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PEGylated chitosan microspheres as mucoadhesive drug-delivery carriers for puerarin

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ABSTRACT: PEGylated chitosans with different degrees of grafting were synthesized, and the application potential of microspheres based on PEGylated chitosan as mucoadhesive drug-delivery carriers for puerarin was investigated. Compared with chitosan microspheres, PEGylated chitosan microspheres (PCMs) exhibited better physical stability and higher swelling capacity, and the amount of water uptake increased as the content of poly(ethylene glycol) methyl ether in the microspheres increased. PCMs showed obviously improved mucoadhesive behavior on a mucosa-like surface. Puerarin was incorporated into the microspheres, and the release experiments *in vitro* showed that the PEGylation of chitosan accelerated puerarin release from the particles and decreased the retention of the drug. The abilities of all of the tested microspheres to open tight junctions and improve the permeability of puerarin were demonstrated with a Caco-2 cell monolayer as an *in vitro* model. The amount of puerarin permeating across the Caco-2 cell monolayer was significantly increased by the incorporation of puerarin into the PCMs. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42623.

KEYWORDS: drug-delivery systems; grafting; polysaccharides; properties and characterization; swelling

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INTRODUCTION

Mucoadhesive drug-delivery systems conceived for mucosal administration have gained increasing attention in recent decades. Drug absorption through a mucosal surface is generally efficient because mucosa usually has a rich blood supply, which provides the means for rapid drug transport to the systemic circulation and prevents degradation by first-pass hepatic metabolism in most cases.[1,2] Various drug-delivery systems with mucoadhesive properties have been reported to increase the mucosal contact time and enhance the penetration of drugs through mucosal tissues.[3–5] Microspheres based on chitosan and its derivatives are currently of considerable interest as a vehicle for mucosal administration because of the unique physicochemical properties of chitosan.[6,7] Chitosan is a well-known mucoadhesive polymer, which has been extensively investigated for drug-delivery applications, both in terms of efficiency but certainly also in terms of safety.[8] The bioadhesive properties of chitosan, stemming from both the electrostatic attraction with negatively charged mucin and the contributions of hydrogen bonding and hydrophobic effects, combined with its ability to transiently open tight junctions in the membrane are beneficial to the delivery of drugs across mucosal tissues.[9]

With the aim of improving and optimizing the characteristics of chitosan, such as its charge density, solubility at higher pH values, mucoadhesiveness, and penetration enhancement ability, numerous chitosan derivatives have been produced, and several studies have been carried out on microspheres based on chitosan derivatives.[10–12] As documented in some studies, in addition to the positively charged surfaces of microspheres at physiological pH, their dehydrating/viscosity effects on mucin play an important role in their mucoadhesiveness and, consequently, in the drug bioavailability.[13] PEGylated chitosan is a reasonably well-explored chemical modified chitosan, which has the advantages of improved aqueous solubility of the polysaccharide, reduced toxicity, and promoted absorption-enhancing properties.[14,15] Jiang *et al.*[16] prepared PEGylated chitosan microspheres (PCMs) through an ionic gelation process with tripolyphosphate as the cross-linking agent to load *Bordetella bronchiseptica* dermonecrotoxin, an atrophic rhinitis vaccine. The PEGylation of chitosan improved the stability of the microspheres and accelerated model vaccine release from *B. bronchiseptica* dermonecrotoxin-loaded microspheres *in vitro*. However, further investigation of the mucoadhesiveness and related properties of PCMs has not been reported.

On the basis of these results, we decided to explore the influence of the PEGylation of chitosan on the properties of the obtained microspheres and their potential use as

mucoadhesive drug-delivery carriers. Puerarin, an effective traditional Chinese medicine treatment of cerebrovascular and cardiovascular diseases, was chosen as the model drug.[17] With the aim of improving the oral absorption of puerarin or reducing the risk of serious adverse effects of its injection, several pharmaceutical techniques, including the fabrication of microemulsions, submicroemulsions, nanoparticles, and prodrugs, have been reported in recent years.[18–22] The mucosal administration of puerarin could probably achieve improved bioavailability and enhanced therapeutic effects, as the permeation of significant amounts of the drug is permitted through the mucosa. In particular, the tissue concentration of puerarin in the target organ (i.e., the brain and heart) may be increased after administration onto the specific mucosal site (e.g., intranasal).[23]

In this study, PCMs were produced via a simple emulsification technique followed by crosslinking with glutaraldehyde. The swelling capacity, drug-release behavior, and mucoadhesiveness of the microsphere formulations combined with their effects on the Caco-2 cell permeability *in vitro* were investigated. As a result, the influence of PEGylation on the properties of chitosan microspheres is discussed, and the application potential of PCMs as mucoadhesive drug-delivery carriers for puerarin are demonstrated.

EXPERIMENTAL

Materials

Poly(ethylene glycol) methyl ether (PEG; number-average molecular weight ≈ 2000) was purchased from Aldrich. Chitosan (200–400 mPa·s, >90% deacetylation) was obtained from Aladdin Reagent Co. Puerarin (>98%) was provided by Nanjing Zelang Medical Technology Co. Glutaraldehyde (50% in H_2O) were obtained from Sinopharm Chemical Reagent Co. Mucin (from porcine stomach) was purchased from Amresco. All of the other reagents used were analytical grade and were used as received.

