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Research paper

Bioprocess of uniform-sized crosslinked chitosan microspheres in rats following oral administration

Wei Wei [a,b], Lian-Yan Wang [a], Lan Yuan [c], Xiao-Da Yang [d], Zhi-Guo Su [a], Guang-Hui Ma [a,*]

[a] Chinese Academy of Sciences, Beijing, PR China
[b] University of the Chinese Academy of Sciences, Beijing, PR China
[c] Peking University Health Science Center, Beijing, PR China
[d] Department of Chemical Biology, Peking University Health Science Center, Beijing, PR China

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Abstract

Chitosan microspheres have a great potential in pharmaceutical application. In this study, uniform-sized chitosan microspheres crosslinked with glutaraldehyde (CG microspheres) were prepared by Shirasu Porous Glass (SPG) membrane emulsification technique. Based on the characterizations of uniform size and autofluorescence, it was possible to develop a new detecting system for observing and quantifying the CG microspheres in rats with three different diameters (2.1, 7.2 and 12.5 μm) synchronously after oral administration. This system was a combination of scanning electron microscopy (SEM), laser scanning confocal microscope (LSCM) and flow cytometer technique, which showed the advantages of being simple, intuitionistic, repeatable and sensitive. After oral administration of three kinds of particles with different diameters, bioadhesion in gastrointestinal tract, absorption in gastrointestinal tract, distribution in systemic tissues, and biodegradation in reticuloendothelial system (RES) were studied firstly in detail. The CG microspheres showed different fates in bioadhesion, absorption and distribution according to their diameters, while the biodegradation also varied due to the different locations in RES. These original results would indicate a better way for the CG microspheres in the clinical application.
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Keywords: Chitosan; Microsphere; Bioadhesion; Absorption; Distribution; Biodegradation

1. Introduction

Chitosan is a copolymer, the deacetylated form of chitin, consisting of 2-amino-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-glucose units linked with β-(1 → 4) bonds. It is the second most abundant polysaccharide in nature. Because of its biocompatibility, biodegradability, bioadhesive ability and low cost, this polymer is considered as a potential carrier for drugs [1–3].

With the development of DNA-recombinant techniques and other modern biotechnologies, a large number of recombinant proteins and peptides are now being investigated for therapeutic applications [4]. Biodegradable microspheres have occupied an important place in the protein and peptide drug delivery system. The use of microsphere-based therapy can protect proteins from degradation, allow the drug to be released to the specific treatment site and control the drug release [5,6].

Chitosan can form microspheres by various methods such as emulsification/solvent evaporation, spray drying, ionotropic gelation, coacervation technique, and more. The size of the microspheres prepared by these methods is difficult to be controlled, and the size distribution is very broad. These disadvantages will bring the following limitations: (1) the reproducibility of microspheres is poor

* Corresponding author. National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, No. 1, Bei-er-tiao, Zhongguancun, Beijing 100080, PR China. Tel.: +86 10 82627072; fax: +86 10 82627072.
E-mail address: ghma@home.ipe.ac.cn (G.-H. Ma).

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among batches; (2) the bioavailability of drug will be decreased and the side-effects of the drug will be increased, especially for anticancer agents, because the accumulated locations also depend on the size of the microspheres; and (3) particles of different size have different effects. The practical and theoretical evaluation such as release rate and biological effect will become complicated, if the size distribution is broad. Therefore, it is necessary to prepare uniform-sized microspheres and control the size of the microspheres for their application in drug delivery system. In the past few decades, chitosan microspheres were always prepared by the facile method of chemical crosslinking with glutaraldehyde (CG microspheres), which will enable us to obtain the microspheres with good sphericity and compact structure. Herein, uniform-sized CG microspheres in this study were obtained by SPG membrane emulsification technique to overcome the disadvantages aforementioned [7].

The oral route, which has the obvious advantage of convenience, is considered to be the preferred route for drug delivery. Orally administered microspheres will follow at least four continuous bioprocesses in vivo: (1) bioadhesion in gastrointestinal tract; (2) absorption in gastrointestinal tract; (3) distribution in systemic tissues; and (4) biodegradation in RES. In the last decade, latex microspheres were always used to study the effects of various parameters on the uptake efficiency: size, hydrophobicity and influence of surface residues following adsorption or covalent binding of specific substrates. These microspheres labeled with fluorochromes are the most used tools to assess the translocation by observing the histological section using fluorescence microscope [8–12]. The histology gives the direct evidence of the presence and location of particles in a tissue, but it is not suitable for quantification, only semi-quantitative information may be obtained. On the other hand, the fluorochromes may influence the charge of microspheres, therefore changing the intrinsic interaction between microspheres and tissues [13]. Radiolabeled microspheres have also been used extensively to evaluate the translocation [14]. The foremost advantage of this method is sensitivity, and a reliable image of microspheres distribution could be given by gamma-scintigraphy camera [15]. However, instability of the labeling could generate false results, especially in oral administration [16]. Furthermore, high radiation doses are required to obtain measurable numbers of microspheres because of the very low efficiency of uptake. In addition, the availability and safety should also be considered before the experiments. It should be also noted that these latex cannot be an ideal model for drug delivery research due to their non-biodegradability and low biocompatibility.

