Carbohydrate Polymers 113 (2014) 304–313

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

In vitro and in vivo evaluation of chitosan microspheres with different deacetylation degree as potential embolic agent

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ARTICLE INFO

Article history:
Received 15 May 2014
Received in revised form 15 June 2014
Accepted 20 June 2014
Available online 11 July 2014

Keywords:
Chitosan
Microspheres
Transcatheter arterial embolization
Embolic agent
Chemoembilization

ABSTRACT

To evaluate the potential of N-acetylated chitosan microspheres used as a chemoembolic agent in vivo and in vitro. Calibrated spherical chitosan microspheres (CMs) were prepared via Water-in-Oil emulsification method and CMs were acetylated (ACMs). The swelling rate of CMs was greatly affected by pH than that of ACMs and both of them affected by temperature. Microspheres with excellent thermal stability demonstrated controllable degradation in lysozyme solution. Doxorubicin was released from microspheres in vitro and exhibited excellent control release profile. ACMs caused hemolysis less than CMs (<5% of the time). Co-culture with mouse embryo fibroblasts revealed that microspheres have non-cytotoxic nature. Microspheres planted in a rat gluteal muscle demonstrated that it were biodegradable and biocompatible. ACMs were performed in rabbit ear embolization model and ischemic necrosis on ear was visible due to the vascular occlusion after 15 days. Acetylated chitosan microspheres could be used as potential biocompatible and biodegradable embolic agents.

1. Introduction

Transcatheter arterial embolization (TAE) is widely employed for clinical interventional therapy, which embolic agents are introduced into the blood vessel through a catheter with a minimally invasive procedure (Oerlemans et al., 2013; Sharafuddin, Sun, & Golzarian, 2006; Weng, Rostamzadeh, Nooryshokry, Le, & Golzarian, 2013). The embolic agents cut off the nutrition and oxygen supply necessary of organ, tissue or tumor by blocking the blood flow, and it can be used in the treatment of many diseases, including hemorrhagic syndrome, vascular lesions and tumors (Ginat, Saad, & Turba, 2009). These embolic agents such as coils, liquid, gelfoam and particles have been researched and most of them are permanent or nonocclusive (Schwarz, Zhang, Metcalfe, Salazkin, & Raymond, 2004). In the early years, Gelfoam, a commercially available biodegradable embolic agent, has been already applied in clinic. It has non-spherical surface and it is difficult to precisely calibrate for embolization because of fast degradation. In addition, polyvinyl alcohol (PVA) microspheres used as non-biodegradable materials are regarded as permanent embolic agents, but PVA particles with irregular shapes cannot be calibrated and tend to aggregate, which causes catheter obstruction and large vessel occlusion (Derdeyn, Moran, Cross, Dietrich, & Dacey, 1995). In present years, only a few types of microspheres are commercially available, i.e., tris-acryl microspheres (Embosphere; Biosphere Medical, Roissy, France), Contour SE (CSE; Target Therapeutics, Boston Scientific Corp., Fremont CA, USA) and Bead Block (DC-bead, Biocompatible Ltd., UK) (Kwak, Shim, Han, & Park, 2005). It seems that they could be used selectively for embolization although each of them has advantages and disadvantages. Therefore, it is crucial to explore a new type of microspheres, including calibrated particle size, non-aggregate, degradation and biocompatibility, which are ideal material for embolization (Jayakrishnan et al., 1994).

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http://dx.doi.org/10.1016/j.carbpol.2014.06.080
0144-8617/

Chitosan, the cationic (1-4)-2-amino-2-deoxy-β-D-glucan partly acetylated to the typical extent close to 0.25, is industrially produced from marine chitin (Muzzarelli, 2011, 2012; Muzzarelli et al., 2012). It has attracted attention owing to its superior features such as biocompatibility, toxicity and biodegradability (Aranaz et al., 2009; Busilacchi, Gigante, Mattioli-Belmonte, Manzotti, & Muzzarelli, 2013; Muzzarelli, 2009). Microspheres made of chitosan are used as drug carriers and anti-hemorrhagic agents (Gavini, Rassu, Muzzarelli, Cossu, & Giunchedi, 2008; Muzzarelli, Stanic, Gobbi, Tosi, & Muzzarelli, 2004; Sinha et al., 2004). Theoretically, the drug-loaded microspheres have the merit of the embolization effect via cutting off vessels supply as well as the treatment effect by prolonging delivery of drug. Thus, chitosan microspheres, a matrix loaded with drug, could be used for embolic materials.

Chitosan microspheres with different degree of deacetylation offer different physicochemical characteristics due to various amounts of amino groups, and they could also be modified the amounts of amino groups according to experimental requirement. In this study, chitosan microspheres (CMs) are prepared by W/O emulsification cross-linking method, and acetylated chitosan microspheres (ACMs) are prepared by acetylating CMs with acetic anhydride. The characteristics, including morphology, welling rate, degradation, drug release, haemocompatibility, cytotoxicityand in vivo feasibility of embolization, have been investigated. The purpose of this paper is to investigate the physicochemical properties of chitosan microspheres with different degree of acetylation and evaluate their potential to be used as embolic agents for vascular embolization.

2. Materials and methods

2.1. Materials

Chitosan (MW 136 kDa and deacetylation degree 90.6%) was obtained from Biochemical Medicine Plant of Qingdao (Qingdao, China). Lysozyme and doxorubicin hydrochloride were purchased from Sigma–Aldrich. DMEM and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were supplied by Amresco (Solon, OH). All the other chemical agents were of analytical grade.

All the experimental animals in this study were supplied by SFDA of Qingdao, consisting of healthy male Wistar rat (200 ± 20 g) and New Zealand white male rabbits (2.0 ± 0.2 kg). The animal protocol was approved by Shandong Medical Laboratorial Animal Administration Committee. All animal studies were performed in compliance with the National institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) revised in 1996.

