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Chitosan–glutathione conjugate-coated poly(butyl cyanoacrylate) nanoparticles: Promising carriers for oral thymopentin delivery

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ABSTRACT

Thymopentin (TP5)-loaded poly(butyl cyanoacrylate) nanoparticles (TP5-PBCA-NPs) were prepared and their efficacies for oral delivery were evaluated before and after coating with chitosan or chitosan–glutathione conjugate (chitosan–GSH). TP5-PBCA-NPs were prepared by using optimized emulsion polymerization. Chitosan–GSH or chitosan was coated onto the surface of TP5-PBCA-NPs utilizing electrostatic interactions. Particle size, zeta potential, entrapment efficiency, TP5 bioactivity and *in vitro* drug release behavior, were examined. The pharmacodynamical studies on oral administration of these nanoparticles were performed using FACScan flow cytometry. All the drug-loaded nanoparticles increased the $CD3^+$, $CD4^+$ and $CD8^+$ T lymphocytes counts of the immune dysfunction rats compared with the TP5 solution but only chitosan–GSH-coated TP5-PBCA-NPs restored the T lymphocytes level to normal ($p < 0.05$). These results suggest that the hydrophobic PBCA-NPs could be used to improve the oral bioavailability of hydrophilic peptides. Chitosan and chitosan–GSH coated nanoparticles probably enhanced the efficacy, because of increased mucoadhesive capacity. This was particularly the case for the chitosan–GSH coated nanoparticles.

1. Introduction

With the rapid development of bioscience and biotechnology, lots of protein and peptide drugs have been developed. In clinical practice, the most common means for administrating these drugs remains injection, which brings patients much inconvenience and pain. Therefore, much attention has been paid to develop non-invasive ways for peptides and proteins administration. Among non-parenteral delivery systems, oral administration is the most attractive route due to its easy handling, high patient compliance and lower costs. However, the development of oral delivery system for proteins or peptides has been hampered by their low stability in gastrointestinal tract and low mucosa permeability.

In recent decades, bioadhesive nanocarriers have been developed for oral delivery of proteins and peptides, such as chitosan or chitosan–aprotinin coated liposomes (Takeuchi, Matsui, Sugihara, Yamamoto, & Kawashima, 2005; Werle and Takeuchi, 2009), lectin-modified liposomes or lectin-conjugated PLGA nanoparticles (Yin, Chen, Qiao, Lu, & Hu, 2006; Zhang et al., 2005). These nanocarriers can protect drugs against premature degradation, increase their absorption by intestinal epithelium and Peyer's patches, and prolong the gastrointestinal residence time through either nonspecific or specific interactions between carriers and mucosal surface of GI tract (Ponchel and Irache, 1998). But for these nanocarriers, there are still some problems to be solved, such as the toxicity and/or immunogenicity of lectin (Lehr, 2000), the low loading capacity or entrapment efficiency for hydrophilic drugs, unsatisfied mucoadhesive capacity of chitosan, and the low stability of liposome in GI tract (Kokkona, Kallinteri, Fatouros, & Antimisiaris, 2000).

The thiolated polymers or designated thiomers, such as sulfhydryl bearing poly(acrylates) or chitosans and thiolated trimethyl chitosan, have shown high bioadhesive ability and specific mucoadhesive property due to the formation of disulfide bonds between the thiolated polymers and cysteinerich subdomains of the mucus gel layer (Leitner, Walker, & Bernkop-Schnurch, 2003). Among these thiomers, chitosan–glutathione conjugate (chitosan–GSH) exhibited improved mucoadhesive properties, and its solution has the excellent enhancement for drug permeation *in vitro* (Kafedjiiski, Foger, Werle, & Bernkop-Schnurch, 2005). So far, the combination of chitosan–GSH and nanocarriers is still developing.

In this study, an assembling strategy for bioadhesive nanocarriers was developed for oral delivery of proteins and peptides. poly(butyl cyanoacrylate) (PBCA), a biocompatible and biodegradable polymer, is used to prepare peptide-loaded and stable nanoparticles in GI tract. Taking advantage of the anionic nature of PBCA nanoparticles (PBCA-NPs), chitosan–GSH or chitosan a positive charge can be easily coated on the surface of PBCA-NPs through the electrostatic interaction, thus forming nanoscale carriers with bioadhesive properties. PBCA has been widely used

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in the preparation of nanoparticles encapsulating various bioactive molecules (Friese, Seiller, Quack, Lorenz, & Kreuter, 2000; Simeonova, Velichkova, Ivanova, Enchev, & Abrahams, 2003; Simeonova et al., 2009; Sullivan and Birkinshaw, 2004; Wilson, Samanta, Santhi, Kumar, Paramakrishnan, & Suresh, 2008), but it is obvious that these nanoparticles do not have satisfactory bioadhesive properties. PBCA-NPs may not be the preferred and attractive candidate for oral bioadhesive drug delivery system.