To our knowledge, few studies are available in the specific area of the behaviors of chitosan microspheres in vivo. In our previous study, CG microspheres were firstly found to possess fluorescent property without conjugating with any fluorescent agent [17]. They could be prepared using a novel membrane emulsification technique to enable a uniform size. Based on the characterizations of uniform size and autofluorescence, a new detecting system was developed in this study. It was a combination of SEM, LSCM and flow cytometer techniques, which are simple, intuitionistic, repeatable and sensitive. The CG microspheres with three different diameters were firstly evaluated in systemic tissues synchronously. The continuous bioprocesses after oral administration were for the first time studied in detail.

2. Experimental section

2.1. Materials

Chitosan (89% for degree of deacetylation, $M_V = 780{,}000$) was purchased from Putian Zhongsheng Weiye Co., Ltd. (Fujian, China). The SPG membrane was bought from SPG Technology Co. (Japan). PO-500 [(Hexaglycerin penta) ester] was bought from Sakamoto Yakuhin Kogyo Co., Ltd. (Japan). KP-18C, a silane coupling agent with C18 hydrophobic chain, was kindly provided by Shin-Etsu Chemical Co. (Japan). Glutaraldehyde was ordered from Sigma–Aldrich Inc. (Germany). Hoechst 33258 was obtained from Molecular Probes (Eugene, OR). Male Sprague–Dawley (SD) rats of narrow weight range (200–250 g) were from the department of laboratory animal science, Peking University health science center (China), and they were permitted to feed ad libitum. All other materials used were of analytical reagent grade.

2.2. Preparation of CG microspheres

The preparation of uniform-sized CG microspheres crosslinked with glutaraldehyde was described in detail by Wang et al. [18]. SPG membranes with a specific pore size were modified hydrophobically with KP-18C before use. The detailed process of CG microsphere preparation is as follows: 2 wt.% Chitosan was dissolved in 1 wt.% aqueous acetic acid containing 0.9 wt.% sodium chloride, which was used as a water phase. A mixture of liquid paraffin and petroleum ether 7:5 (v/v) containing 4 wt.% PO-500 emulsifier was used as an oil phase. Then the water phase was permeated through the uniform pores of SPG membrane into the oil phase by the pressure of nitrogen gas to form W/O emulsion. After that GST (glutaraldehyde saturated toluene) as a crosslinking agent was slowly dropped into the W/O emulsion to solidify the chitosan droplets. Finally, the chitosan microspheres were collected and washed two times with petroleum ether, acetone and ethanol under centrifugation of 3000*g*, and then the microspheres were lyophilized. A JEM-6700F (JEOL, Japan) scanning electron microscopy (SEM) was used to observe the shape and surface feature of CG microspheres. The mean size and size distribution of CG microspheres were determined by Mastersizer 2000 laser diffractometry (Malvern, United Kingdom). The size distribution was defined

by CV (coefficient variation) value, and the corresponding formula is as follows:

$$\mathrm{CV} = \left(\sum_{i=1}^{n} \frac{(di - \overline{d})^2}{N} \right)^{\frac{1}{2}} \Big/ \overline{d} \times 100\%,$$

where di is the diameter of the ith diameter, $\overline{d}$ is the mean diameter, and N is the total number of microspheres.

2.3. Bioadhesion in gastrointestinal tract

A modified everted sac experiment was selected to study the intestinal bioadhesion. After dissecting the rats, intestinal tract tissues (duodenum, jejunum, ileum and colon) were removed. 3-cm segments of duodenum and 6-cm segments of jejunum, ileum and colon were everted with glass rod, respectively, and lightly washed with PBSG (phosphate buffered saline, pH 7.2, containing 2 g/L glucose) to remove the contents. Knots were placed at both ends of the segment. The sac was filled up with PBSG and introduced in a tube containing PBSG with the three different diameters of microspheres, the concentration of which was 2×10^6 equally. The tube was agitated end over end for 30 min at 37 °C, and then the sac was immersed twice in fresh PBSG for 5 min to remove the unbound microspheres. The sac was cut into small pieces and completely disrupted for 3 min using a S-450D digital sonifier (Branson, USA). KOH should be added into the disrupted sample suspensions at 1 wt.% to reduce the fluorescent background before analysis using a flow cytometer. The repetition number (n) was 6 in each group.

The method to study the bioadhesion of CG microspheres in the stomach is similar to that mentioned above. The stomach was excised from abdomen, and then opened to remove the contents. Both sides of the stomach were flushed with ice-old artificial gastric juice (AGJ, hydrochloric acid solution, pH 1.2), and the lumen was turned out. The stomach was introduced in a tube containing AGJ with the three different diameters of microspheres, the concentration of which was 2×10^6 equally. The tube was agitated end over end for 30 min at 37 °C, and then the stomach was immersed twice in fresh AGJ for 5 min to remove the unbound microspheres. The lumen layer was detached from the stomach, and cut into small pieces. The sample was completely disrupted and then analyzed by flow cytometer. A similar experiment was performed using PBS (phosphate buffered saline, pH 7.2) instead of AGJ. The following process is similar to that mentioned above. The repetition number (n) was 6 in each group.

