at 37°C in a DMEM medium (Gibco) supplied with FBS (10%), 100U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 humidified atmosphere. After the cells were cultured in a 24-well plate at a density of 8×10^4 cells/well overnight, 50 μL of microcapsules in sterile water ($\sim 8 \times 10^5$ capsules/well, i.e., the cell/capsule ratio was around 10) was added to each well. The cells were continually cultured in an incubator at 37°C for 16 h, and then washed with phosphate buffered saline to remove the free microcapsules. The fluorescence photo images were taken by a fluorescence microscope (Olympus IX81, Japan). The cells were then detached and resuspended in PBS and analyzed by FCM (FACS Calibur, Becton Dickinson BD) at an excitation wavelength of 488 nm to measure the percentage of cells internalizing capsules. Linear amplification was used for forward scatter (FSC) and side scatter (SSC) parameters. Log amplification was used for fluorescence signals. The threshold (the lower limit of FSC) was set to be >80. The negative control cells without coincubated microcapsules were used to adjust FSC (E-1) and SSC voltage so that the cell population was placed in the center of FSC-SSC dot-plot. And the voltage on FL1 was adjusted to keep the auto-fluorescence intensity of the negative control cells run in the middle of the first decade (between 10^0 and 10^1). 10,000 events were measured to obtain the mean fluorescence.

Characterization

For scanning electron microscopy (SEM, ULTRA 55, ZEISS, Germany) observation, the microcapsules suspension was dropped on a silicon slide, air dried and then sputtered with gold. The acceleration voltage was set at 3 kV. Fourier transform infrared spectroscopy (FTIR, Vector 22, Bruker optics, Switzerland) was recorded after the microcapsules were dried in air and pelleted with KBr powder. The amount of FA on the microcapsules was determined from the absorbance at 280 nm of UV–Vis spectra (shimadazu UV2550), using known concentrations of FA as standards. ζ-Potential of the microcapsules was measured by a ζ-sizer (Delsa™ Nano, Beckman Coulter), each datum was averaged from three measurements.

RESULTS AND DISCUSSION

Preparation of $CaCO_3$(DS) microparticles and microcapsules

Utilizing the $CaCO_3$(DS) microparticles as template, the $(PAH/GA)_3$ microcapsules [Fig. 1(a)], capsules grafted with PEG [Fig. 1(b)] and capsules conjugated with FA via the PEG spacer [Fig. 1(c,d)] were fabricated. These dried hollow microcapsules collapsed