2.2. Preparation of CMs and ACMs

Chitosan microspheres (CMs) were prepared using Water-in-Oil (W/O) emulsion technique (Kim et al., 2007). In briefly, chitosan–acetic acid solution (2%, w/v) was added drop-wise into liquid paraffin (1:5, V/V) including span-80 and tween-80. The mixtures were stirred at 1100 rpm for 60 min (RW-20 stirrer, IKA Co., Ltd., Germany). Next, formaldehyde solution was added drop-wise for another 60 min. The suspension was then filtered through a Sub-sieve (120 mesh, U.S.P Standard Sieves) and fully rinsed by isopropyl alcohol. After that, the CMs were suspended in NaH_4B solution for 12 h and then successively dehydrated in ethanol, and dried.

Acetylated chitosan microspheres (ACMs) were prepared by acetylating CMs with acetic anhydride. In briefly, CMs were dispersed in methanol, and then excessive acetic anhydride was added into the mixture and stirred for 16 h. Microspheres were immersed in KOH–alcohol (0.5 mol/L) for 1 h, washed and dried to acquire ACMs.

2.3. Characteristics analysis

Surface features of dried microspheres (CMs/ACMs) were observed by optical microscopy (OM) (CKX-31, Olympus Co., Ltd.) and scanning electron microscopy (SEM) (KYKY-2800B, Scientific Instrument Co., Ltd.). Size distribution was measured using Mastersizer 2000 (Malvern Instruments Ltd., UK). The infrared spectra of samples (chitosan, CMs and ACMs) were recorded by a Nicolet FTIR 5700 spectrophotometer (Madison, USA) at room temperature (25 °C). The degree of deacetylation (DD) of microspheres (chitosan, CMs and ACMs) was determined by potentiometric titration according to the method of Wang et al. (Kang et al., 2010). The deacetylation degree (DD) of chitosan, CMs and ACMs were 90.6%, 84.8% and 20.9%, respectively.

2.4. Measurement of the swelling ratio

The swelling ratio (SR) of microspheres (CMs/ACMs) was measured by quantifying the average particle size changes before and after the experiment. (1) Samples were immersed in buffer solution (pH 4.0, pH 7.2 and pH 10.0) at 37 °C with gentle shaking, respectively. (2) Samples were immersed in pH 7.2 buffer solutions at different temperatures (4 °C, 37 °C, 60 °C) with gentle shaking, respectively. Average CMs/ACMs particle sizes were measured at different predetermined time intervals (0, 15, 30, 60, and 90 min), and the SR was calculated using the following Eq. (1):

$$SR\% = \frac{D_t - D_0}{D_0} \times 100\% \quad (1)$$

where D_0 is the initial average particle size of microspheres and D_t is the average particle size of microspheres after swelling.

2.5. Thermal stability and degradation in vitro

Sample (CMs/ACMs) was sterilized in PBS under 121 °C for 1 h and then placed in room temperature (25 °C) for 3 months. The morphology and thermal stability of sample was observed by microscope.

The degradations of sample (CMs/ACMs) in vitro were measured by following method: the sample (100 mg) was soaked in HAc-NaAc buffering solution (pH 6.0) containing 0.5 mg/mL lysozyme at 45 °C with gentle shaking. Fifty percent lysozyme solution was refreshed daily in order to ensure optimal enzyme activity. Sample was collected, rinsed, dried and weighed after 1, 2, 4 and 8 weeks. The degradation rate of sample in vitro was calculated as the percentage of mass loss of the dried sample after lysozyme treatment. The sample treated in HAc-NaAc buffering solution (pH 6.0) without lysozyme serves was used as the control.

2.6. In vitro drug release studies

Doxorubicin-loading chitosan (DOX-CMs) and reacetylated chitosan microspheres (DOX-ACMs) were prepared by following method. In briefly, a certain amount of doxorubicin hydrochloride (DOX) was mixed into chitosan–acetic acid solution and the mixture was used as water phase. The subsequent process was basically the same as above-mentioned preparation of blank microspheres. In order to improve the encapsulation efficiency, the drug-loading microspheres (DOX-CMs and DOX-ACMs) were immersed in a certain concentration DOX solution for 24 h at 4 °C and excess DOX solution was removed before experiment. The encapsulation efficiency (EE %) and drug-loading capacity (LC %) for microspheres

were performed according to the procedure reported earlier (Choi, Kim, Pak, Yoo, & Chung, 2007; Rinaudo, 2006). The concentration of the DOX solution before and after loading was measured by spectrophotometer (Shimadzu, Japan) at 481 nm.

In vitro release of DOX from microspheres was determined as follows. The loaded microspheres (20 mg) were immersed in 1 mL PBS (pH 7.2) and then placed in a dialysis bag (MW cutoff 8000–12,000), after that, the dialysis bag was placed in a brown bottle with 50 mL PBS (pH 7.2) and wrapped by tin foil. The bottle was put in shaking water bath at 37 °C; 1 mL release medium was collected for test and replaced with the same quantity of PBS at predetermined time intervals. The DOX released from microspheres was determined by the assay described above. All experiments were carried out in triplicate. In order to evaluate the release behavior of DOX in different pH value, sodium citrate buffer (pH 4.0) was used for testing and the procedure was the same as described above.

2.7. Hemolysis test

The hemolytic activity of the samples (CMs/ACMs) was evaluated by Singhal's method (Zhou et al., 2013). Prior to testing, the samples (CMs/ACMs) (10 mg, 50 mg) were soaked into equilibrium in normal saline at 37 °C for 24 h. The blood drawn from a healthy rabbit was anticoagulated by adding acid citrate dextrose (ACD) solution as anticoagulant blood (1:9, V/V). 1 mL of diluted ACD blood was added to each sample and incubated in shaking water bath at 37 °C for 0.5 and 1 h. After that, samples were centrifuged. Supernatant absorbance was measured at 545 nm and the hemolysis rate (HR %) was calculated. Normal saline treatment was used as a negative control (0% lysis) and distilled water was positive control (100% lysis).

$$HR\% = \frac{A_{\text{sample}} - A_{\text{nc}}}{A_{\text{pc}} - A_{\text{nc}}} \times 100\% \quad (2)$$

where HR% is the hemolysis rate of samples, A_{sample}, A_{nc} and A_{pc} is sample absorbance, negative control and positive control, respectively.