Synthesis of PEGylated Chitosan

PEG (2.00 g) was dissolved in acetonitrile (50 mL), and an excess of triethylamine (0.2 mL) was added. 4-Nitrophenyl chloroformate (0.40 g) in an acetonitrile solution (10 mL) was added dropwise to the mixture and then allowed to react overnight with stirring. The resulting solution was filtered and concentrated through rotary evaporation. The product (PEG–NO_2) was purified by precipitation from diethyl ether three times to remove the unreacted 4-nitrophenyl chloroformate and dried *in vacuo*.

PEGylated chitosan was prepared from the reaction between the *p*-nitrophenyl oxycarbonyl group of PEG–NO_2 and the primary amino group of chitosan. A certain amount of PEG–NO_2 and chitosan was dissolved in a 3% v/v acetic acid solution. The pH value of the mixture was adjusted to 7 by the addition of an NaOH aqueous solution. The precipitate was filtered, washed with water three times, and lyophilized.

Preparation and Characterization of the Microspheres

The microspheres were produced by emulsification and a crosslinking process. Briefly, PEGylated chitosan was dissolved in a 3% v/v acetic acid solution to obtain a polymer solution at a concentration of 1.5% w/v. This solution (3.3 mL) was emulsified in 100 mL of liquid paraffin (the oil phase) containing 2% v/v Span 80 with a homogenizer (RW20 digital mechanical overhead stirrer, IKA, Germany). The mixture was initially stirred at 1000 rpm for 10 min to form a water-in-oil emulsion, and then, the rate was decreased to 450 rpm. Afterward, 8.3% v/v glutaraldehyde in a 1,4-dioxane solution (1.5 mL) was slowly added with constant stirring for 1.5 h. The formed microspheres were filtered, washed several times with petroleum ether and then with isopropyl alcohol, and dried in a vacuum oven overnight. Chitosan microparticles were prepared as a comparison.

The size and shape of the microspheres were examined under a scanning electron microscope (S-3000N, Hitachi, Japan), and the size distribution was determined with a WQL particle size analyzer (LKY-2, SPSIC, China).

Swelling Experiments

The swelling properties of the microspheres were evaluated in deionized water at 37°C. At predetermined times (6, 12, and 24 h), the samples were centrifuged, and the excess water was removed carefully. The swollen microspheres were weighed and then dried in an oven at 60°C until there was no change in the dried mass of the microspheres. Each experiment was repeated six times, and the average weight of the microspheres was used to calculated the swelling capacity (%) with the following equation:

$$\text{Swelling capacity}\,(\%) = \frac{W_s - W_d}{W_d} \times 100\% \qquad (1)$$

where W_s is the weight of the swollen microspheres and W_d is the weight of the dried samples.

Evaluation of the Drug-Loading Capacity (LC) of the Microspheres

A certain amount of puerarin-dissolved acetic acid solution was used to prepare the drug-containing polymer solution. Then, the puerarin-loaded microspheres were prepared via the same emulsification and crosslinking process mentioned previously. The drug-loading capacity of the microspheres was determined when the samples were dissolved in a hydrochloric acid solution. An amount of 10 mg of the puerarin-loaded microspheres was suspended in a 3% v/v hydrochloric acid solution and stirred under reflux for 3 h until the microspheres were completely dissolved. The pH value of the mixture was adjusted to 4 by the addition of an NaOH aqueous solution before the quantitative determination of puerarin in the solution with Agilent 1200 series high performance liquid chromatography (HPLC) instrument (Chemstation System, Diode Array Detector, Eclipse XDB-C18 column, 4.6 × 150 mm, 5 μm). The mobile phase consisted of methanol and water (20/80 v/v). The flow rate was 1 mL/min, the oven temperature was maintained at 30°C, and the sample injection volume was 20 μL. The column eluent was monitored at 250 nm, and puerarin was separated with a retention time of 14.1 min. All of the measurements were performed in triplicate and averaged.

LC was then calculated with the following equation:

$$\text{LC}(\%) = \frac{\text{Incorporated puerarin weight}}{\text{Microsphere weight}} \times 100\% \quad (2)$$

In Vitro Release Studies

The release of puerarin from the microspheres was assessed by the incubation of 10 mg of sample at 37°C in 5 mL of pure water with horizontal shaking. At specific intervals of time, the samples were centrifuged at 4000 rpm for 4 min. Then, 1 mL of supernatant was taken, and it was replenished by the same volume of fresh water. The collected solutions were subjected to further HPLC analysis. All of the experiments were carried out in triplicate.

Adsorption of Mucin on the Microspheres

The microspheres (10 mg) were dispersed in a mucin aqueous solution (1 mg/mL, 6 mL). After incubation in shaker at 37°C for 2 h, the dispersion was centrifuged at 4000 rpm for 2 min, and the supernatant was removed to measure the free mucin content with the periodic acid/Schiff colorimetric method.[24] Briefly, a mixture of 2 mL of supernatant and 0.2 mL of periodic acid reagent was incubated in a water bath at 37°C for 2 h. Then, 0.2 mL of Schiff reagent was added at room temperature. The absorbance of the solution was detected at 555 nm with an ultraviolet–visible (UV–vis) spectrophotometer after 30 min, and the amount of free mucin in the dispersion was determined. All of the experiments were carried out in triplicate. The adsorption of mucin on the microspheres was assessed as follows:

$$\text{Adsorption ratio}\,(\%) = \frac{\text{Amount of total mucin} - \text{Amount of free mucin}}{\text{Amount of total mucin}} \times 100\% \quad (3)$$