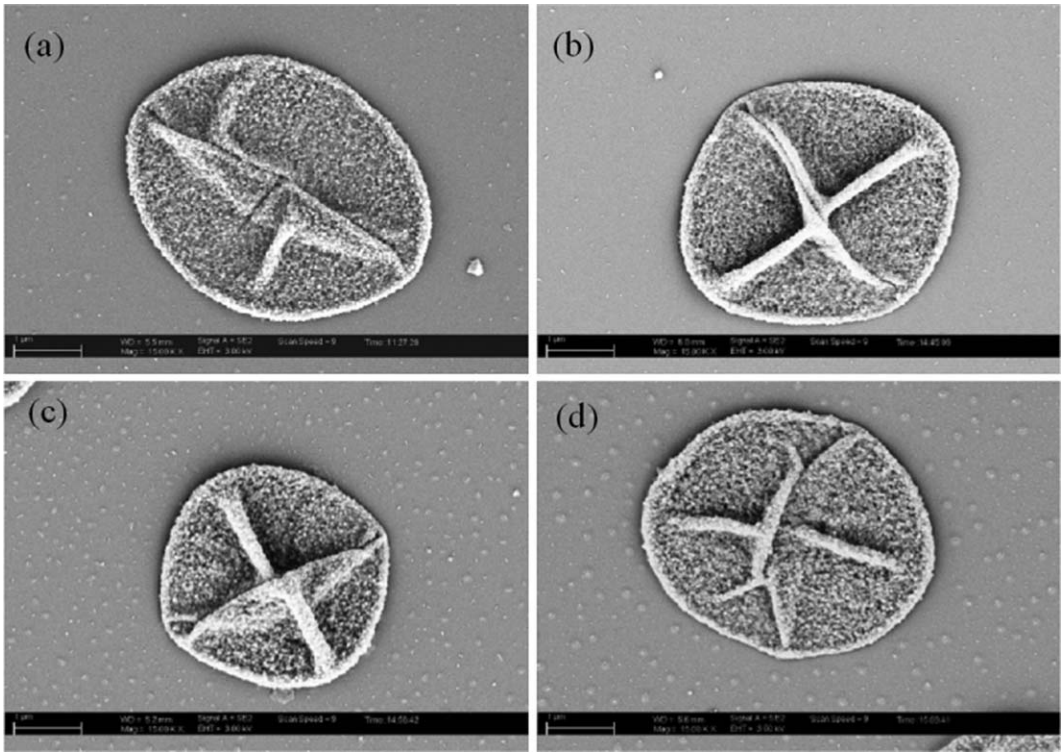


Figure 1 SEM images of (a) $(PAH/GA)_3$, (b) $(PAH/GA)_3$-PEG, (c) $(PAH/GA)_3$-PEG-FA 4 h, and (d) $(PAH/GA)_3$-PEG-FA 54 h microcapsules.

and were folded due to the volatilization of the water inside. Comparatively, there was no obvious change among the surface morphology of these capsules, indicating that the conjugation reaction of PEG or FA did not bring apparent topographical influence. Therefore, the influence of the surface morphology of the microcapsules modified with or without FA on the cellular uptake can be excluded.

Characterization of capsules conjugated with FA

To confirm the conjugation of PEG and FA, the hollow microcapsules were characterized by FTIR [Fig. 2(a)]. The absorbance at 3434 cm^{-1} is assigned to the stretching vibration of —NH bond of PAH, which is the main components of the microcapsules and thus universally exits in all the capsules. The absorption peaks at 1637 cm^{-1} and 1124 cm^{-1} are assigned to the