2.4. Absorption in gastrointestinal tract

The absorption of CG microspheres in the gastrointestinal tract was observed by SEM. Rats were given a single oral gavage of 2 ml suspension containing chitosan microspheres of the three different diameters, the concentration of which was 2×10^6 equally. At 8 h after dosing, tissues from the gastrointestinal tract (stomach, intestine and colon) were collected and washed with PBS. The samples were fixed in 5% glutaraldehyde and then in 3% osmium tetroxide. After being immersed in glycerin, the samples were dehydrated by a freeze dry system (Labconco, USA). Finally, the samples were coated with gold by an ion coater and examined by SEM.

2.5. Distribution of CG microspheres in systemic tissue

The distribution of CG microspheres in systemic tissue was also observed by LSCM. Single oral gavage was performed on rats similarly. On day 3 after dosing, rats were sacrificed and dissected. Systemic tissues (stomach, duodenum, jejunum, ileum, colon, mesenteric vessel, mesenteric lymph node, liver, spleen, heart, kidney, muscle and brain) were removed and washed with PBS. Tissue samples were fixed in 4% formaldehyde and then sliced into 4-mm-sections. After being dehydrated in ethanol gradient solution, the samples were embedded in paraffin, respectively. Thin (5 μm) paraffin sections of the aforementioned tissues were cut, deparaffinized and rehydrated in ethanol gradient solution. The slices were stained with Hochest33258, and then observed by LSCM. Three fluorescent images at different wavelengths (420–450, 520–540 and 580–600 nm) were taken with the excitation at 364 and 488 nm.

Microsphere counting was accomplished using flow cytometer. Liver and spleen were collected on different days after different doses. The method was similar to the process mentioned above. The repetition number (n) was 6 in each group.

2.6. Biodegradation in liver and spleen

Microspheres were collected from liver and spleen at a specific day after dosing. The decrease of fluorescence intensity was evaluated by flow cytometer, the fluorescence spectrum was accomplished by LSCM, and the surface feature of the microspheres was observed by SEM.

3. Results and discussions

3.1. Characterization of CG microspheres

By SEM examination, the CG microspheres prepared by SPG membrane emulsification technique were spherical and smooth, no pore was observed on their surface (Fig. 1). Uniform-sized CG microspheres with yellow fluorescence are displayed in overlay images (Fig. 2). The mean diameters of CG microspheres in three groups were 2.1, 7.2 and 12.5 μm, and the CV values were 16.34%, 12.32% and 11.09%, respectively. Obviously, the characterizations (such as good sphericity, compact structure autofluorescent property and uniform size) mentioned above made these CG microspheres an ideal model to track their behaviors in vivo.

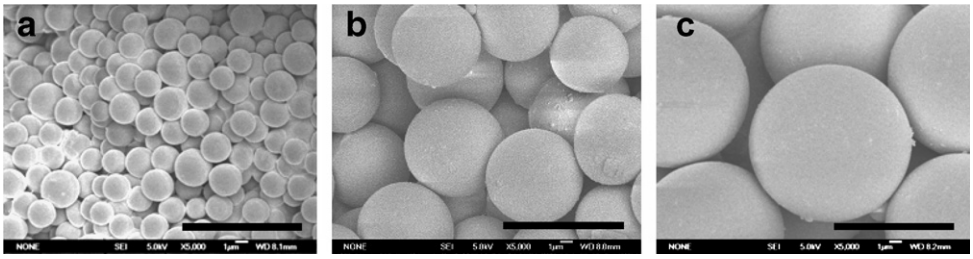

Fig. 1. SEM photographs of CG microspheres in the diameters of (a) 2.1, (b) 7.2 and (c) 12.5 μm. Black scale bars represent 10 μm in each case.

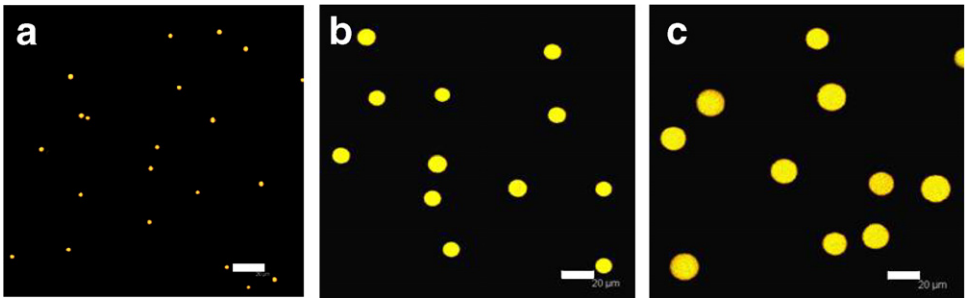

Fig. 2. LSCM images of CG microspheres in the diameters of (a) 2.1, (b) 7.2 and (c) 12.5 μm. Scale bars represent 20 μm in each case.