In Vitro Mucoadhesion Studies

The quantity of the drug-loaded microspheres adhering to the surface of the mucin saturated filter paper was determined to assess the *in vitro* mucoadhesive properties of the microspheres according to the previously described method.[25] The puerarin-loaded microspheres (10 mg) were spread out onto a filter paper (2.5 × 2.5 cm) wetted completely with a mucin solution (2% w/v in pure water). The filter paper was fixed with the samples face downward inside a tube under controlled conditions of humidity (75.8 ± 1.6%), temperature (28 ± 1°C), and wind speed (6.2 ± 1 m/s) for 15 s. Then, the microparticles that remained adherent to the surface were recovered by washing the filter paper with a 3% v/v hydrochloric acid solution. The amount of drug in the adhered microspheres was quantified via HPLC as mentioned previously. The *in vitro* mucoadhesion behavior of the microspheres was expressed with the following equation:

$$\text{Mucoadhesivity}\,(\%) = \frac{\text{Amount of puerarin in the adhered microspheres}}{\text{Total amount of puerain}} \quad (4)$$

Caco-2 Cell Culture

Caco-2 cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 1% nonessential amino acids, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C and 90% humidity in a 5% CO_2 atmosphere. The growth medium was changed every other day. The cells were passaged at 80–90% confluency with a 0.25% trypsin/0.20% ethylenediamine tetraacetic acid solution.

Cytotoxicity Assay

Caco-2 cells were seeded in 96-well plates at a density of 1×10^5 cells/cm^2 and cultured for 40 h in incubator at 37°C and 90% humidity under 5% CO_2. The culture medium was replaced with a Dulbecco's Hanks balanced salt solution containing microspheres at a concentration of 3 mg/mL and incubated for 4 h. Thereafter, the microspheres were removed, and each well was washed twice by phosphate-buffered saline. The Dulbecco's Hanks balanced salt solution (100 μL) and a methylthiazolyldiphenyl-tetrazolium bromide solution (20 μL, 0.5 mg/mL in phosphate-buffered saline) were then introduced. After incubation (37°C, 90% humidity, 5% CO_2) for another 4 h, 150 μL of dimethyl sulfoxide was added to solubilize the resulting formazan crystals, and the absorbance intensity was measured by a microplate reader (SpectraMax Puls384, Molecular Devices) at 490 nm. The relative cell viability (%) was expressed as a percentage relative to the untreated control cells.

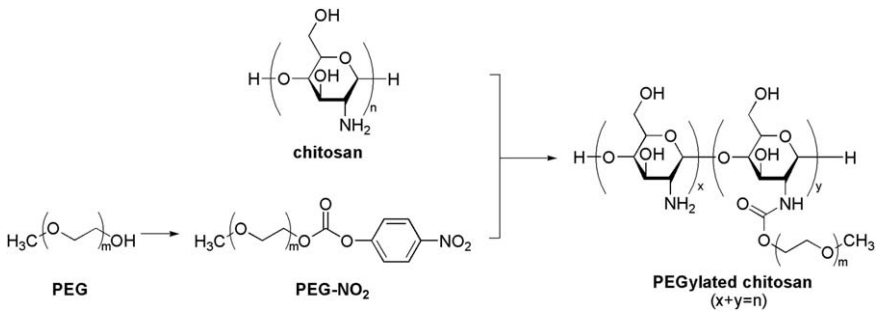
Caco-2 Cell Monolayer Permeability Studies

Caco-2 cells were seeded at a density of 1×10^5 cells/cm^2 in Millicell 24-well cell culture inserts having a poly(ethylene terephthalate) membrane (area = 0.3 cm^2, pore diameter = 0.4 μm). The culture medium was added to both the apical and basolateral compartments and was changed every alternate day for at least 21 days to allow the cells to mature and form a monolayer. Then, the culture medium was aspirated. A D-hanks solution was added to both the apical (0.2 mL) and basolateral (0.9 mL) compartments, and the cells were incubated for 30 min (37°C, 90% humidity, 5% CO_2). After the equilibration period, the transepithelial electrical resistance (TEER) of the monolayer in the D-hanks solution was measured, and the apical chamber medium was replaced with microspheres suspensions in the D-hanks solution (3 mg/mL, 0.2 mL). The TEER values were monitored at 30-min intervals.

The Caco-2 cell monolayer used for transport studies was prepared as mentioned previously. After the equilibration, the apical chamber medium was replaced with 0.2 mL of D-hanks solution containing puerarin alone or puerarin-loaded microspheres, and 0.9 mL of D-hanks solution was supplied into the basolateral side. At predetermined times of 30, 60, 90, and 120 min, aliquots were withdrawn from the basolateral part, and equal volumes of fresh transport medium were supplied. The samples were spectrometrically assayed for drug content at 250 nm (UV–vis, TU-1900, PERSEE, China). Each experiment was performed six times. The apparent permeability coefficient (P_{app}; cm/s) of puerarin was calculated according to the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{AC_0} \quad (5)$$

where dQ/dt is the permeability rate (μg/s), A is the surface area of the monolayer (0.3 cm^2), and C_0 is the initial concentration of puerarin in the apical side (μg/mL).



Scheme 1. Synthesis of the PEGylated chitosan.