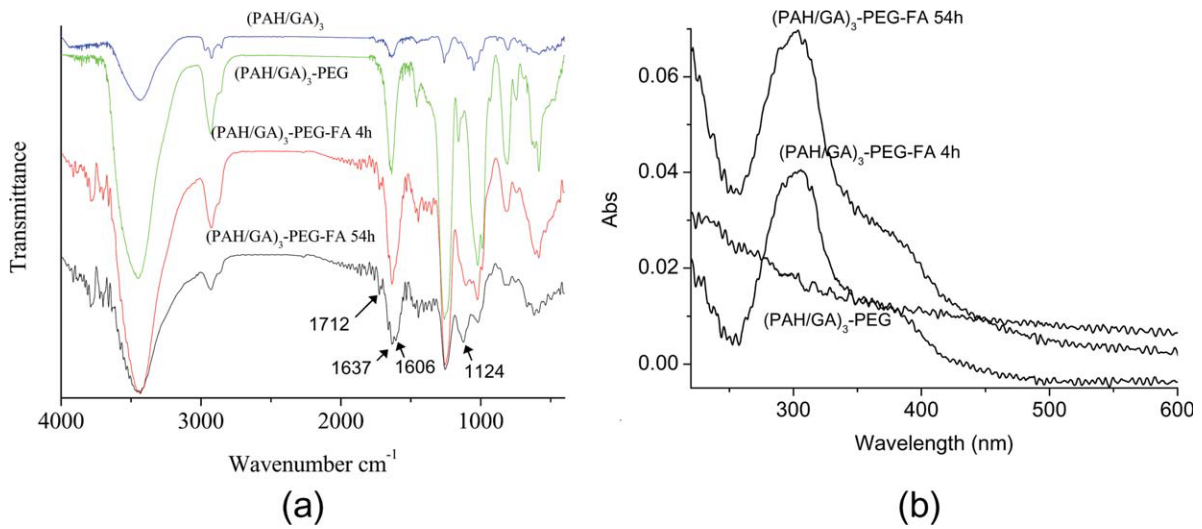


Figure 2 (a) FTIR spectra of $(PAH/GA)_3$, $(PAH/GA)_3$-PEG, $(PAH/GA)_3$-PEG-FA 4 h, and $(PAH/GA)_3$-PEG-FA 54 h microcapsules. (b) UV–vis spectra of $(PAH/GA)_3$-PEG-FA 54 h and $(PAH/GA)_3$-PEG-FA 4 h microcapsules after subtraction of the spectrum of $(PAH/GA)_3$-PEG microcapsules. The capsule concentration was $(1.0 \pm 0.1) \times 10^7$/mL. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

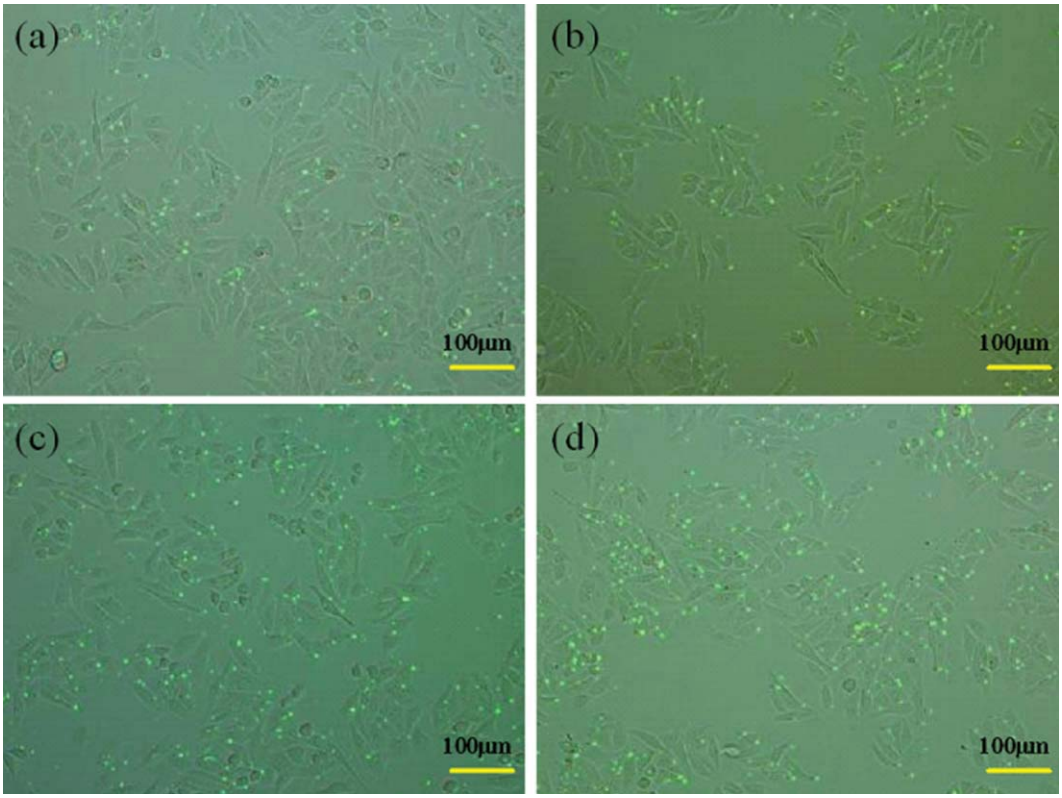


Figure 3 Merge images of fluorescence and transmission modes after the HepG2 cells were incubated with (a) $(PAH/GA)_3$, (b) $(PAH/GA)_3$-PEG, (c) $(PAH/GA)_3$-PEG-FA 4 h, and (d) $(PAH/GA)_3$-PEG-FA 54 h microcapsules for 16 h, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