Thymopentin (TP5) was chosen as a model peptide in this study. TP5 is a synthetic pentapeptide (Arg-Lys-Asp-Val-Tyr) corresponding to amino acid residues 32–36 of the thymic hormone thymopoietin (Goldstein and Boyse, 1979). It possesses all the biological activities of a native hormone, for example, induction of early T cell differentiation and immune regulation (Goldstein, 1985). The pentapeptide has been used for the treatment of some diseases, such as acquired immunodeficiency syndrome (AIDS), cutaneous T-cell lymphoma/cancer immunodeficiency and rheumatoid arthritis (Wang et al., 2006). Due to poor membrane permeability, extensive metabolism in the GI tract and extremely short half-life of 30 s, repeated injections or *i.v.* infusions of TP5 are necessary, which greatly limit its clinical applications.

In the present study, the characteristics of these coated or non-coated PBCA-NPs, including particle size, zeta potential, bioactivity, entrapment efficiency and *in vitro* drug release were investigated. Using immune dysfunction rats as the pharmacodynamic model *in vivo*, the immunomodulatory potency was studied after oral administration of these nanoparticles and TP5 solution, in which T lymphocyte subsets population was analyzed and evaluated as the efficacy index by FACScan flow cytometry.

2. Materials and methods

2.1. Materials

Chitosan (viscosity average molecular weight 90 kDa, degree of N-deacetylation > 95%) was obtained from Zhejiang Aoxing Biotechnology Co. (China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), L-glutathione reduced form (GSH), N-hydroxysuccinimide (NHS) and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were all purchased from Bio Basic Inc. (Canada). Porcine stomach mucin (type III), MTT and concanavalin A (Con A) were provided by Sigma (USA). α-Butyl cyanoacrylate monomer was kindly provided by Beijing Suncon Medical Adhesive Co. (China). Thymopentin was purchased from Shanghai Soho-Yiming Pharmaceuticals Co. (China). Cyclophosphamide was provided by Jiangsu Hengrui Medicine Co. (China). Fluorescein isothiocyanate (FITC) antirat CD3, phycoerythrin (PE) antirat CD8a and hemolysin were supplied by eBioscience (USA). Allophycocyanin (APC) antirat CD4 was supplied by Biolegend (USA). All other chemicals were of analytical grade.

2.2. Synthesis of chitosan–GSH

The covalent attachment of reduced glutathione to chitosan was carried out according to the method described by Kafedjiiski et al. (2005). In brief, 0.5 g of chitosan was hydrated in 1 M HCl and then dissolved by the addition of demineralized water to obtain 1% (w/v) polymer solution. Under continuous stirring, 1 g of reduced glutathione in demineralized water was added to the polymer solution. Then, EDAC and NHS were added into the solution to give a final concentration of 200 mM for each component. The reaction mixture was adjusted to pH 6.0 with 5 M NaOH and incubated for 6 h at room temperature until conjugate formation. The product was then dialyzed in tubing (molecular weight cutoff 12 kDa) to eliminate unbound reagents. Finally, the conjugate solutions were lyophilized at −50 °C and 0.01 mbar (FD2.5, Heto, Denmark) and stored at 4 °C until use. The controls were prepared in the same way but omitting EDAC and NHS during the coupling reaction.

2.3. Determination of the thiol group content of chitosan–GSH conjugate

The amount of thiol groups immobilized on chitosan–GSH conjugate was determined spectrometrically with Ellman's reagent as described previously (Atyabi, 2008). 0.5 mg conjugate was dissolved in 250 μl of demineralised water. Then, 250 μl PBS (0.5 M, pH 8.0) and 500 μl Ellman's reagent were added. The samples were incubated for 3 h at room temperature. After centrifugation, the absorbency of supernatant was measured at wavelength of 450 nm, with the correction wavelength set at 620 nm. GSH standards were used to calculate the amount of thiol groups immobilized on the polymer.

2.4. Measurement of mucoadhesive capacity of chitosan and chitosan–GSH in vitro

The mucoadhesive capacity of chitosan and chitosan–GSH with mucin particles was evaluated by measuring the changes of zeta potential and mean particle size of mucin particles (Shen, Wang, Ping, Xiao, & Huang, 2009; Takeuchi, Thongborisute, et al., 2005). The chitosan and chitosan–GSH were completely dissolved in phosphate buffer (0.05 M, pH 5.8) and filtered through 0.22 μm of cellulose acetate membrane before use. The mucin was hydrated and diluted with phosphate buffer (0.05 M, pH 5.8) to obtain a 0.5% (w/v) suspension. Followed by ultrasonication and filtering through 0.80 μm of cellulose acetate membrane, the filtrate was collected for use.

Equal volumes of polymers solution and the mucin filterer suspension were mixed and incubated for 30 min with continuously stirring. The zeta potential and mean particle size of the filtrate were measured by Zetasizer (3000HS, Malvern Instruments Ltd., UK). Each test was performed in triplicate.