2.8. Cytotoxicity tests

Mouse embryo fibroblasts (MEFs) were used for general cytotoxicity test and the specific process of MEFs culture could refer to J. Zhang method (Wang et al., 2006). The cell viability was determined using the MTT method (Wang et al., 2009). The samples (CMs/ACMs) were soaked in DMEM medium to get equilibrium swelling and were steam-sterilized (121 °C, 150 kPa, 20 min). Then these spheres were planted into culture plates. Only cells of 4–7 generations were used in this experiment in order to ensure the optimal cell activity. MEFs were seeded into 96 well culture plates (3×10^3 cell/well) and incubated overnight at 37 °C, in a 5% CO_2 and 95% relative humidity environment. And then, the DMEM medium was replaced with preprocessed sample medium (200 μL). Subsequently, the plates were incubated for 24, 48 and 72 h. At predetermined time, MTT solution (20 μL) was added and incubated for another 4 h. Supernatant was discarded and DMSO (150 μL) was mixed. Plates were placed on shaking water bath at 37 °C for 10 min and then absorbance was measured at 490 nm. Cells cultured without microspheres serves were control and six replicates per sample were tested. The relative growth rate (RGR %) was expressed as the percentages of viable cells compared to the control group survival.

2.9. Muscle implantation in vivo

Wistar rats were employed for the evaluation. The rats were divided into four groups with randomized block design. The first group was control (5 rats). The second group was sham-operated group (5 rats). The third group was implanted CMs (17 rats). The last group was implanted ACMs (17 rats). The rats were anesthetized by injection of pentobarbital sodium (30 mg/kg) and then were fixed on the animal operating table in prone position. The fur in the gluteal region was cleared and then sterilized with 1% iodine and 75% alcohol. The gluteal muscle was incised about 1 cm on either side of spine. After that, the swelled CMs/ACMs (20 mg) were implanted into the skeletal muscle and the wound was stitched. Blood was drawn from rats in time interval (0, 3, 7 and 14 d) for

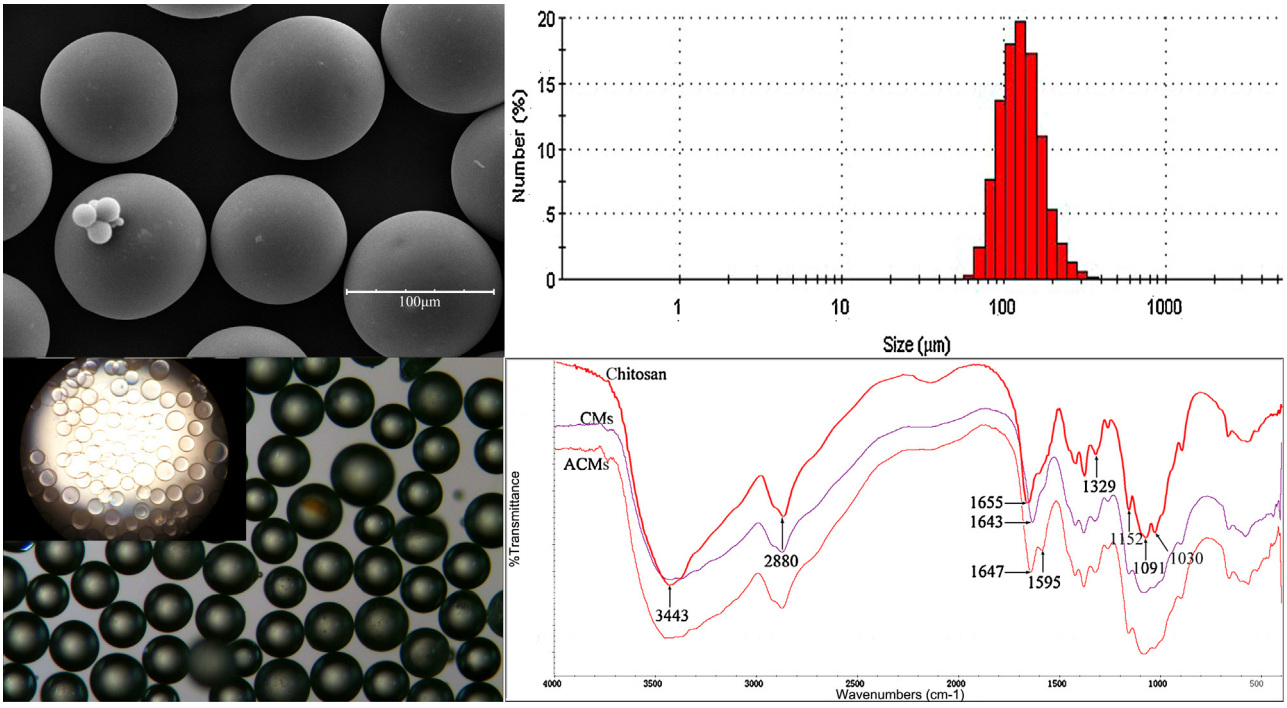

Fig. 1. Morphology, particle diameter distribution and FTIR spectra of the chitosan microspheres.