stretching vibration of $-NH_2$ group and the C—O—C vibration of PEG, respectively. The absorption peaks at 1712 cm^{-1} and 1606 cm^{-1} are assigned to the vibration of the —COOH group and the benzene ring of FA, respectively. Therefore, the FTIR results identify that PEG and FA had been immobilized on the microcapsules successfully. The immobilized amount of FA was further quantified by UV–Vis absorption spectroscopy [Fig. 2(b)]. By subtracting the UV absorbance of $(PAH/GA)_3$-PEG capsules from that of the $(PAH/GA)_3$-PEG-FA capsules, the grafting density of FA on the microcapsule surface were found to be $(8.4 \pm 1.2) \times 10^9$ and $(1.5 \pm 0.1) \times 10^{10}$ FA molecules/capsule for the $(PAH/GA)_3$-PEG-FA 4 h and $(PAH/GA)_3$-PEG-FA 54 h, respectively.

The prepared capsules were further characterized by ζ-potential. Although the main component of the capsules was positively charged PAH, the potentials of all these capsules were negatively charged due to the embedded DS (negatively charged), which was initially doped inside the $CaCO_3$ particles. Recently Ting et al. examined the cytocompatibility of the PSS/PAH polyelectrolyte films and reported that neither the PAH-terminated nor the PSS-terminated polyelectrolyte films were cytotoxic. However, the PSS-terminated film enhanced the proliferation of the cells compared with the PAH-terminated film.[19] Therefore, in this work, the negatively charged DS was designed to be embedded in the PAH capsules to modulate the surface charge and endow with low cytotoxicity. After grafting of PEG, the potential value was increased from -21.3 ± 1.7 mV (for the $(PAH/GA)_3$ capsules) to -12.3 ± 1.5 mV. It is known that the PEG molecules are highly hydrophilic and flexible, and thereby can screen surface charge of the capsules to some extent. Utilizing the PEG spacer, the potential values of the capsules conjugated with FA (-32.2 ± 0.9 mV for $(PAH/GA)_3$/PEG/FA 4 h capsules, and -34.8 ± 0.1 mV for $(PAH/GA)_3$/PEG/FA 54 h capsules) decreased obviously. The conjugation of FA will consume the positively charged $-NH_2$ groups of PAH, leading to the relative increase of the negatively charged groups and thereby the more negatively charged capsule surface. This explains also the higher amount of FA conjugation produced more negative surface. All the results confirm that the microcapsules with desired structures and properties have been successfully prepared.

Cells cocultured with capsules

The four types of capsules, i.e., $(PAH/GA)_3$,$(PAH/GA)_3$-PEG, $(PAH/GA)_3$-PEG-FA 4 h, and (PAH/

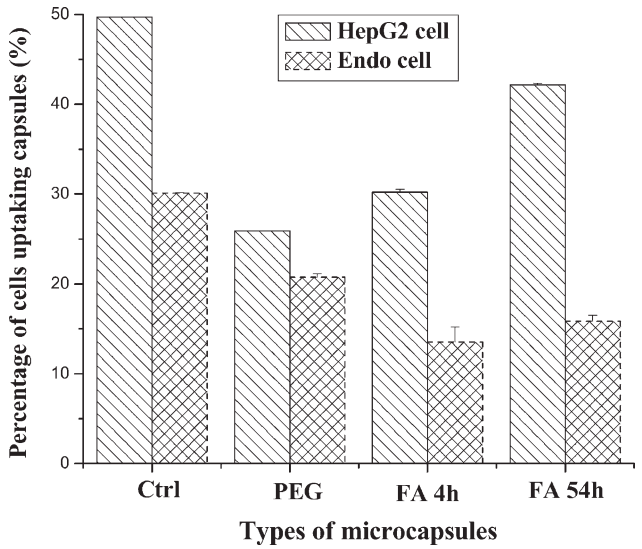


Figure 4 FCM characterization of cells internalizing the microcapsules. Ctrl, PEG, FA 4 h and FA 54 h denote $(PAH/GA)_3$, $(PAH/GA)_3$-PEG, $(PAH/GA)_3$-PEG-FA 4 h, and $(PAH/GA)_3$-PEG-FA 54 h microcapsules, respectively.