2.5. Preparation and coating of TP5-loaded PBCA nanoparticles (TP5-PBCA-NPs)

TP5-PBCA-NPs were prepared by emulsion polymerization method (Bootz, Vogel, Schubert, & Kreuter, 2004; Mulik, Mahadik, & Paradkar, 2009; Niall Behan, 2001) with modification. Briefly, under constant stirring, 100 μl BCA was dropped into 10 ml solution containing 0.6% dextran-70, 0.7% poloxamer 188 and 0.1% sodium metabisulfite (pH 2.0 adjusted by HCl). 20 min later, 10 mg TP5 dissolved in 1 ml de-ionized water was introduced. Keeping the polymerization for 4 h under stirring at 800 rpm, the reaction solution was neutralized to pH 4.5 and polymerized for another 1 h. The resulting suspension was filtered to remove agglomerates. Subsequently, the suspension was freeze-dried (FD2.5, Heto, Denmark) with additional trehalose as a cryoprotectant.

For the preparation of chitosan-coated or chitosan–GSH-coated PBCA-NPs, 10 ml chitosan or chitosan–GSH solution with various concentrations (0.1, 0.3, and 0.5%) was added to 0.75 g lyophilized powder of TP5-PBCA-NPs, respectively. Then, the mixture was incubated for 30 min to obtain chitosan-coated TP5-PBCA-NPs (chi-TP5-PBCA-NPs) and chitosan–GSH coated TP5-PBCA-NPs (chi_{GSH}-TP5-PBCA-NPs).

The amount of thiol groups modified onto TP5-PBCA-NPs was calculated, which was equal to the difference between the total amount of thiol groups in the mixture and the amount of thiol groups in the supernatant after centrifugation of the mixture at

15,000 rpm for 30 min. Both amounts were determined spectrometrically with Ellman's reagent as described in Section 2.3.

2.6. Nanoparticles characterization

2.6.1. Morphology, particle size and zeta potential

The morphology of three nanoparticles was characterized with electronic transmission microscope (TEM) (H-700; Hitachi, Japan) using negative-staining method. The size distribution and zeta potential were determined by Zetasizer (3000HS, Malvern Instruments, UK).

2.6.2. Assay of drug entrapment efficiency (DEE)

Nanoparticles suspension 0.2 ml was diluted with 0.8 ml demineralized water, and then was centrifuged at 15,000 rpm for 30 min. The unloaded drug amount in the supernatant was determined by HPLC (1100 LC, Agilent, USA) at 275 nm through RP C18 column (150 mm × 6.0 mm, 5 μm, Shimpack, Japan). Mobile phase was consisted of PBS (0.02 mol/l, pH 7.0)–MeOH (87:13, v/v), and the flow rate was 1 ml/min. Instead of demineralized water, tetrahydrofuran was used to mix with the nanoparticles suspension for the assay of total drug amount. DEE was calculated as follows:

$$\text{DEE\%} = \frac{(\text{total drug amount} - \text{unloaded drug amount})}{\text{total drug amount}} \times 100\%$$

2.6.3. In vitro drug release studies

In vitro release studies of TP5 from these nanoparticles were performed as follows. The nanoparticles suspension 2 ml or the drug solution containing 2.0 mg/ml of TP5 was transferred into a dialysis bag. The bags were then dipped into 100 ml phosphate buffer (0.05 M, pH 6.8) or 0.1 M HCl (pH 1.2), 37 °C with stirring 60 rpm. At specified time intervals, 1 ml of release medium were withdrawn for HPLC assay and 1 ml fresh media was added. The cumulative release of TP5 from the nanoparticles was calculated. All the operations were performed in triplicate.

2.7. Bioactivity assay in vitro of TP5-PBCA-NPs

T cell proliferation assay was utilized for the bioactivity identification of TP5 entrapped in PBCA-NPs (Chi, Xie, Zhang, Yang, Li, & Mei, 2008). The TP5-PBCA-NPs suspension was centrifuged at 15,000 rpm for 30 min to remove the free drug in supernatant, and then the precipitate was re-suspended in 0.05 M PBS (pH 6.8). The release study of re-suspended TP5-PBCA-NPs was carried out as described in Section 2.6.3. The release medium was taken and diluted to 1 μg/ml of TP5 as the bioactivity test solution. The fresh prepared TP5 solution with same concentration was used as the reference.