Statistical Analysis

Statistical analysis was performed with the Student *t* test, and the differences were judged to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

Synthesis and Characterization of the PEGylated Chitosan

The *p*-nitrophenyl oxycarbonyl group terminated compound could react readily with primary amines under mild conditions. PEGylated chitosan was synthesized as shown in Scheme 1. First, the terminal hydroxyl groups of PEG were reacted with 4-nitrophenyl chloroformate in tetrahydrofuran. The chemical structure of PEG–NO_2 was characterized via a UV–vis spectrometer, ^{1}H-NMR, and Fourier infrared transform (FTIR) spectroscopic studies. Compared with PEG, PEG–NO_2 had specific ultraviolet absorption at 270 nm, and the protons of the phenyl ring from the *p*-nitrophenyl oxycarbonyl group gave two NMR signals at $\delta = 7.50$ and 8.37 ppm (Figure 1). The absorption peaks at 1774 and 1527 cm^{-1} corresponding to terminal ester group and the nitro groups were obvious in the FTIR spectrum of PEG–NO_2, respectively (Figure 2).

Sun *et al.*[26] investigated the characteristics of microspheres composed of different (low, medium, and high) chitosan and reported that medium-molecular-weight chitosan microspheres exhibited the strongest adhesion to the mucosal surface. In this study, chitosan with a medium molecular weight (weight-average molecular weight ≈ 200,000–250,000) was used to prepare the microspheres. The formation of *p*-nitrophenol, a bright yellow byproduct, demonstrated the successful grafting of PEG onto the chitosan chain, and the PEGylated chitosan precipitated from the mixture as a product. Unfortunately, because of the low degree of PEGylation, there were no obvious specific peaks corresponding to the grafted PEG chains in the ^{1}H-NMR and FTIR spectra of the PEGylated chitosan (Figures 1 and 2). The grafting degree of the PEGylated chitosan was demonstrated by elemental analysis, and the influence of the proportion of reactant molecules was investigated. As the PEG–NO_2/chitosan mass ratio in the reaction mixture increased from 1 : 1 to 2 : 1, the PEGylation degree increased distinctly from 0.15 to 0.23%. A further increase in the amount of PEG–NO_2 had less effect on improving the grafting degree (Table I).

Preparation and Characterization of the Microspheres

The PEGylated chitosans P1C and P2C were used to prepare the P1CM and P2CM microspheres, respectively, via the emulsification and crosslinking processes. The chitosan microspheres (P0CMs) were prepared with unmodified chitosan through the same process as a comparison. Compared with the preparation of the chitosan microspheres reported previously, the amount of crosslinker glutaraldehyde used in this study increased; this was probably due to the steric hindrance of the PEG chains. As shown in Figure 3, the PCMs had a broader particle size

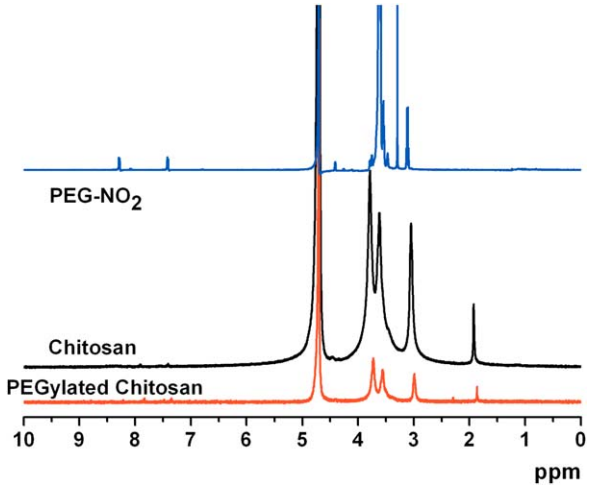


Figure 1. ^{1}H-NMR spectra of chitosan, PEG–NO_2, and PEGylated chitosan (P3C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

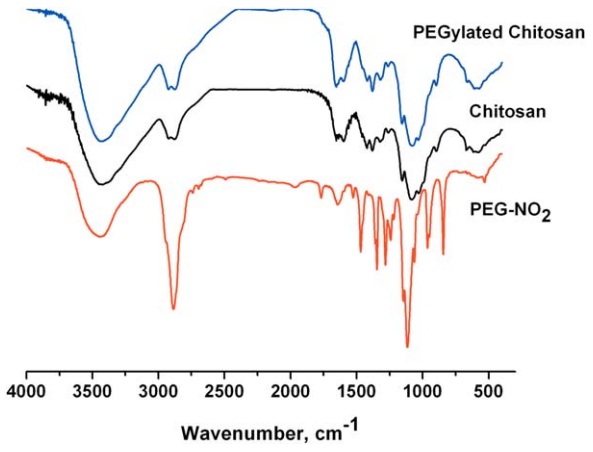


Figure 2. FTIR spectra of chitosan, PEG–NO_2, and PEGylated chitosan (P3C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table I. Degree of PEGylation Calculated from Elemental Analysis

Sample	PEG-NO_2/ chitosan (w/w)	C/N	PEGylation degree (%)
Chitosan	—	5.467	—
P1C	1/1	5.583	0.15
P2C	2/1	5.647	0.23
P3C	3/1	5.655	0.24

distribution than the P0CMs. The mean diameters of the P0CMs, P1CMs, and P2CMs were 2.48 ± 0.09, 2.73 ± 0.14, and 3.22 ± 0.26 μm, respectively. Moreover, scanning electron microscopy revealed that both the P0CMs and PCMs had a spherical shape and a smooth surface (Figure 4). PEGylation played a positive role in promoting the dispersion of the microspheres and decreasing the degree of aggregation.