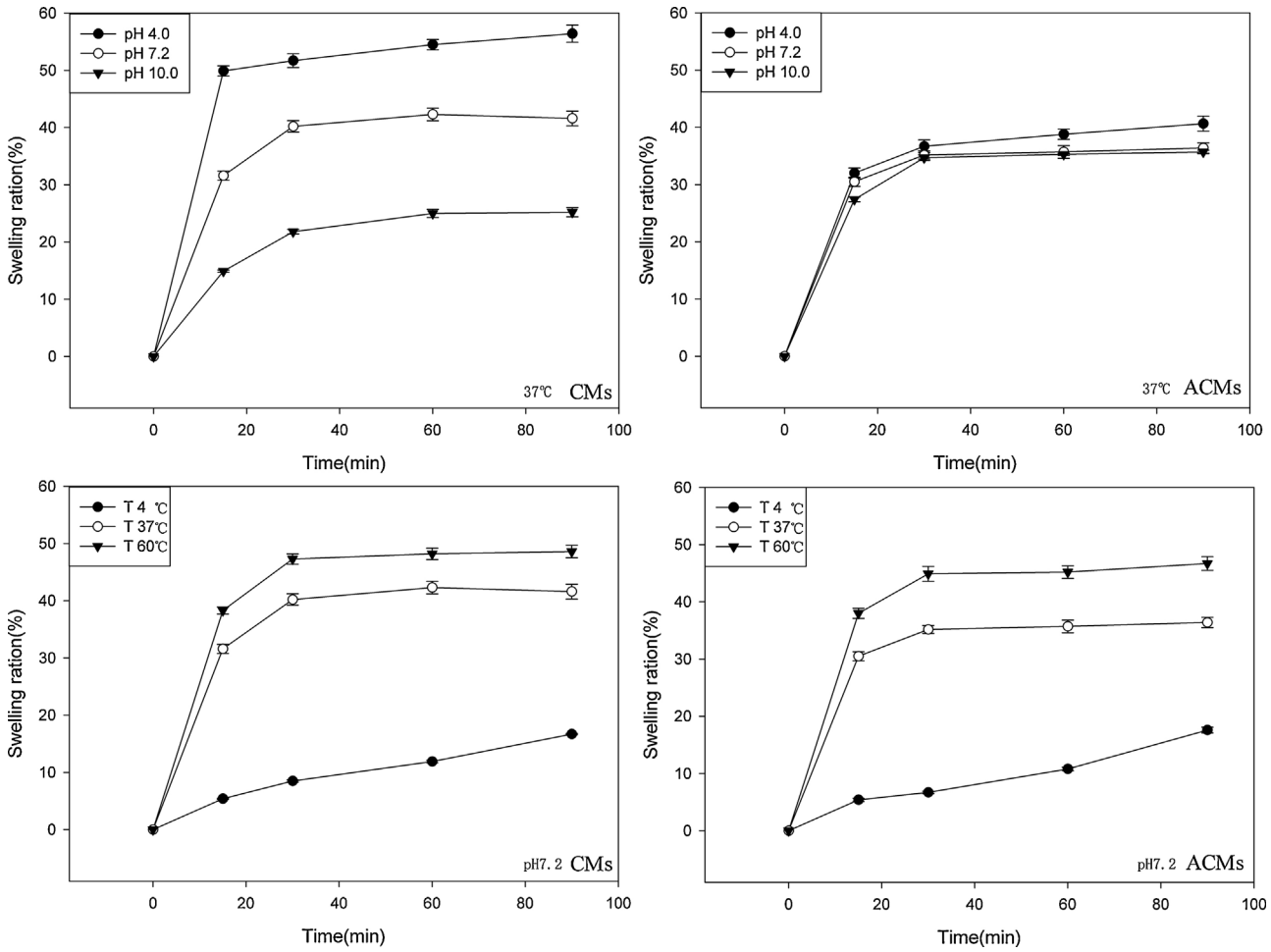

Fig. 2. Swelling ratio (SR) of CMs/ACMs at different pH (4.0, 7.2 and 10.0; 37 °C fixed) and at different temperature (4, 37 and 60 °C; pH 7.2 fixed). Data were represented as mean ± SD (n = 5).

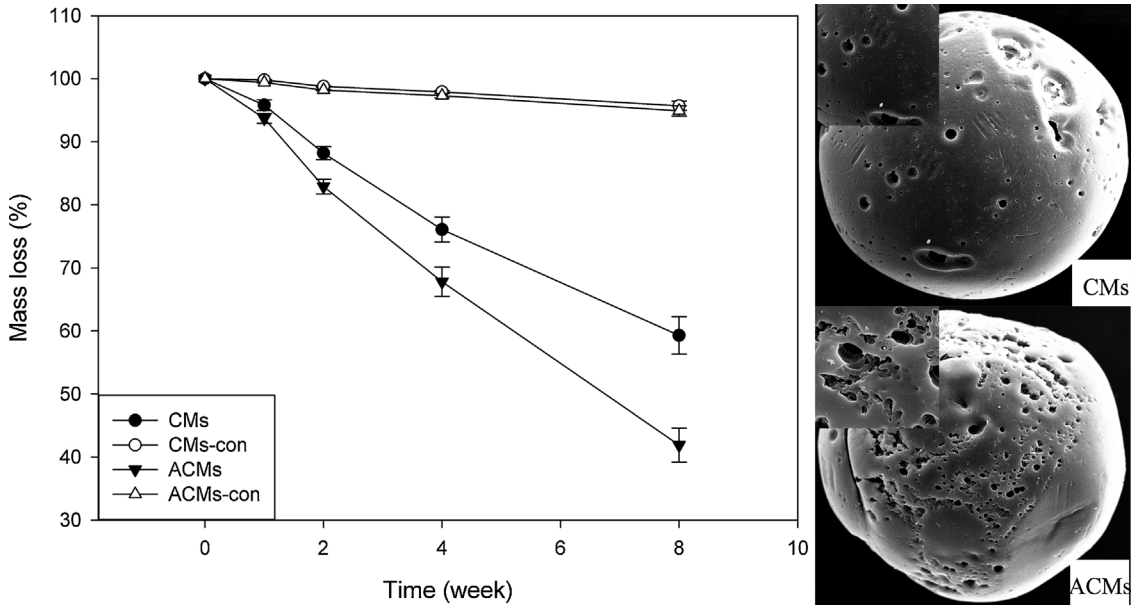

Fig. 3. Degradation curve of CMs/ACMs in vitro and SEM micrograph of CMs/ACMs after degradation. Data were represented as mean ± SD (n = 3).