Spleen cells were collected from BALB/c mice and suspended with RPMI 1640 to the concentration of 5.0×10^6 cell/ml. The test solution or reference solution of TP5 were added in 96-well plate (10 μl/well) with or without 10 μl concanavalin A (ConA, 50 μg/ml) in triplicate. RPMI 1640 containing 10% fetal bovine serum was added to each well till 100 μl/well. Then 100 μl of cell suspension above was added into each well and mixed. The cells well treated with 100 μl RPMI 1640 containing 10% fetal bovine serum were regarded as the blank control. All the cells were kept for 48 h at 37 °C in a 5% CO_2 incubator. MTT (5 mg/ml) was added into each well at 4 h before the end of culture. After incubation, the cells were centrifuged in a microtiter plate centrifuge (TD5A-WS, Changsha Xiangyi Centrifuge Instrument Co., China) for 15 min at 3,000 rpm. The supernatant was removed. Then 200 μl of DMSO was added to each well to dissolve formazan crystals. Absorbance was read at 570 and 630 nm on a spectrophotometric plate reader (Thermo Labsystems MK3, USA), respectively. The TP5 bioactivity was expressed as the stimulation index (SI) and calculated as follows:

$$\text{SI} = \frac{(A_{570} - A_{630})_{\text{TP5 sample}}}{(A_{570} - A_{630})_{\text{control}}}$$

2.8. In vivo pharmacodynamic studies

2.8.1. Immunosuppression model rats

All animal experiments complied with the requirements of the National Act on the use of experimental animals (China). 30 normal female Sprague–Dawley rats (180–210 g, provided by the experimental animal center of Zhejiang province) were equally divided into six groups (five each) and fasted for 12 h with free access to water before the experiments. Group 1 as a normal control was per orally given physiological saline once daily for 10 days. Group 2 through Group 6 were intraperitoneally primed with cyclophosphamide in a dose of 35 mg kg^{-1} d^{-1} at first 3 days for construction of immunosuppression model (Li, Wang, Song, Fan, & Mei, 1996; Sun et al., 2000). From the fourth day, once daily for 7 days, Group 2 as an immunosuppression control were orally given physiological saline, and Group 3 through Group 6 were orally administered with TP5 dose of 2 mg kg^{-1} d^{-1} of following formulations: (1) TP5 solution; (2) TP5-PBCA-NPs; (3) chi-TP5-PBCA-NPs; (4) chi_{GSH}-TP5-PBCA-NPs. 200 μl blood of each rat in all groups was collected via the caudal vein at eleventh day. All the blood samples were placed into heparinized tubes and stored at 4 °C until flow cytometry analysis.

2.8.2. Flow cytometry analysis

Lymphocyte populations were determined by multiparameter flow cytometry with three-color analyses as follows (Wang et al., 2006; Yin et al., 2006). Anticoagulant blood 100 μl, 2 μl CD3-FITC, 5 μl CD4-APC and 5 μl CD8a-PE were added to test tube, mixed by vortex for 30 s and incubated at room temperature and darkness for 20 min. Then 2 ml hemolysin was added and incubated for another 10 min. After red cells were lysed completely, the specimens were centrifuged at 2000 rpm for 5 min. The sediment cells were collected, washed with 2 ml physiological saline twice, fixed in 0.5 ml of 1% paraformaldehyde, and then the $CD3^+$, $CD4^+$ and $CD8^+$ T-lymphocyte subsets were measured within 4 h using FACSCalibur flow cytometer with CELLQuest software (Becton Dickinson, USA) for acquisition and analysis. The values of $CD3^+$ T cell counts, $CD4^+$ T cell counts and $CD8^+$ T cell counts, as well as the ratios of $CD4^+$ and $CD8^+$ were calculated for the evaluation of immunocompetence.

2.8.3. Statistical analysis

Mean values and standard deviations (SD) of recorded parameters were calculated. For comparison between the experimental groups and corresponding controls, the analysis of variance ANOVA followed by post hoc Student–Newman–Keuls test (SPSS 13; SPSS Int., Chicago, IL, USA) was applied. A value of $p < 0.05$ was considered to be significant.

3. Results and discussion

3.1. Interaction between mucin particles and chitosan–GSH or chitosan

Thiolated chitosan, chitosan–GSH, was synthesized by the amide bond formation between glycine carboxylic acid groups of glutathione and amine groups of chitosan. The free thiol groups per gram conjugate was determined to be 271.5 ± 6.2 μmol, which was similar to the result (265 μmol/g) published by Kafedjiiski et al. (2005). It was expected that these free thiol groups of the chitosan

Table 1
Changes in size and zeta potential of the mucin particles when mixed with chitosan–GSH or chitosan solution.

Kind of particles	Mean particles size (nm)	Polydispersion index	Zeta potential (mV)
Mucin	380.7 ± 10.3	0.357 ± 0.013	−5.7 ± 0.12
Mucin + chitosan	468.7 ± 4.1*	0.334 ± 0.011	+3.3 ± 0.15*
Mucin + chitosan–GSH	600.5 ± 6.2*	0.187 ± 0.031	+13.5 ± 0.75*

Data are represented with mean ± SD ($n = 3$).
* Differs from mucin, $p < 0.05$.

Table 2
Characterization of coated and uncoated TP5-PBCA-NPS.