Figure 5 shows the swelling behaviors of the microspheres in aqueous solutions at 37°C. All of the investigated samples exhibited considerable swelling properties, and a significant amount of water was absorbed within the first 6 h. After immersion for 6 h, the swelling capacities of the P0CMs, P1CMs, and P2CMs were 174.8 ± 10.9, 203.0 ± 8.4, and $250.6 \pm 6.2\%$, respectively. After 24 h, the water uptake of the P2CMs approached saturation, and the swelling capacity increased to $312.8 \pm 20.5\%$. From these results, we deduced that the PCMs presented a better performance in water-uptake capability than the P0CMs, especially with respect to quick swelling. The soft and hydrophilic PEG chains grafted onto chitosan introduced a relatively loose structure of the microspheres to hold water, and the swelling capacity of the microspheres increased with increasing degree of PEGylation.

LC of the Microspheres

The concentration of drug in the puerarin-loaded microspheres was quantified by the dissolution of particles in acidic solutions followed by HPLC measurement, and the LC values of the samples were calculated according to eq. (2). The amount of puerarin involved in the preparation of the drug-loaded microspheres was adjusted and optimized. It was apparent from the inspection of Table II that the LCs of the P0CMs, which changed from 0.17 ± 0.04 to $16.73 \pm 1.77\%$, were affected by the mass ratio of puerarin to chitosan. The drug loading increased significantly with increasing mass ratio from 3 : 5 to 4 : 5 because of the visible redundant drug solidified on the surface of the microspheres. A similar phenomena occurred when the drug loading of the P1CMs was investigated. Therefore, the puerarin-loaded microspheres formulated with a drug/polymer mass ratio of 3 : 5 was used to investigate the influence of PEGylation on the properties of the microspheres. Puerarin was

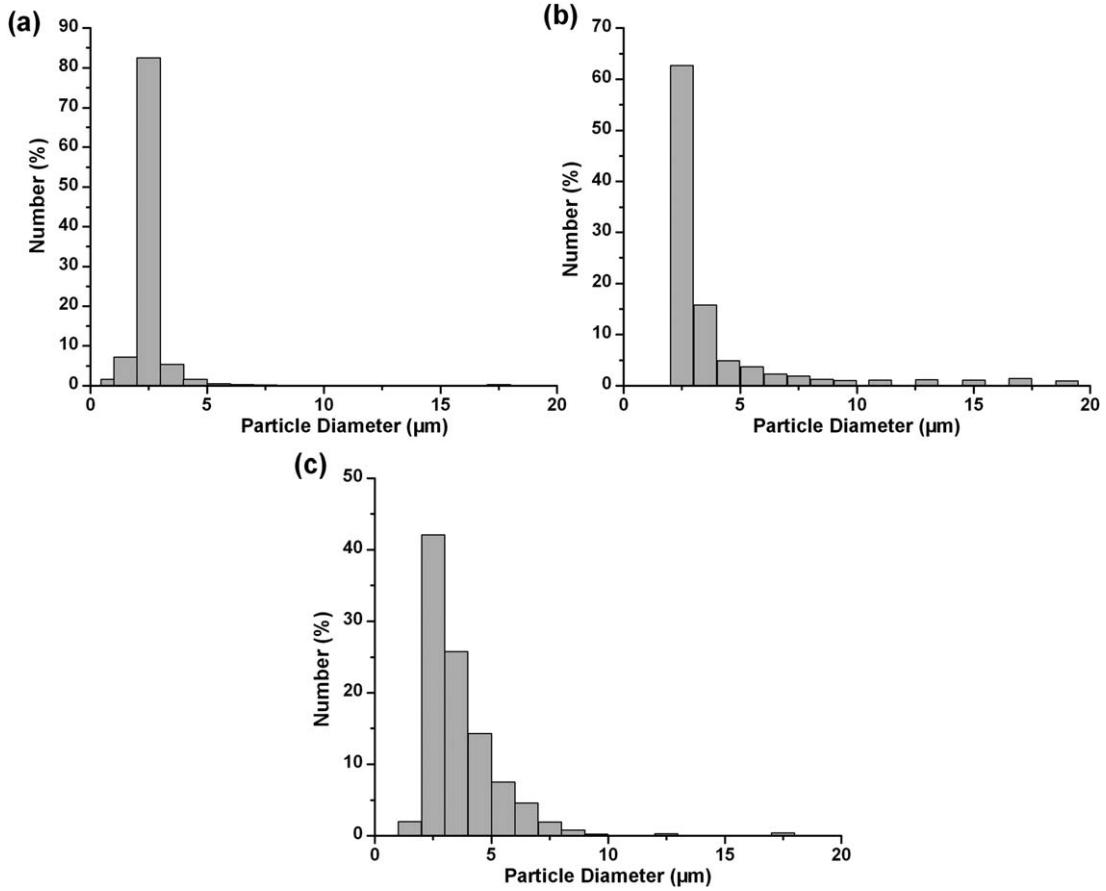


Figure 3. Particle size distributions of the (a) P0CMs, (b) P1CMs, and (c) P2CMs.