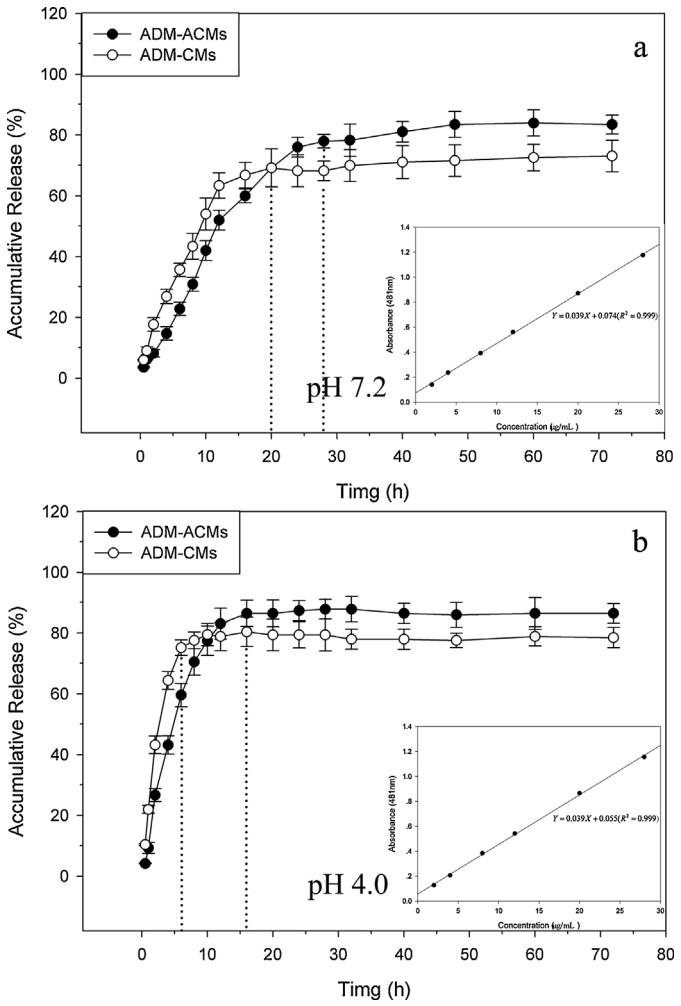

Fig. 4. Doxorubicin release profiles of the DOX-CMs and DOX-ACMs at different pH PBS at 37 °C. (a: pH 7.2; b: pH 4.0).

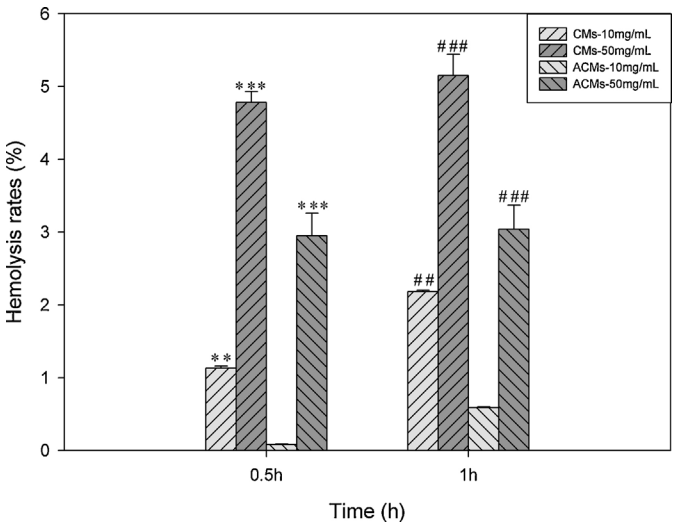

Fig. 5. Graph showing in vivo hemolysis rate of CMs/ACMs at different time points. Data were represented as mean ± SD ($n = 3$). *Over bars indicated degree of significance as compare to ACMs-10 mg/mL at 0.5 h (unless indicated), where $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns, not significant. #Over bars indicated degree of significance as compared to ACMs-10 mg/mL at 1 h (unless indicated), where $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; ns, not significant.

blood analysis (COULTER® LH 750 Hematology Analyzer, Beckman-coulter Co., Ltd.). Animals were killed at different time intervals (1, 4, 12 and 24 w) and the implanted tissue was removed, fixed in 10% buffered formalin and examined using paraffin section method. The biodegradation and biocompatibility of microsphere were evaluated.

2.10. Embolization performed in vivo

The rabbits were anesthetized by injection of pentobarbital sodium (40 mg/kg) and then were fixed supine on the animal operating table. The fur was removed from both ears with depilatory at least 8 h before the rabbits were trialed. The central auricular artery of rabbits was clamped temporarily for puncturing. ACMs suspended with glycerol (20 mg/mL) were slowly injected into branch vessel (0.3 mL/ear) from the proximal part to the distal part. After that, the clamp was released and the wound was handled well. The embolization effects of rabbit ear were observed at time intervals (0, 3, 7, 15 d).

2.11. Statistical analysis

All experiments and measurements were at least performed in triplicates. Data were mean ± standard deviation. Data were analyzed with software SigmaPlot 10.0.

3. Results and discussion

3.1. Characteristics of CMs/ACMs

Chitosan microspheres with uniform size and smooth surface were successfully prepared using W/O emulsification cross-linking technique. The microspheres (CMs/ACMs) had spherical shell with mean diameter of 132 μm and could maintain the shapes after drying (Fig. 1). FTIR spectra of chitosan, CMs and ACMs were showed in Fig. 1. In the FTIR spectra, The peak at 1595 cm^{-1} existed in ACMs spectra but not obviously in chitosan and CMs spectra, indicating that acetyl groups increased in ACMs (Singhal & Ray, 2002).

3.2. The swelling ratio (SR) of microspheres

Swelling ratio (SR) of CMs/ACMs at different pH (4.0, 7.2 and 10.0; 37 °C fixed) and different temperature (4 °C, 37 °C and 60 °C; pH 7.2 fixed) was shown in Fig. 2. The SR of CMs decreased significantly as pH increased (from 4.0 to 10.0), but no significant change in that of ACMs at different pH. The SR of CMs/ACMs increased significantly as temperature increased (from 4 °C to 60 °C). It could be concluded that the SR of CMs were more greatly affected by pH than that of ACMs. However, both SR of CMs/ACMs were affected by temperature and showed a similar profile. Acetyl group introduction decreased the dependency of pH on ACMs swelling property. Compared to CMs, it was assumed that ACMs had a stable swelling rate and were less affected by pH, which was related with the amount of amino groups on surface of microspheres (Zhang, Chen, Li, & Liu, 2007). It was implied that ACMs could maintain a certain swelling pressure under changing environmental condition in body. When temperature changes (4–37–60 °C), SR of CMs/ACMs increased significantly. The Brownian motion of water molecules was active as the temperature increased. The water molecules could easily enter the inner of microspheres when the hydrogen bond was broken in high temperature. Therefore, both of ACMs/CMs have similar swelling profile in different temperature.