$GA)_3$-PEG-FA 54 h, were then subject to culture *in vitro* with the HepG2 cells and endothelial cells for 16 h, respectively. Results showed that all the four types of capsules could adhere onto or be internalized by the HepG2 cells (Fig. 3) and the endothelial cells (images not shown), but their numbers were different. More numbers of the FA-modified microcapsules adhered onto or internalized by the HepG2 cells [Fig. 3(c,d)] than those of the $(PAH/GA)_3$ [Fig. 3(a)] and $(PAH/GA)_3$-PEG microcapsules [Fig. 3(b)]. No apparent alteration of the cell morphology was observed.

Quantification of cellular uptake by FCM

The specific delivery of the FA conjugated microcapsules to the HepG2 cells, which overexpress the FA receptor, was quantitatively analyzed by FCM (Fig. 4). Comparison of the uptake percentage by the endothelial cells, which do not overexpress the FA receptor, is also summarized in Figure 4. It shows that the $(PAH/GA)_3$ (Ctrl) microcapsules had the maximum percentages of the cellular uptake for both the HepG2 and endothelial cells, which is basically attributed to the unspecific adhesion of the microcapsules. After decorated with PEG, the uptake percentages were significantly decreased to 1/2 and 2/5 for the HepG2 and endothelial cells, respectively. The PEG molecules are highly hydrated and thus have a large steric volume to repel the unspecific adhesion of the microcapsules, leading to the reduction of cellular uptake. Further conjugation of the FA molecules to the PEG spacers, however, resulted in completely reverse effect on the uptake of the microcapsules by the HepG2 and endothelial cells. For the endothelial (Endo) cells with scarce FA receptors, the cellular uptake percentages of the FA-conjugated capsules were further reduced to some extent compared with that of the $(PAH/GA)_3$-PEG capsules. The reason might be attributed to the more negatively charged microcapsule surface which is unfavorable for the unspecific adhesion. For the HepG2 cells overexpressing FA receptors, the cellular uptake percentages of the capsules conjugated with FA increased significantly compared to that of the $(PAH/GA)_3$-PEG capsules due to the obvious ligand–receptor binding. Moreover, unlike the case of the endothelial cells, the microcapsules having a higher number of the FA molecules showed significant improvement of the uptake percentage. All these results confirm that the FA conjugated capsules have a higher selectivity to be delivered to the cells overexpressing FA receptors, for example, HepG2.

CONCLUSIONS

Four types of covalently cross-linked PAH microcapsules, i.e., $(PAH/GA)_3$, $(PAH/GA)_3$-PEG, $(PAH/GA)_3$-PEG-FA 4 h, and $(PAH/GA)_3$-PEG-FA 54 h were prepared. Conjugation of the PEG or FA molecules did not change the morphology of the capsules. FTIR and UV–vis characterizations confirmed the successful linking of PEG and FA, which was found to be $(8.4 \pm 1.2) \times 10^9$ and $(1.5 \pm 0.1) \times 10^{10}$, FA molecules/capsule for the $(PAH/GA)_3$-PEG-FA 4 h and $(PAH/GA)_3$-PEG-FA 54 h, respectively. After grafting with PEG, the unspecific adhesion of $(PAH/GA)_3$/PEG capsules was reduced obviously. Selective delivery of the FA-conjugated capsules to the FA receptors overexpressing cells, for example, HepG2 cells was demonstrated by comparison with the endothelial cells which do not overexpress FA receptors. The percentage of HepG2 cells internalizing the FA modified capsules increased with the increase of FA conjugation ratio too.

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