3.2. Bioadhesion in gastrointestinal tract

The bioadhesion of CG microspheres with three different diameters in the gastrointestinal tract was evaluated by modified everted sac experiments, combining with flow cytometer analysis. The uniformity in size of the CG microspheres resulted in narrow fluorescence signals both in FLH-1 and FLH-2 channels following flow cytometer analysis (only FLH-2 displayed in Fig. 3). In our previous study, we found the linear relationship between fluorescence intensity and volume of CG microspheres with the same crosslinking degree. Thus, the CG microspheres with different diameters showed different fluorescence intensities (Fig. 3a–c), and we could locate them at different position on the histogram when they were mixed (Fig. 3d). Analysis of tissues from untreated rats was used to set a background value. The efficiency of the tissue disruption and particle counting process was determined by adding known numbers of microspheres to tissue samples. The recovery was found to be 81 ± 8% for stomach; 87 ± 6% for duodenum; 89 ± 4% for jejunum; 87 ± 5% for ileum; and 82 ± 6% for colon. Compensatory adjustments were made to the particle counting results. Fig. 4b displays results of bioadhesion of CG microspheres in different segments of the intestinal tract. It could be seen that of the three different diameters of microspheres used in this study, 2.1 μm showed the most bioadhesion due to their small particle size. Furthermore, ileum was the most bioadhesive segment for these chitosan microspheres. In addition, 2.1 μm also showed much higher bioadhesion in colon, which might indicate a better way for oral colon drug delivery system (OCDDS). The results of CG microspheres adhering on the stomach in AGJ and PBS are shown in Fig. 4a, which indicates that acid environment could somewhat enhance the bioadhesion of CG microspheres, because the free amino group, which was not involving in the crosslining reaction, could become quaternary ammonium group at this low pH value to enhance the effect of hydrogen bond between the microspheres and the mucus.

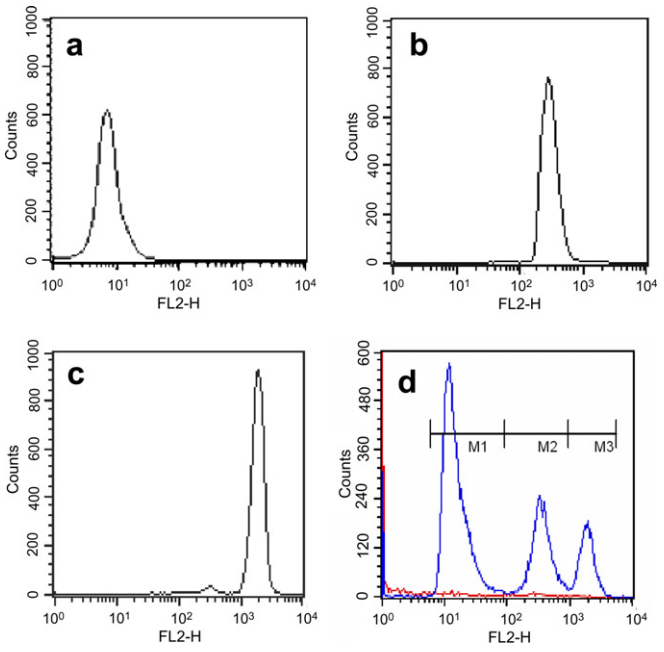

Fig. 3. Fluorescence intensity of CG microspheres with different diameters in FL2-H channel (a) 2.1, (b) 7.2 and (c) 12.5 μm. Histogram (d) was from the everted sac experiments measured on flow cytometer. The red line indicates the control group, while the blue line indicates the experimental group.

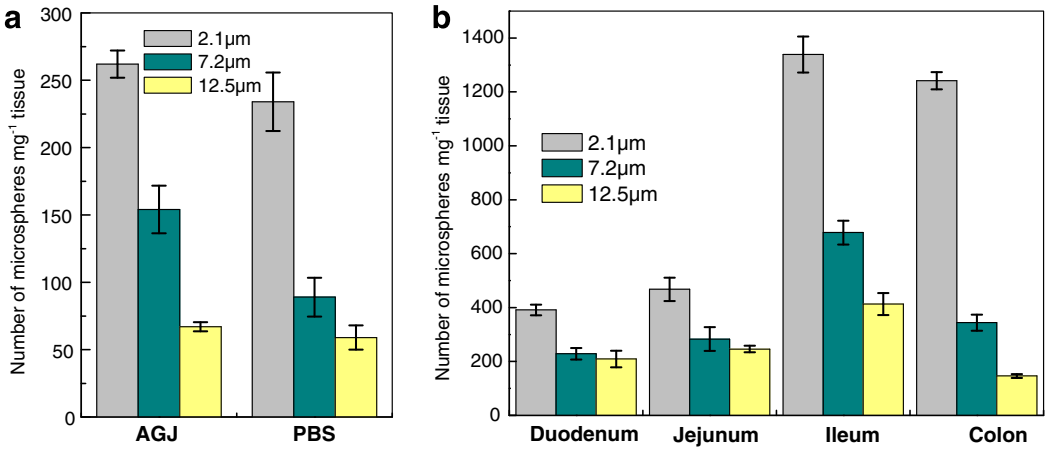

Fig. 4. Bioadhesion of CG microspheres with different diameters in the stomach (a) and the intestinal tract (b) (means ± SD, $n = 6$).

3.3. Absorption of CG microspheres in gastrointestinal tract

The absorption of CG microspheres in the gastrointestinal tract was observed by SEM (Fig. 5). Both 2.1 and 7.2 μm CG microspheres were found in the stomach and small intestine, but only 2.1 μm CG microspheres were found in the colon due to their higher bioadhesion at the segment mentioned above. CG microspheres with the diameters of 12.5 μm were not present, indicating that they were oversized for the absorption in the gastrointestinal tract. Taken together, to achieve better utilization, the particle size in the food or as a drug carrier should be controlled below 12.5 μm at least during practical application.