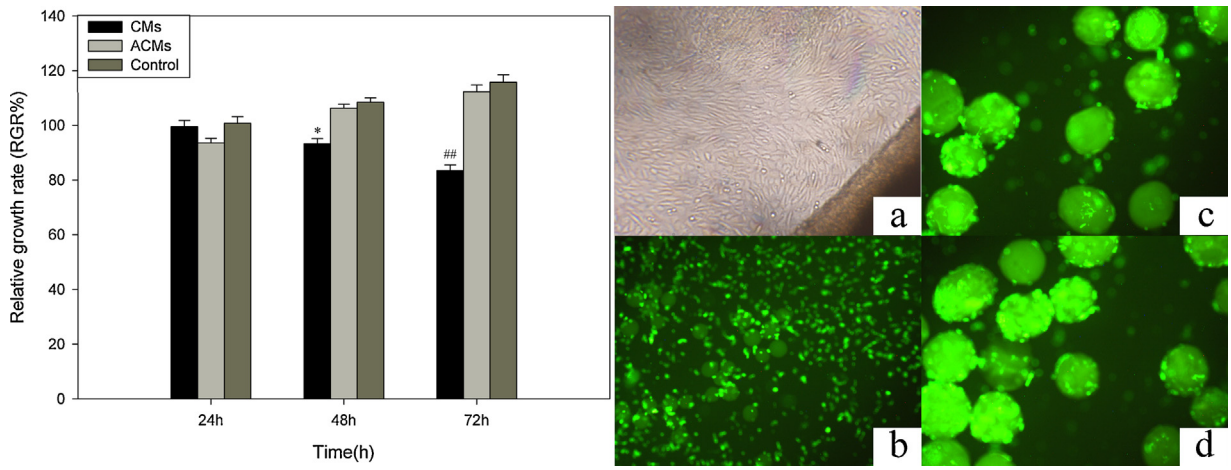

Fig. 6. In vitro cytotoxicity of CMs/ACMs on mouse embryo fibroblasts (MEFs). Data were represented as mean ± SD ($n = 3$). (a) Micrograph of MEFs; (b) Fluorescence micrograph of MEFs; (c): MEFs adhesion to CMs; (d): MEFs adhesion to ACMs. The green spots are MEFs. *Over bars indicated degree of significance as compared to Control at 48 h (unless indicated), where $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns, not significant. #Over bars indicated degree of significance as compared to Control at 72 h (unless indicated), where $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; ns, not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Thermal stability and degradation in vitro

The structures of microspheres remained stable in PBS after steam autoclaving at 121 °C, 150 kPa for 1 h (Fig. A.1). Microspheres were yellow in color and partly aggregation after autoclaving. However, these microspheres were quickly dispersed after shaking and kept a complete spherical shell; there was no rupture and fragment in system (Fig. A.1-a). The sterilized microspheres were pale yellow and had good dispersibility after being placed at room temperature for 3 months, which also showed smooth surface and perfect spherical under microscope (Fig. A.1-b). The results indicated that microspheres have a good thermal stability and still maintain the structural integrity under extreme conditions. It was also one of the items about evaluating the stability and safety testing of medical materials.

Degradation curve of CMs/ACMs in vitro and SEM micrographs of CMs/ACMs after degrading showed in Fig. 3. The mass loss of microsphere (CMs/ACMs) increased with incubation time. The mass loss rate has no obvious difference between two kinds of microspheres in the first week (CMs: 4.2%, ACMs: 6.3%). However, the difference gradually increased with time, and 40.7% of CMs, 58.1% of ACMs were degraded in the eighth week. Degradation curve of CMs/ACMs showed that the degradation velocity was slow in first two weeks but fast after that. The mass loss in the control group of CMs/ACMs was 4.3% and 5.1%, respectively. The result indicated that there were little relation between degradation of microsphere and HAc-NaAc buffering solution (pH 6.0). The SEM micrographs of CMs/ACMs after degrading were shown in Fig. 3 (right). There were rough surface filled with different sizes of holes on CMs/ACMs, and the holes will enlarge with time and finally complete collapse. The mass loss of ACMs was more than that of CMs, which implied that ACMs have greater degradation rate than that of CMs in lysozyme solutions in the same period. Chitosan could be degraded by many kinds of enzymes such as chitosanase, cellulose, lysozyme, papain, pectinase (Hansen, Nielsen, & Berg, 1989). Lysozyme plays a crucial role in the degradation of chitosan because our body lacks chitosanase or cellulose. Nordtveit (Wang et al., 2006) had reported that the degradation of chitosan could be influenced and regulated by the deacetylation degree of chitosan, which was confirmed by our experimental results. In the lysozyme solution, the degradation process of ACMs was faster than that of CMs due to the different amino group proportion between them.

3.4. In vitro drug release

The encapsulation efficiency (EE %) and drug-loading capacity (LC %) of microspheres were determined by the above method. The microsphere had a high EE % (CM: 55.68%, ACMs: 53.94%) and LC % (CM: 11.54%, ACMs: 10.68%), which was attributed to wrapping and adsorption by microspheres. The mean of EE % and LC % in CMs was higher than that of ACMs, but no statistics difference between them ($p > 0.05$, the detail data is not shown as Table). The cumulative release profile of DOX from microspheres in different pH (pH 4.0, pH 7.2) was recorded in Fig. 4. DOX release curves of two microspheres exhibited a similar biphasic pattern. On the pH 7.2 medium, most of DOX were released from CMs in 0–20 h and then steady released at low rate. However, DOX was released from ACMs in 0–28 h and subsequently steady. On the pH 4.0 medium, most of DOX were released from CMs with the burst release in 0–6 h and that of ACMs in 0–16 h. Compared to the release curve of CMs, it is seemed that ACMs had more advantages on drug control release. Apparently, DOX release behavior from microspheres was closely connected with the swelling of microspheres mentioned above. The swelling rate of CMs was more sensitively affected by pH value than that of ACMs; therefore, DOX was easier released from CMs than that of ACMs accompanied by swelling process. At predetermined time intervals, DOX release of the two microspheres started to burst release in initial period and then reached a plateau after that. These results indicated that the drug-loading chitosan microspheres had a good controlled-release effect and could be used for drug carrier delivery.