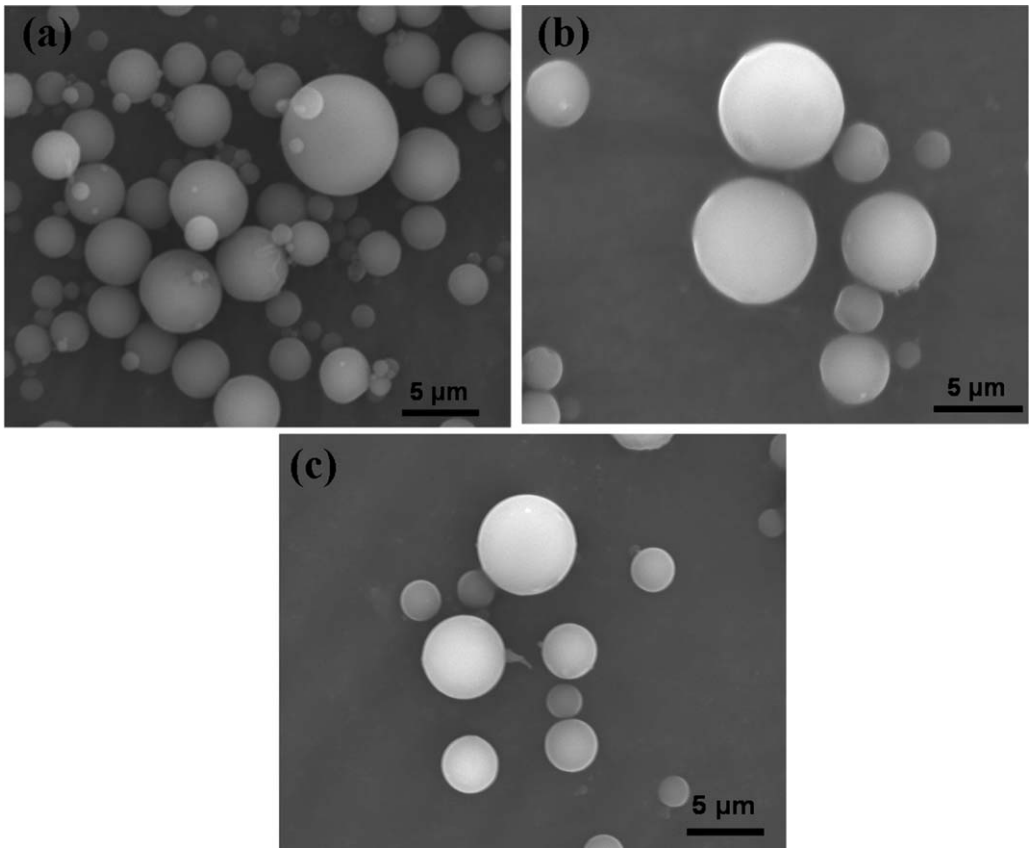


Figure 4. Scanning electron microscopy images of the (a) P0CMs, (b) P1CMs, and (c) P2CMs.

incorporated in the P2CMs under the condition mentioned previously, and the corresponding LC was 4.16 ± 0.10%.

In Vitro Release of Puerarin from the Microspheres

The release behavior of puerarin-loaded microspheres was investigated in aqueous solution at 37°C. A burst release of puerarin from both PEGylated and unmodified chitosan microspheres within the first 30 min was present in Figure 6. Subsequently, the release of the drug was dramatically slowed. Approximately 78% of the puerarin was released from the P0CMs in 6 h, and the drug amounts released from the PEGylated microspheres were higher, about 91 and 93% from the P1CMs and P2CMs, respectively, in the selected experimental time window. As mentioned previously, the increased PEGylation degree promoted the water uptake of the microspheres. Hydration of the microspheres played an important role in their drug-release behaviors. As a result, PEGylation accelerated puerarin release from the particles and decreased the retention of the drug. A rapid release profile is desirable for mucoadhesive drug-delivery systems in order to increase the absorption of drug before the formulation is removed from the administration site by the physiological clearance mechanisms.[11]

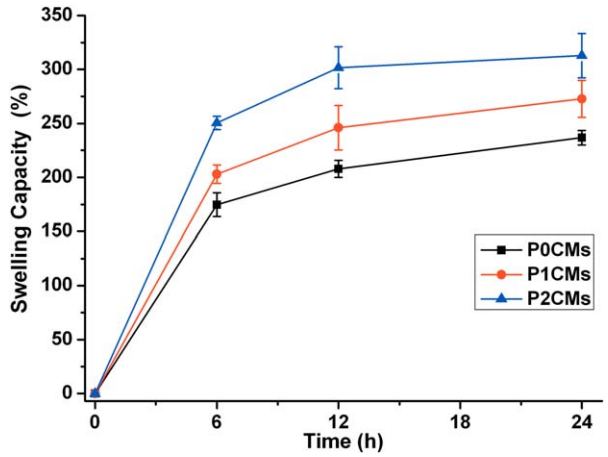


Figure 5. Swelling capacities of the P0CMs, P1CMs, and P2CMs with respect to time. Data are reported as mean values ($n = 6$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Assessment of the Mucoadhesive Behavior of the Microspheres

The mucoadhesive properties of chitosan microspheres are thought to be the results of the combined effects of electrostatic interaction between chitosan and mucin and the increased viscosity of mucus via a dehydration process.[13] In this study, the influence of PEGylation on the mucoadhesive behavior of the microspheres was investigated via the evaluation of the interactions between the microspheres and mucin both in the aqueous solution and on the surface.