3.4. Distribution of CG microspheres in systemic tissues

Many factors are known to affect the uptake of microspheres, such as particle size and characteristics, animal species and age, and administered dose. With the advantage of autofluorescence, the distribution of CG microspheres was firstly observed by LSCM through three color channels in systemic tissues (Fig. 6). The CG microspheres could be obviously differentiated by their strong yellow fluorescent events even though at a high background induced by the tissues. In the gastrointestinal tract, the same results were obtained with the SEM observation aforementioned. After being absorbed, the microspheres in the small intestine were delivered in the vessel or located in the lymph node, while others still stayed in mucosa layer of the gastrointestinal tract. Several reports have focused on the size dependence of particles uptake by using labeled particles with broad size distribution. According to Eldridge et al. [19], the microspheres in a size range of 5–10 μm remained fixed in the Peyer's patches, while the smaller (1–5 μm) particles were transported through the efferent lymphatics with the macrophages and by targeting

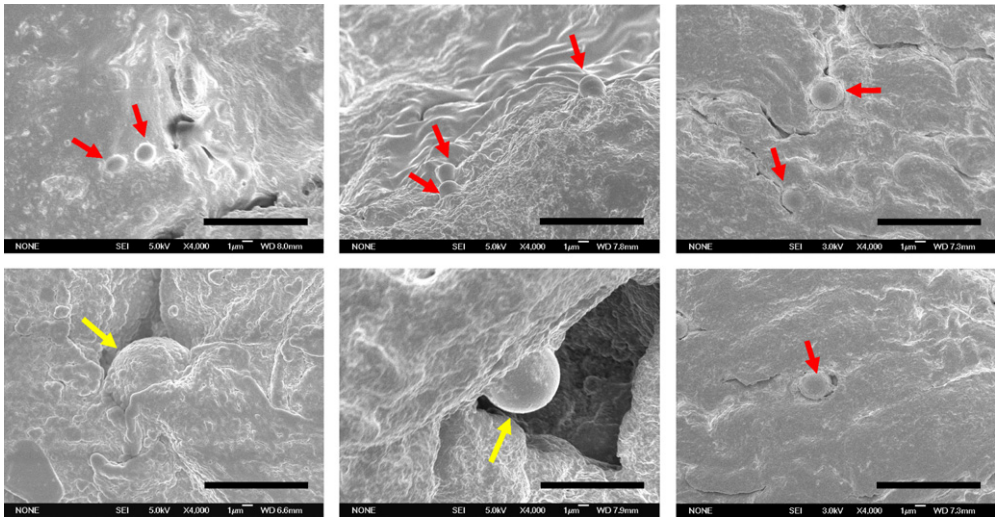

Fig. 5. SEM photographs of CG microspheres with different diameters in the gastrointestinal tract: stomach (left), intestine (middle) and colon (right). Black scale bars represent 10 μm in each case. Microspheres with a diameter of 2.1 μm are indicated by red arrows, while those with a diameter of 7.2 μm are indicated by yellow arrows.

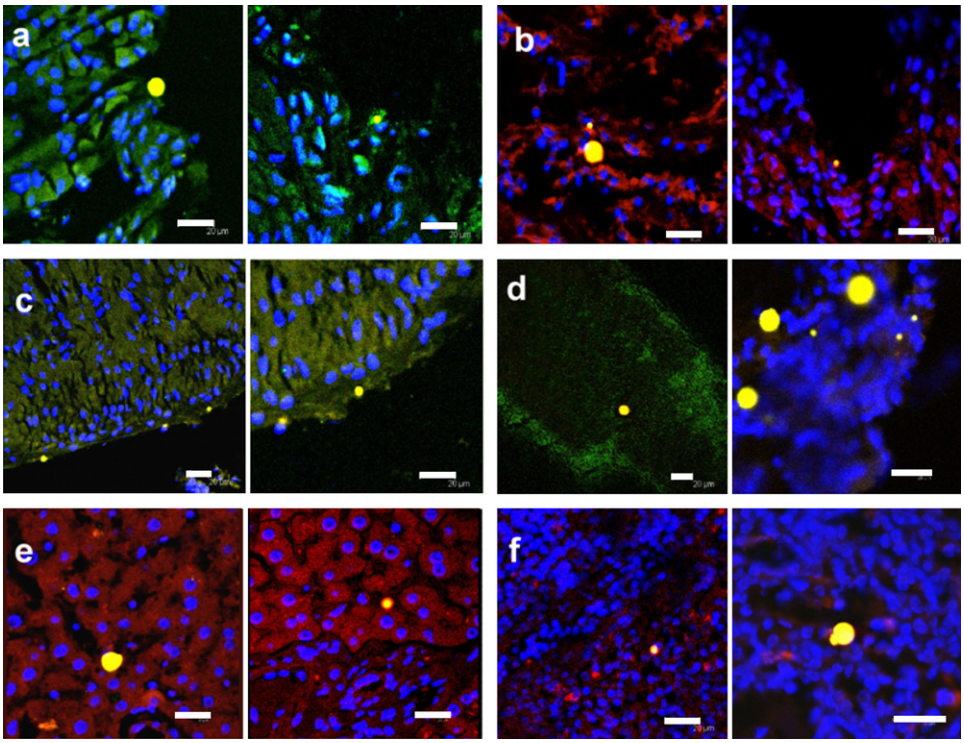

Fig. 6. Distribution of CG microspheres with different diameters in systemic tissues: (a) stomach, (b) small intestine, (c) colon, (d) mesenteric vessel and lymph node, (e) liver and (f) spleen. Scale bars represent 20 μm in each case.