3.5. In vitro hemolysis assay

One of the important evaluations for the development of medical materials for body implantation was that the materials should not induce hemolysis between material and blood. Thus, hemolysis behavior of the ACMs/CMs was performed by incubating microspheres with blood. Hemolysis rates (HR %) of fresh rabbit blood with microspheres were shown in Fig. 5. HR % of ACMs (<5%) with 10 mg and 50 mg was lower than that of CMs in different time points (0.5 h and 1 h). According to the standard of ASTM-F/756-08 (2000) (Gupta & Jabrail, 2006): HR % in the range of 0–2% was considered non-hemolytic and the range of 2–5% was slightly hemolytic; rate exceeding 5% were considered hemolytic. In 0.5 h time point, HR % of ACMs/CMs with 10 mg were non-hemolytic (<2%) but slightly hemolytic in 50 mg dosage (2.95%/4.78%), and HR % of CMs-50 mg

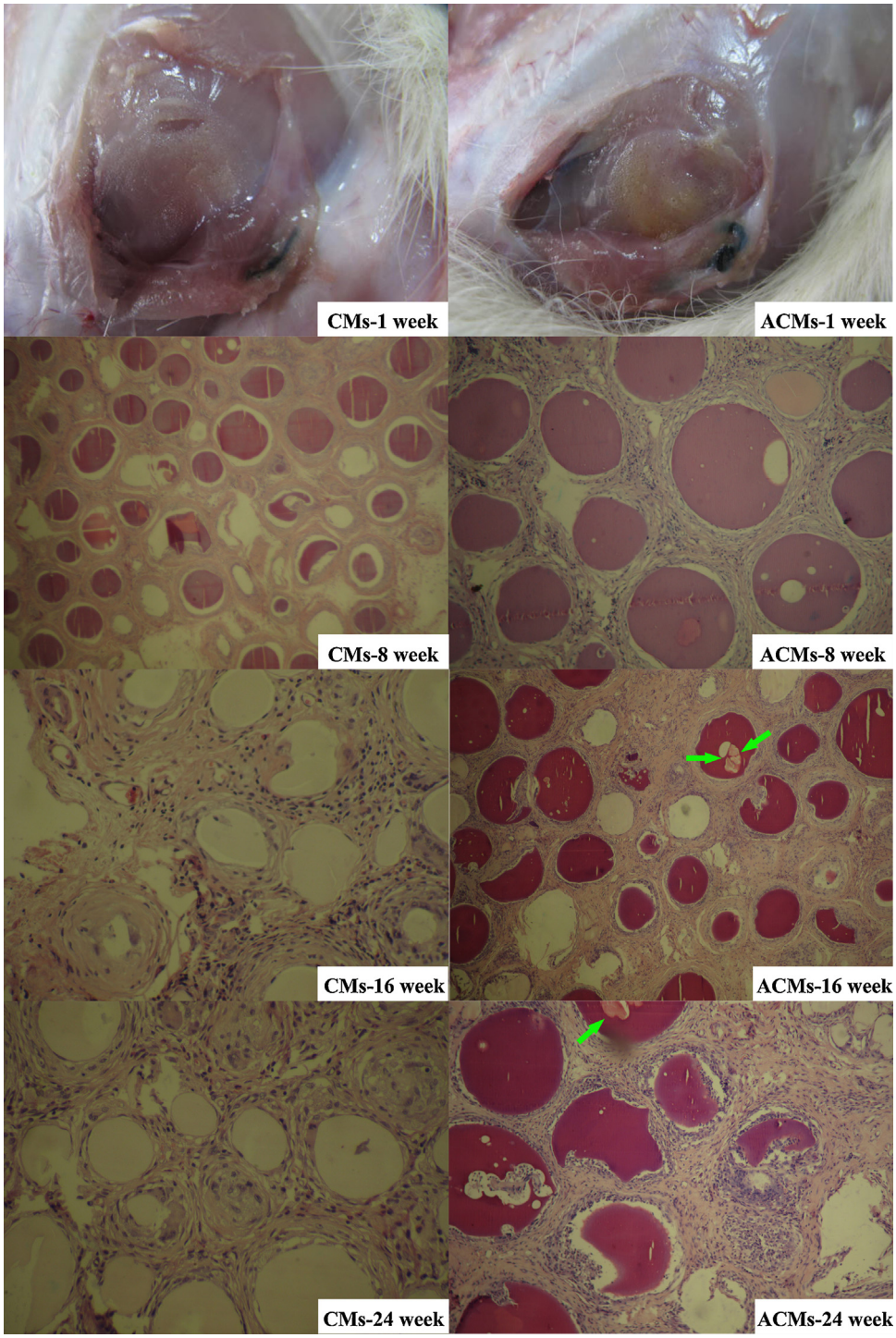

Fig. 7. Histological sections of rat skeletal muscle after implanting with microspheres (ACMs/CMs) in different time (1, 8, 16 and 24 weeks). (On the first week, the section did not stain; on the 8th–24th week, the section stained with H&E). Arrow: granulation tissue grown in the degrading microsphere.

was approached to hemolytic (4.78 %≈ 5%). In 1 h time point, the HR % of ACMs-10 mg was also non-hemolytic (<2%), that of ACMs-50 mg and CMs-10 mg were slightly hemolytic (3.04%/2.18%), and HR % of CMs-50 mg has already induced in hemolytic (5.15%). These results showed that ACMs exhibited better haemocompatibility than that of CMs. Previous studies (Pantaleone Yalpani, & Scollar, 1992) indicated that chitosan could induce hemolysis via electrostatic interactions. In aqueous solution, the amino groups on chitosan were protonated in the form of $-NH^{3+}$. Thus, negatively charged molecule (glycoprotein on red cell membrane) could be attracted by electrostatic interaction. The excessive attraction between CMs and red cell might induce red cell membrane bending and rupturing, and further, lead to hemoglobin release and hemolysis. The appropriate introduction of acetyl group would reduce the amount of amino groups and positive charges, which could reduce rates of hemolysis and improve the blood compatibility of ACMs.