Nanoparticles	Mean particles size (nm)	Polydispersion index	Zeta potential (mV)	Drug entrapment efficiency (%)
TP5-PBCA-NPs	217.1 ± 7.7	0.038 ± 0.007	-21.9 ± 1.8	81.99 ± 3.48
chi-TP5-PBCA-NPs	275.9 ± 3.3	0.114 ± 0.009	+23.3 ± 1.1	73.96 ± 1.97
chi_{GSH}-TP5-PBCA-NP_S	308.0 ± 6.7	0.152 ± 0.016	+34.4 ± 0.9	71.51 ± 2.61

0.3% polymer concentration was used in the coating.

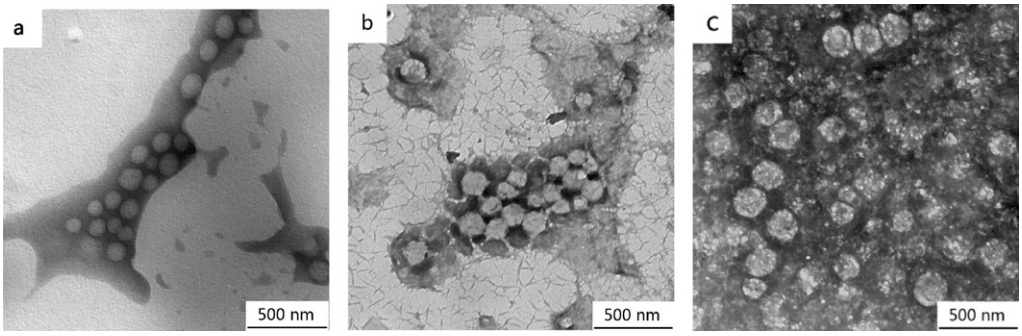

Fig. 1. Transmission electron micrographs of TP5-loaded nanoparticles: (a) TP5-PBCA-NPs, (b) chi-TP5-PBCA-NPs and (c) chi_{GSH}-TP5-PBCA-NPs.

conjugate might have stronger mucoadhesive property than chitosan. As shown in Table 1, the zeta potential of mucin particles changed from negative to positive and the particle size increased significantly after incubated with both of the chitosan–GSH and chitosan solution ($p < 0.05$), indicating the interaction between the polymers and mucin particles. The particle size increment of mucin particles after incubated with chitosan–GSH was larger than that incubated with chitosan. Accordingly, the zeta potential changed by the former was higher than that by the latter. Indeed, chitosan–GSH has stronger interaction with mucin than chitosan due to disulfide bonds formation between thiol groups of chitosan–GSH and cysteine residues of the mucin.

3.2. Preparation and characteristics of chi-TP5-PBCA-NPs and chi_{GSH}-TP5-PBCA-NPs

In the present study, TP5-PBCA-NPs were prepared by emulsion polymerization of n-butyl-2-cyanoacrylate monomers in the presence of TP5. As same as the results indicated by Douglas et al. (1985), the homogeneous nanoparticles (217.1 ± 7.7 nm) were obtained when both dextran-70 and poloxamer 188 were used as the stabilizers in the process. However, the entrapment efficiency of TP5 was low as 6.13%. It was very interesting that the encapsulation efficiency could be significantly increased to 81.99% when sodium metabisulfite, a common antioxidant in pharmaceutical formulations, was used together with these stabilizers (Table 2). Meanwhile, the drug loading capacity of TP5-PBCA-NPs was about 3.2% (w/w), which was greatly higher than that published by other researchers (less than 0.33%) (He, Jiang, & Zhang, 2008). It is very challenging to incorporate the hydrophilic molecule like TP5 into hydrophobic nanoparticles. The entrapment efficiency of TP5 in lipid nanoparticles prepared from O/W or W/O/W microemulsion methods were reported to be 5.2% and 1.7%, respectively (Silvia Morel, Cavalli, & Gasco, 1996). As for lectin-conjugated PLGA nanoparticles, the entrapment efficiency was below 33% (Yin et al., 2006). For multilamellar and plurilamellar liposomes, the encapsulation efficiency were above 75%, but the mass ratio of drug to lipids was about 1.4% (w/w) (Panico, Pignatello, Cardile, & Puglisi, 1997). Our study showed that the presence of sodium metabisulfite in the acid aqueous solution could reduce the zeta potential of PBCA-NPs from −0.8 ± 0.25 mV (without sodium metabisulfite) to −21.9 ± 1.80 mV. The more negative charges probably enhanced the incorporation of TP5 in PBCA-NPs because TP5 molecules with PI value of 8.59 carried positive charges in the acidic condition (Xin Li et al., 2007).