to the liver and spleen passively. Several observations in rodents showed that this transcytosis occurred for PLGA and polystyrene particles in the same range. Obviously, the uniformity of these CG microspheres made it more satisfying for this evaluation. Therefore, we carried out the experiment with three particle sizes, one above 10 μm, one below 5 μm and one between 5 and 10 μm with a narrow size distribution. To our great surprise, the 7.2 μm (>5 μm) in diameter was firstly found in the liver and spleen as well as 2.1 μm, which disagreed with the previous findings on the size dependence of distribution in vivo. Other tissues, such as heart, kidney and lung, showed no uptake (images not displayed). These interesting results may be due to many factors. The polysaccharide structure of chitosan may play an important role during the location in vivo. Different techniques, such as fluorescent labeling, radio labeling and tissue dissociation, would also be a good explanation for these contradictory results.

It has been proved that most of the microspheres in the systemic circulation would lodge in the RES liver and spleen. Based on the uniform size and autofluorescent characterizations of CG microspheres, we were able to measure small numbers of these particles with three different diameters synchronously in liver and spleen following an oral gavage. Size, dose and time dependences of the number of microspheres found in these two organs of the reticuloendothelial system were analyzed, respectively. Compensatory adjustments were made as mentioned above. The CG microspheres in the sizes of 2.1 and 7.2 μm were discussed in the following study, which could transfer from the gastrointestinal tract to the systemic circulation.

The dose responses of absorption into the liver and spleen given as 10^6, 10^7 and 10^8 of mixed microspheres are shown in Figs. 7 and 8. The absorption appeared to be unsaturated over the 10^8 dose. Greater numbers of smaller microspheres were found than larger microspheres. While the maximum number of microspheres found increased with increasing dose, the absorption percent

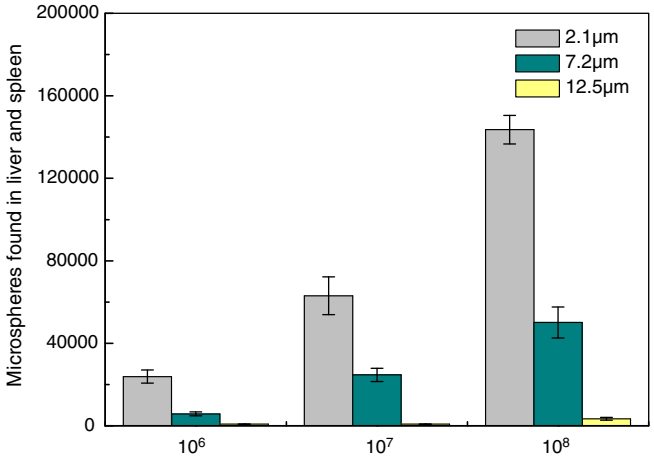

Fig. 7. Dose dependence of the number found in liver and spleen at 3 days after a single gavage. Rats were administered 1 ml suspension containing CG microspheres with the three diameters at a concentration of 10^6, 10^7, and 10^8 equally (means ± SD, $n = 6$).

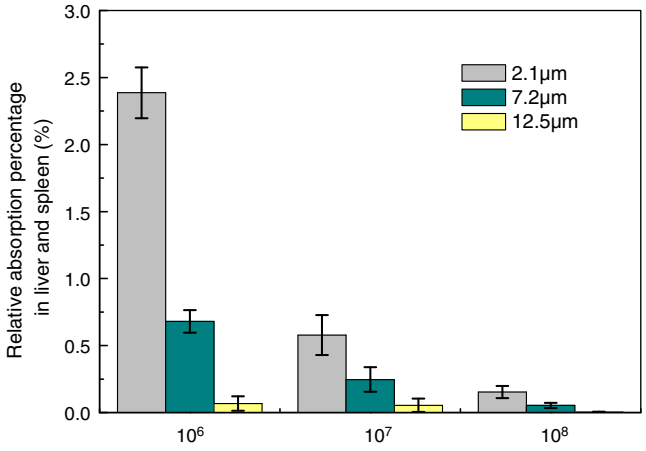

Fig. 8. Dose dependence of the relative absorption rate in liver and spleen at 3 days after a single gavage. Rats were administered 1 ml suspension containing CG microspheres with the three diameters at a concentration of 10^6, 10^7, and 10^8 equally (means ± SD, $n = 6$).

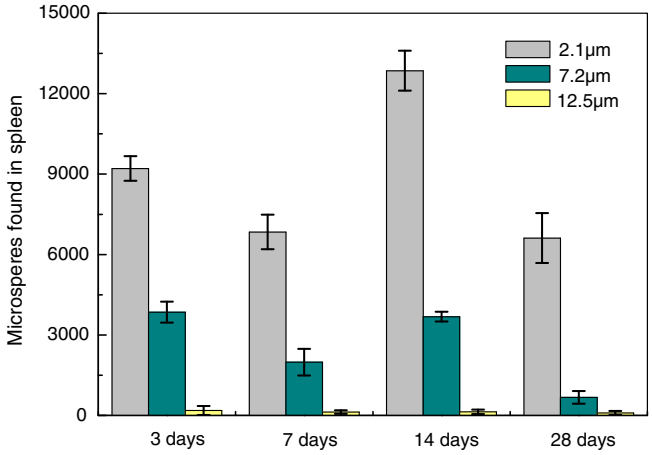

Fig. 10. Time dependence of the number of CG microspheres found in the spleen. The single oral administration of 1 ml suspension containing CG microspheres with the three diameters at a concentration of 10^8 equally. The time points are 3, 7, 14 and 28 days after dosing (means ± SD, $n = 6$).