Because of the strong electrostatic interaction, mucin in solution could be adsorbed onto the surface of the chitosan microspheres. The amount of mucin adsorbed was determined from

Table II. Influence of the Mass Ratio of Puerarin to Chitosan (or P1C) in the Preparation Process of the Drug-Loaded Microspheres on LC

	LC (%)				
Sample	1/5	2/5	3/5	4/5	5/5
P0CMs	0.17 ± 0.04	1.80 ± 0.21	3.52 ± 0.22	14.38 ± 1.25	16.73 ± 1.77
P1CMs	0.16 ± 0.03	1.22 ± 0.11	3.56 ± 0.14	8.09 ± 1.08	12.58 ± 1.47

the change in the free concentration of mucin in the reaction solution according to eq. (3). The adsorption ratio of mucin on the P0CMs was 67.42 ± 0.53%. A wealth of studies have shown that PEG-based polymers exhibit excellent protein-resistant properties because of the PEG chain mobility and steric stabilization force. PEG chains grafted onto the chitosan backbone decreased the interaction of the microspheres and mucin; this resulted in a dramatic decrease in the adsorption. As the PEGylation degree increased, the adsorption ratios of mucin onto the P1CMs and P2CMs decreased to 34.56 ± 0.76 and 29.39 ± 0.47%, respectively.

The amount of puerarin-loaded microspheres adhering to the mucin saturated surface after the application of a stream of air was further determined. The mucoadhesiveness of the microspheres was evaluated through HPLC measurement of the concentration of the drug in the particles adhered on the surface. The results showed some differences in the mucoadhesive properties, which were dependent on the PEGylation degree of chitosan because the adhesion ratios of the P0CMs, P1CMs, and P2CMs on mucosa-like surfaces were 75.55 ± 2.61, 95.23 ± 1.63, and 92.28 ± 2.13%, respectively. The PCMs were characterized by an obviously improved mucoadhesive behavior on a mucosa-like surface with respect to the P0CMs. On the basis of the results of the adsorption ratio of mucin onto microspheres in solutions and the results from the experiments of water uptake, the improved dehydration of mucus should be the main reason for the better mucoadhesiveness of PEGylated samples on the surface.

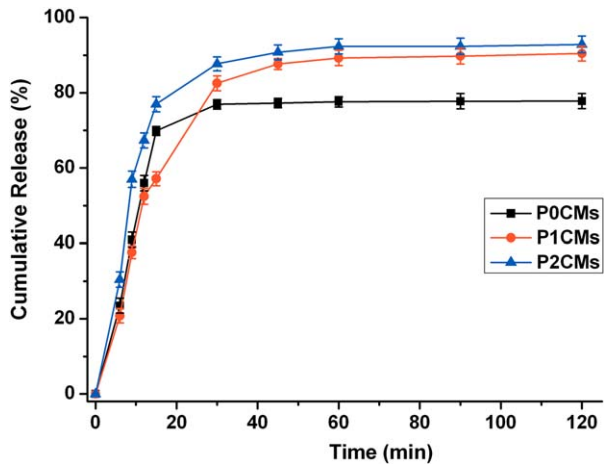


Figure 6. Release profiles of puerarin from the P0CMs, P1CMs, and P2CMs. Data are reported as mean values ($n = 3$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Studies with the Caco-2 Cell Line

The influence of the PEGylation of microspheres on the drug permeability was investigated on the Caco-2 cell monolayer. The Caco-2 cell line has been widely reported as a relatively simple and well-characterized *in vitro* model for drug-transport studies, specifically for the research on the drug-delivery systems intended for oral and nasal administration.

One of the major requirements for the drug-delivery systems is low cytotoxicity. In this study, all of the microspheres were found to be nontoxic to Caco-2 cells. A reduced cytotoxicity attributed to PEGylation, as previously reported, was observed.[15] The cell viabilities after particle exposure for 4 h at a concentrations of 3 mg/mL were 85.5 ± 2.1, 91.8 ± 1.3, and 97.2 ± 1.6%, respectively, for the P0CMs, P1CMs, and P2CMs.

Then, the ability of the microspheres to open monolayer tight junctions was tested by measurement of the TEER values. Smith *et al.*[27] reported that chitosan-mediated tight-junction disruption was caused by a translocation of the tight-junction protein ZO1 from the membrane to the cytoskeleton. In comparison with chitosan in free-solution form, particles based on chitosan had a much lower effect on opening the tight junctions, and the available amount of positive charge at the surface of the particles played an important role in determining the extent to which the particles can modulate paracellular permeability.[28] As evident from Figure 7, the microsphere-mediated reduction in TEER was observed, and the magnitude of decrease in TEER ranged around 35% of the initial value. This was comparable with the previous reports on the effect of the chitosan particles on TEER of the Caco-2 cell monolayer. The P2CMs induced a greater drop in TEER than the P0CMs and P1CMs after the first 60 min of the experiment, and then, the TEER value began to stabilize. The quick and significant decrease in the TEER values observed in case of the P2CMs might have been due to their excellent swelling capacity because the hydrogel drug carriers exhibited a remarkable ability to open the paracellular tight junctions.[29–31] The large water-absorption characteristics of the drug carriers resulted in cell dehydration and subsequent cell shrinking, which ultimately led to the expansion of tight junctions. After 120 min of incubation, the difference in the reduction of TEER induced by the tested samples was insignificant, and this phenomenon probably resulted from the negative effect of PEGylation on decreasing the TEER values caused by the lower chitosan content in the particles counteracting the positive effect mentioned previously.