After incubated with chitosan or chitosan–GSH, these polymers could be confirmed presence on the surface of TP5-PBCA-NPs as shown in Figs. 1–3. In acidic aqueous solution (pH < 6.5), the glucosamine units of chitosan can be converted to the form $R–NH_3^+$ form (Thomas Chandy, 1990), and interacts with negatively charged surfaces of particles (Fukuda, 1980; Garcia-Fuentes, Prego, Torres, & Alonso, 2005; Kawashima, Yamamoto, Takeuchi,

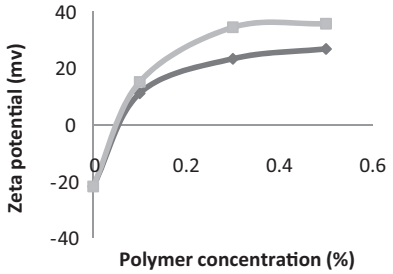

Fig. 2. The influence of chitosan (Diamond) or chitosan–GSH (Box) concentration on zeta of polymer-coated PBCA-NPs (mean ± SD, $n = 3$).

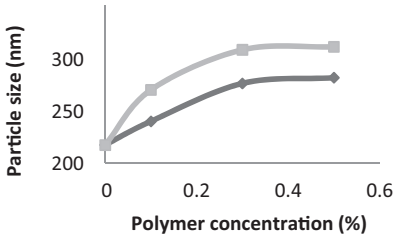

Fig. 3. The influence of chitosan (Diamond) or chitosan–GSH (Box) concentration on sizes of polymer-coated PBCA-NPs (mean ± SD, $n = 3$).

& Kuno, 2000; Takeuchi, Matsui, et al., 2005). The results indicated that the zeta potentials of TP5-PBCA-NPs were converted from negative charge (−21.9 mV) to positive and the sizes were increased by approximately 60–100 nm (Table 2).

When the chitosan or chitosan–GSH concentration in the coating was increased to 0.3%, both the particle size and zeta potential reached to maximum (Figs. 2 and 3). About 36% of the total thiol groups existed on the surface of chi_{GSH}-TP5-PBCA-NPs. At same concentrations of chitosan and chitosan–GSH, chi_{GSH}-TP5-PBCA-NPs showed larger particle size and higher zeta potential. This was consistent with the results of mucin adhesion test *in vitro* where the higher cohesive property of chitosan–GSH was found. The size, zeta potential and drug entrapment efficiency of TP5-PBCA-NPS before and after coating were shown in Table 2. It could be seen that the drug entrapment efficiency of chitosan or chitosan–GSH coated nanoparticles was decreased to some extent because of the drug leaching in the incubation procedure.

3.3. In vitro drug release studies

The *in vitro* release profiles of TP5 from nanoparticles formulations were pH dependent. In the simulated gastric fluid at first 3 h, as shown in Fig. 4, less than 20% TP5 released from PBCA-NPs, and about 35% TP5 released from chi-TP5-PBCA-NPs and chi_{GSH}-TP5-PBCA-NPs. It seems that the release amounts were mainly contributed from the free drugs in these systems (Table 2). For TP5 solution, the release percentage was 95.33 ± 2.2% at first 3 h. The low release rate of TP5 from NPs was probably due to the high affinity of TP5 with NPs under low pH condition.

However, in pH 6.8 PBS at first 3 h, as shown in Fig. 5, the percentage of TP5 released from the solution, PBCA-NPs, chi-PBCA-NPs and chi_{GSH}-PBCA-NPs were 94.63 ± 1.7%, 78.45 ± 2.3%, 65.60 ± 1.9% and 66.93 ± 2.1%, respectively. At 6 h, more than 80% TP5 was released for these nanoparticles. The release patterns of chi-TP5-PBCA-NPs and chi_{GSH}-TP5-PBCA-NPs were very similar. The coating layers of chitosan or chitosan–GSH on the surface of TP-PBCA-NPs reduced drug diffusion rate. The results suggested that most of the drug

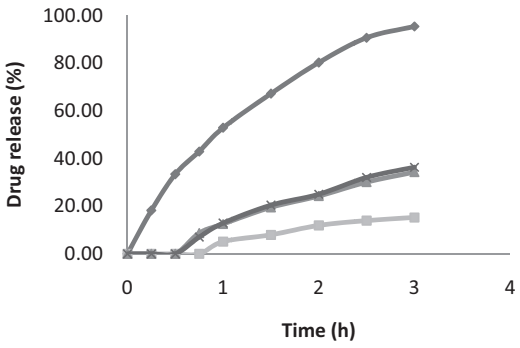

Fig. 4. *In vitro* release profiles of TP5 solution (Diamond), TP5-PBCA-NPs (Box); chi-TP5-PBCA-NPs (Triangle); chi_{GSH}-TP5-PBCA-NPs (Cross). Dialysis method, 37 °C, 0.1 M HCl (pH 1.2) (mean ± SD, $n = 3$).