decreased. Although the total counts of particles in the body were not conducted, the results indicate that more than 2.0% CG microspheres in the size of 2.1 μm were absorbed in the dose of 10^6. The absorption percentage of 7.2 μm-diameter microspheres was about half due to their larger size. Obviously, the numbers would increase if the microspheres were in a single size range. An effective and economical dose should be considered in the clinical applications.

The time dependences of translocation in liver and spleen during 28 days given as 10^8 of mixed microspheres are shown in Figs. 9 and 10, respectively. The number of microspheres in the diameter of 7.2 μm decreased gradually in the liver, but the maximum number of 2.1 μm microspheres found in the liver occurred on day 7. Both the microspheres in the sizes of 2.1 and 7.2 μm have the similar fate in the spleen. The number was lower in the spleen than in the liver and this fluctuated with time. Given the general observation mentioned above about the time course, the elimination of the CG microspheres varied with the particle size and RES organs, which indicated that more than one mechanism of particle transported to, lodged in, or cleared from the liver and spleen.

3.5. Biodegradation in liver and spleen

A study was conducted in the following part to investigate the degradation of CG microspheres. Our previous study found that CG microspheres showed great fluorescence around 520 (band II) and 580 nm (band III) with the excitation at 488 nm due to the presence of C=N bonds from schiff base. Quite a little fluorescence between 420 and 480 nm (band I) could be detected from C=C bonds with the excitation at 364 nm, because C=N bond may function as a repressor for suppression of fluorescence from C=C bond when both are present in the CG microspheres [17].

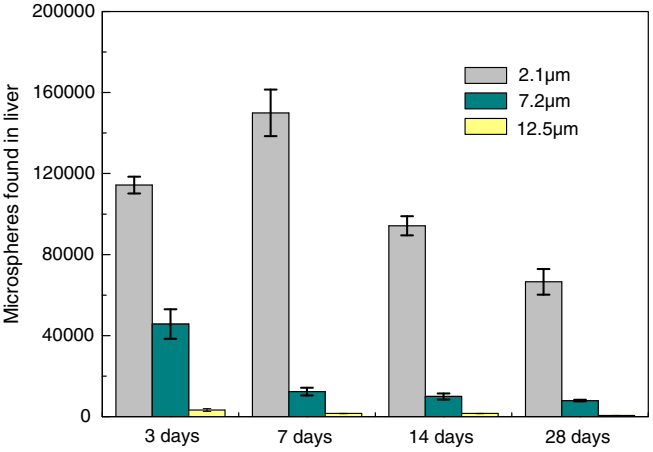

Fig. 9. Time dependence of the number of CG microspheres found in the liver. The single oral administration of 1 ml suspension containing CG microspheres with the three diameters at a concentration of 10^8 equally. The time points are 3, 7, 14 and 28 days after dosing (means ± SD, $n = 6$).

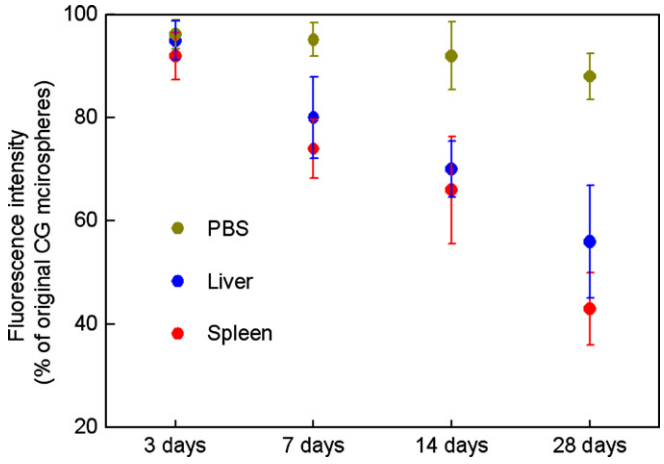

Fig. 11. Decrease of fluorescence intensity of CG microspheres on band III in PBS, liver and spleen. The samples were excited at 488 nm, and the time points are 3, 7, 14 and 28 days after dosing (means ± SD, $n = 6$).