The permeation of puerarin from solution and microsphere formulations across the Caco-2 cell monolayer was demonstrated by the permeation profiles (Figure 8) and the calculated P_{app}

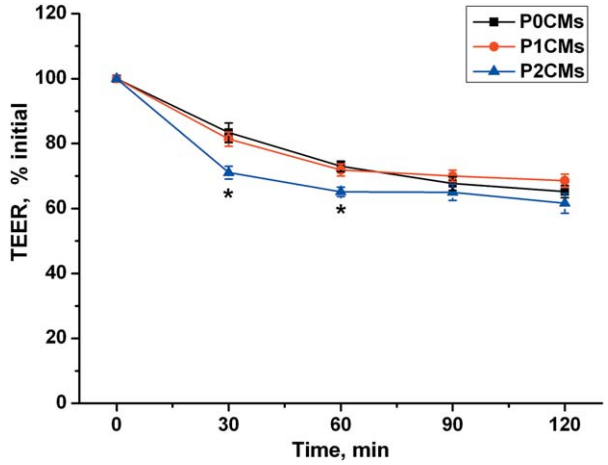


Figure 7. Effects of the P0CMs, P1CMs, and P2CMs on the TEER of a Caco-2 cell monolayer. Data are reported as mean values ($n = 6$). *Differs from the P0CMs ($p < 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

values. The results obtained indicate that the incorporation of puerarin into the microspheres significantly affected its permeability. The puerarin solution showed about 7.52% of the drug permeated across the Caco-2 cell monolayer in 120 min. As expected, the amounts of puerarin permeated were significantly increased up to about 13.97, 16.41, and 17.97% after incorporation into the P0CMs, P1CMs, and P2CMs, respectively. The puerarin-loaded P0CMs showed a P_{app} value of 19.22×10^{-6} cm/s and caused a 2.7-fold enhancement of the P_{app} value compared with that of the puerarin solution (7.03×10^{-6} cm/s). In particular, for the puerarin-loaded P1CMs and P2CMs (23.35×10^{-6} and 24.80×10^{-6} cm/s, respectively), PEGylation yielded 21.5 and 27.4% enhancements compared with the P_{app} value of the puerarin-loaded P0CMs. The effectiveness of chitosan-based drug carriers in improving the drug-permeation properties,

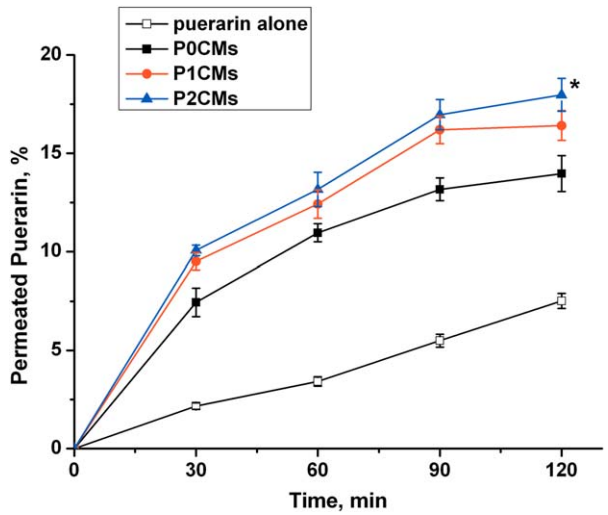


Figure 8. Permeation profiles through the Caco-2 cells of puerarin alone and puerarin-loaded microspheres. Each point is the mean of six experiments. *Differs from the P0CMs ($p < 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

which is related to their abilities in opening the cellular tight junctions and their mucoadhesive properties allowing a prolonged intimate contact between the biological membranes and the carriers, has been studied in depth. In this study, the relationship between the mucoadhesion of the microspheres and the drug permeability was not taken into account because the Caco-2 cells could not secrete mucin. Because the ability of the tested microsphere formulations in the tight-junction opening had no significant difference after 120 min of incubation, we hypothesized that the excellent swelling capacity of the PCMs, with a consequent rapid and efficient drug-release behavior, was responsible for the improved permeability of puerarin.

CONCLUSIONS

PEGylated chitosan derivatives with different degrees of substitution (0.15 and 0.23%) were synthesized and used to prepare microspheres for the transmucosal delivery of puerarin. The PEGylation of chitosan conferred valuable benefits to the properties of the microspheres even at a low degree of PEG substitution. In addition to preventing the aggregation of particles and the efficient release of puerarin *in vitro*, microspheres based on PEGylated chitosan exhibited improved mucoadhesive properties and decreased cytotoxicity. Moreover, the permeation-enhancing capabilities of the particles were clearly observed with the Caco-2 cell monolayer model. From our detailed investigation, the noticeable and positive effects of the swelling capacity of the microspheres on their drug-release behavior, mucoadhesiveness, and opening of tight junctions were demonstrated. With these considerations taken into account, for the transmucosal delivery of puerarin or other hydrophilic therapeutic agents, PCMs are suitable as promising candidates for the development of mucoadhesive microparticulate drug-delivery systems.

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