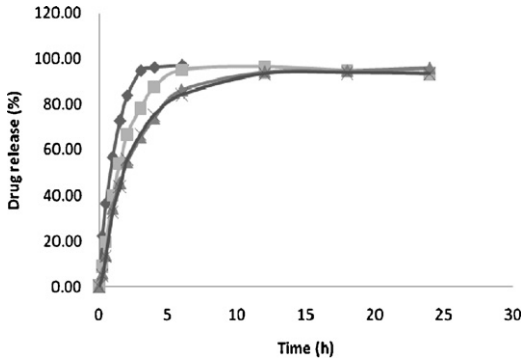

Fig. 5. *In vitro* release profiles of TP5 solution (Diamond), TP5-PBCA-NPs (Box); chi-TP5-PBCA-NPs (Triangle); chi_{GSH}-TP5-PBCA-NPs (Cross). Dialysis method, 37 °C, pH 6.8 PBS (mean ± SD, $n = 3$).

could be entrapped stably in the nanoparticles in stomach environment and then released in intestinal tract for absorption. It is especially valuable for these coated nanoparticles because the mucosadhesion behavior may be expected to occur simultaneously.

3.4. Bioactivity assay in vitro of TP5-PBCA-NPs

The classical assay of T cell proliferation has been utilized to determine the bioactivity of TP5 entrapped in PBCA-NPs. The bioactive TP5 is one kind of synergetic agents, which can strengthen the stimulation function of ConA to T cells proliferation. Such a synergetic effect of the bioactivity test solution (TP5 solution from TP5-PBCA-NPs) was observed in this study (Fig. 6). Comparing with ConA itself, the amount of spleen cells expressed as the stimulation index was significantly increased ($p < 0.05$) when the bioactivity test solution was used together with ConA. It was noticed that the increase of SI was very similar to that of TP5 solution ($p > 0.05$). The results showed that the polymerization process of TP5-PBCA-NPs had no influence on bioactivity of TP5 in the nanoparticles.

3.5. Pharmacodynamic effect of oral TP5-nanoparticles on immune dysfunction rats

Cyclophosphamide, a known immunosuppressant, was extensively used to establish immune dysfunction models of rats (Doi, Nagai, Tsukuda, & Suzuki, 1996; Fen Xia Hou et al., 2007; Gao et al., 2009; Muruganandan, Lal, & Gupta, 2005). The changes of $CD3^+$, $CD4^+$ and $CD8^+$ T cell population and the $CD4^+/CD8^+$ ratios are generally recognized as the index of immune function. After the rats were intraperitoneally primed with cyclophosphamide for 3 days, a series of immune dysfunction symptom was observed (Group 2–6), such as upright hair, reduced appetite, slight diarrhea and diminished activity. As shown in Table 3, the T-lymphocyte percentages of $CD3^+$, $CD4^+$ and $CD8^+$ of the pathological control rats (Group 2) were significantly lowered, and the $CD4^+/CD8^+$ ratio was markedly increased, as compared with those of the normal control rats (Group 1). These results indicated that the T-lymphocyte level of the pathological rats was significantly affected by the immunosuppressant and the pathological model was successfully established.

The level changes of $CD3^+$, $CD4^+$ and $CD8^+$ T-lymphocyte subsets in immune dysfunction rats following administrations with various TP5 formulations with the dose 2 mg kg^{-1} d^{-1} for 7 days (Group 3, 4, 5 and 6) are also shown in Table 3. Compared with those of Group 2, the oral administration of TP5 solution (Group 3) was nearly ineffective, and the $CD3^+$, $CD4^+$ and $CD8^+$ percentages changed little. For TP5-PBCA-NPs (Group 4) and chi-TP5-PBCA-NPs (Group5), the levels were not recovered to the normal values of

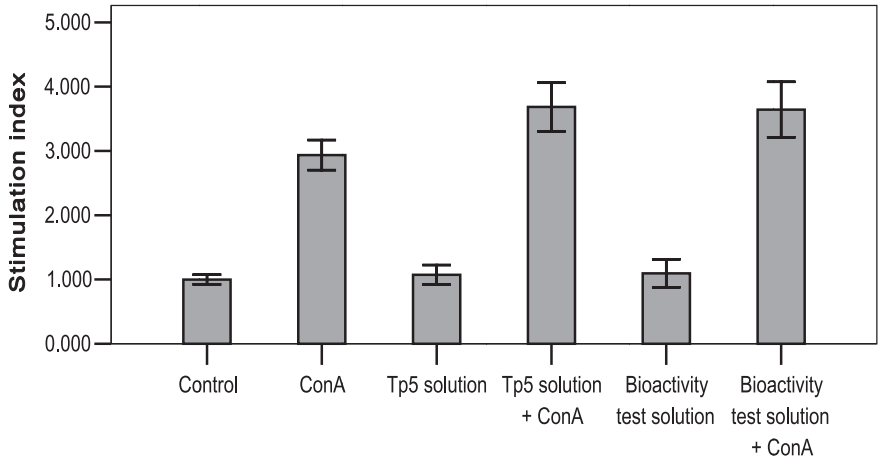

Fig. 6. The stimulation index of ConA with or without TP5 (1 μg/ml) ($n = 3$).

Table 3
$CD3^+$, $CD4^+$ and $CD8^+$ T cells population in immunosuppression rats following oral administrations of TP5 formulations with the dose of 2 mg kg^{-1} d^{-1} for 7 days.