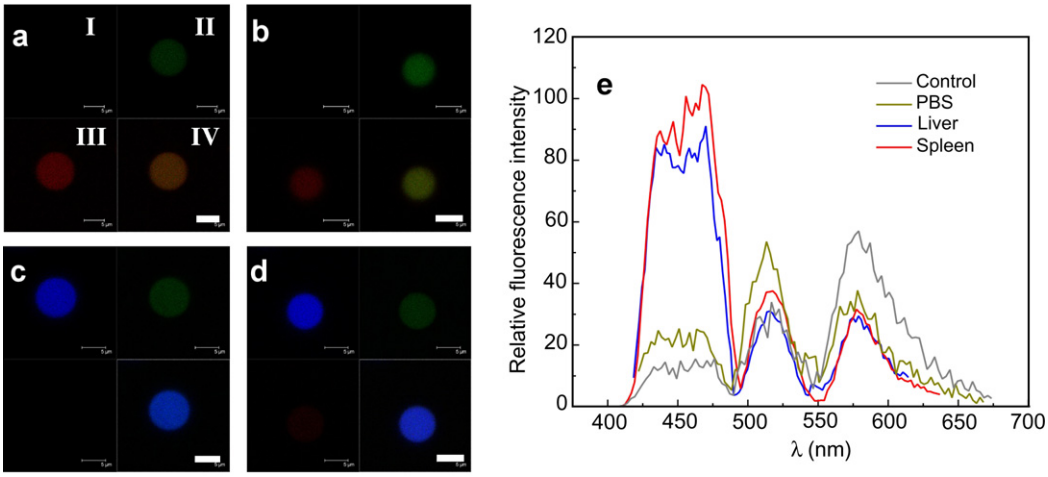

Fig. 12. LSCM images of CG microspheres (a) after preparation (b) incubation in PBS after 28 days, (c) in liver and (d) spleen at 28 days after oral administration. Each image consists of four parts: band I, band II, band III and IV (overlay). Scale bars represent 5 μm in each case. Fluorescence spectrum of CG microspheres is shown in (e) excited at 364 nm, at 28 days in vivo and vitro.

To our surprise, the fluorescence intensity at band II and III of the CG microspheres depicted in Figs. 9 and 10 decreased during 28 days (Fig. 11), while it increased obviously at band I (Fig. 12). Based on the previous research mentioned above, we could conclude that more C═N groups were reduced in the liver and spleen than in the PBS. One possible reason was that Cytochrome P450 (CYP450), a family of isoenzymes, may play a central role in the biodegradation of C═N groups.

To date, although the native glutaraldehyde is somewhat toxic, several blood substitutes crosslinked with glutaraldehyde are available in the market or in the clinical phase III evaluation (Hemopure, Hemolink and PolyHeme), and no obvious toxicity has been found.[20,21] Our results obtained with the fluorescence analysis showed that the Schiff base C═N bond involved with glutaraldehyde during the crosslinking reaction could turn into a stable C—N form in vivo, which is difficult to transform back into the aldehyde group. Such biodegradation would reduce the side effects and confirm the potential use of glutaraldehyde as a crosslinking reagent in the clinical application.

Fig. 13 shows the SEM photograph of CG microspheres collected after 28 days in vivo and in vitro. The original CG microspheres were smooth and spherical. After being incubated in PBS after 28 days, on the contrary, many protuberances were found on the surface due to the effect of swelling. The microspheres in the liver were degraded into those with pores on the surface, but slits in the spleen. Several studies have shown that one of the features of chitosan is its susceptibility to specific hydrolytic enzymes, such as lysozyme, which can be found in body fluid, intestinal tract, liver, spleen, etc. [22]. The oligosaccharides are being released during the enzymatic degradation of chitosan, which seem to play an important role in the biodegradation of CG microspheres. Further study should focus on different forms of biodegradation in the liver and spleen. The presence of different enzyme system in vivo may have made the difference.

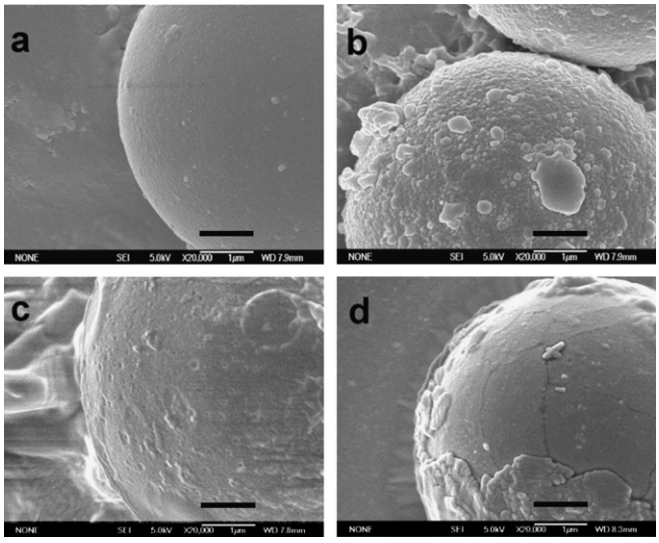

Fig. 13. SEM photographs of CG microspheres (a) after preparation (b) incubation in PBS after 28 days, (c) in liver and (d) spleen at 28 days after oral administration. Black scale bars represent 1 μm in each case.

Given the general observation cited above, it could be indicated that (1) the two main biodegradations were focused on the C═N bonds from the Schiff base and the β-(1 → 4) bonds from chitosan; (2) the biodegradation of CG microspheres was different due to the location in RES; (3) the CG microspheres hardly degraded during 28 days, which will be favorable for the long-term oral immunization.

4. Conclusion

In this paper, a new detecting system has been developed to track the CG microspheres following oral administration. Based on the characterizations of uniform size and autofluorescent property, the CG microspheres with three different

diameters were firstly evaluated in systemic tissues synchronously. Results showed that the bioprocess of these CG microspheres highly depended on the particle size, dose, time and tissues. These original works may give us a better understanding of the behaviors of CG microspheres as a drug carrier in further studies and applications.

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