Groups	Treatment	$CD3^+$ (%)	$CD4^+$ (%)	$CD8^+$ (%)	$CD4^+/CD8^+$
1	Saline	64.74 ± 3.52	42.78 ± 1.94	23.54 ± 1.66	1.82 ± 0.15
2	Saline	31.68 ± 2.45[a]	23.76 ± 2.43[a]	7.38 ± 0.70[a]	3.22 ± 0.22[a]
3	TP5 solution	35.84 ± 3.37[a]	24.32 ± 2.75[a]	8.52 ± 1.02[a]	2.87 ± 0.30[a]
4	TP5-PBCA-NP_S	48.74 ± 1.53[a,b,c]	33.76 ± 1.32[a,b,c]	15.88 ± 1.58[a,b,c]	2.15 ± 0.28[b,c]
5	chi-TP5-PBCA-NP_S	55.32 ± 4.00[a,b,c]	38.00 ± 2.74[a,b,c]	17.62 ± 1.31[a,b,c]	2.16 ± 0.15[b,c]
6	chi_{GSH}-TP5-PBCA-NP_S	60.40 ± 2.06[b,c]	41.00 ± 3.95[b,c]	22.50 ± 1.75[b,c]	1.84 ± 0.29[b,c]

Group 1 and Group 2 were the normal control and pathological control, respectively. Each value represents the mean ± SD ($n = 5$).
[a] $p < 0.05$ vs. Group 1.
[b] $p < 0.05$ vs. Group 2.
[c] $p < 0.05$ vs. Group 3.

Group 1 ($p < 0.05$), but the lowered $CD3^+$, $CD4^+$ and $CD8^+$ percentages were significantly increased and the ratio of $CD4^+/CD8^+$ were significantly decreased. Nevertheless, for the rats of Group 6 after oral administration of chi_{GSH}-PBCA-NPs, all lowered $CD3^+$, $CD4^+$ and $CD8^+$ percentages were significantly increased and close to the normal values ($p > 0.05$). The ratio of $CD4^+/CD8^+$ was also restored to the normal level.

TP5 is the active sequence of the natural thymopoietin, which enhances the production of thymic T cells and helps to restore immunocompetence. The results indicate that the oral administration of these TP5 loaded nanoparticles is effective for improving the $CD3^+$, $CD4^+$ and $CD8^+$ population and reversing the irregular $CD4^+/CD8^+$ ratio to the normal values. Vauthier et al. (2003) suggested that the uptake of nanoparticles by Peyer's patches may be the major reason for improving oral bioavailability of drugs. In this study, the TP5-PBCA-NPs showed better pharmacodynamic effect than the free drug solution. Moreover, it was noticed that the nanoparticles with bioadhesive ability could further improve therapeutic efficacy. The largest pharmacodynamic efficacy was found when the nanoparticles were coated by thiolated chitosan. The similar pharmacological effect could be compared in the published study, where TP5-loaded pH-sensitive chitosan nanoparticles coated with Eudragit S100 were orally administered with TP5 dose of 10 mg kg^{-1} d^{-1} for 6 days (Zheng et al., 2006); while the dose was only 2 mg kg^{-1} d^{-1} in our study. It was possible that enteric coating might interfere with the adhesive capacity of the chitosan nanoparticles. The largely improved efficacy of chi_{GSH}-TP5-PBCA-NP_S may contribute to its advanced mucoadhesive properties of chitosan–GSH coating layers and the stability protection of the PBCA-PNs in GI tract. It is well known that the interaction of thiolated polymer with cysteinerich subdomains of intestine mucin results in the disulfide bonds formation in the intestinal mucus layer. Moreover, the inhibition effect of chitosan–GSH for enzyme protein tyrosine phosphatase was also reported (Kafedjiiski et al., 2005). Both the properties of nanoparticles and chitosan–GSH probably enhance the oral absorption of TP5.

4. Conclusion

In this study, hydrophobic PBCA nanoparticle, was used for oral delivery of water soluble peptide, TP5. The entrapment efficiency was greatly increased by the optimized emulsion polymerization. Meanwhile, the biological activity of TP5 could be maintained during the polymerization process. The PBCA-NPs with anionic property were easily assembled with mucosadhesive polymers, chitosan and chitosan–GSH, by the electrostatic interaction to form the nanoscale bioadhesive carriers. All the TP5-loaded nanoparticles have pharmacodynamic effect on the immunosuppression rats following the oral administration, which suggests that these nano-carriers can improve the absorption of peptide. The combination of nanoparticles with mucosadhesive polymers is advantageous. Our study proves chitosan–GSH is a more valuable polymer than chitosan for the nanoparticles coating and implies that chitosan–GSH-coated PBCA-NPs offer an effective and promising oral delivery system for improving absorption of peptides and proteins. The bioavailability study of the nano-systems is being studied.

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