3.6. Cytotoxicity tests

To evaluate cytotoxicity of microsphere (ACMs/CMs), mouse embryo fibroblasts (MEFs) were chosen to incubate with

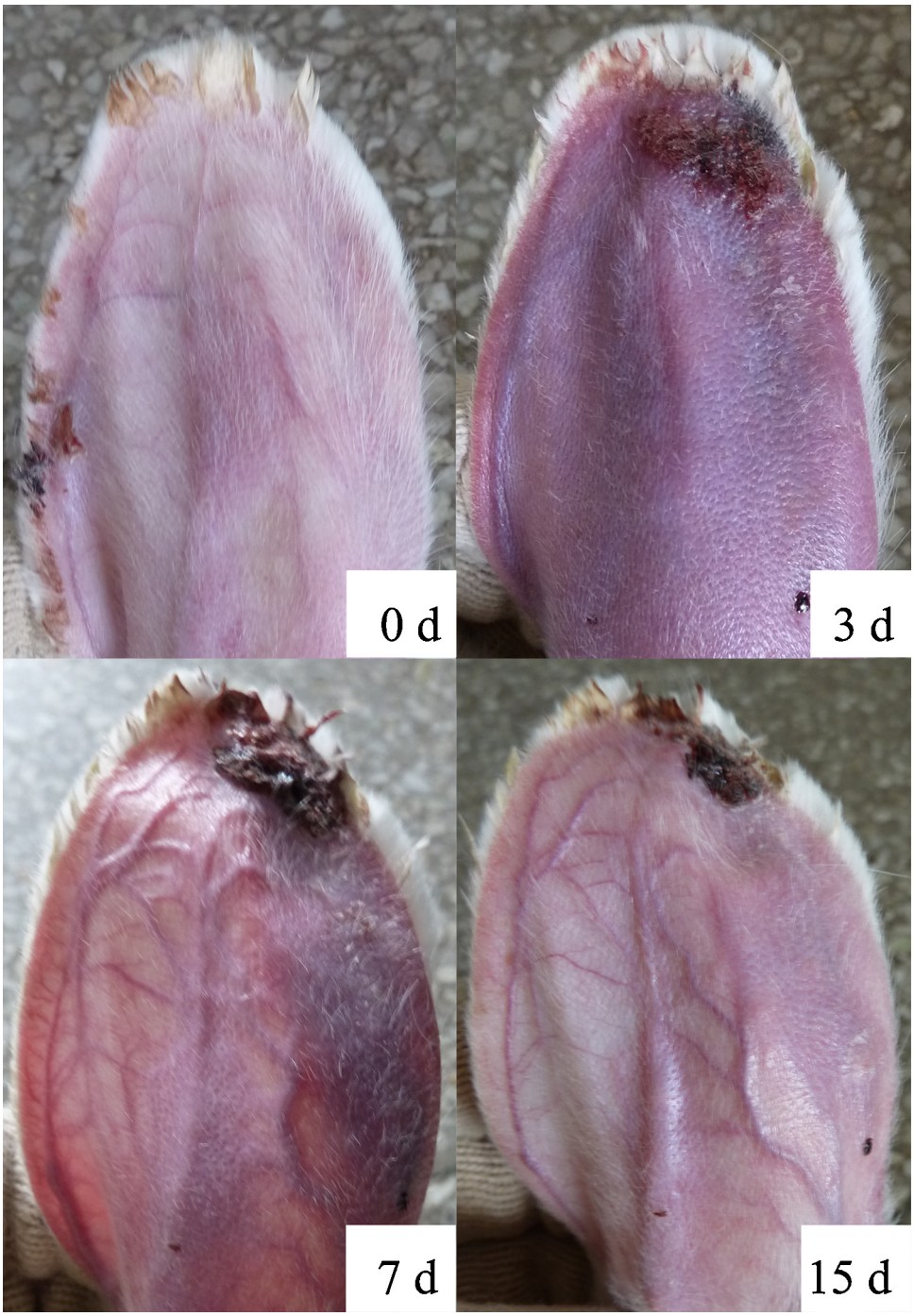

Fig. 8. The pre- and post-embolization with ACMs comparison of the rabbits' ears in different time (0, 3, 7 and 15 days).

microsphere in vitro. Cytotoxicity in MEFs was shown in Fig. 6. The results showed that relative growth rates (RGR) of MEFs on CMs decreased with incubation time, but, that of ACMs increased with incubation time although this was less than observed at early time (24 h) (the detailed data is not shown in Figure). It was implied that ACMs could stimulate the growth of MEFs and CMs were not cytotoxicity to MEFs. Some researchers reported that chitosan had good mucoadhesive properties due to it was a cationic polymer that could attract the cell by electrostatic interaction (Nordtveit, Vårum, & Smidsrød, 1994). However, some studies (Seyfert, Biehl, & Schenk, 2002) have reported that chitosan could induce poor cell proliferation because of high charge density, which would disequilibrate the charge on cytomembrane. In our paper, the results of RGRs of MEFs on CMs decreased with incubation time were consistent with the former studies. Appropriate acetyl group introduction could adjust the charge density on the surface of ACMs and benefit the cell-attraction. Cell attachment rates were less in ACMs initially, but RGR of ACMs increased gradually, perhaps due to stretch and proliferation of MEFs at the surface. These results further confirmed that chitosan microspheres with proper acetylation have potential to be used as a vascular chemoembolization agent.

3.7. *Muscle implantation in vivo*

Rat muscular implant test in our study was used for evaluating the security and degradation of embolic materials. The

microspheres (ACMs/CMs) were implanted in gluteal region of rat and its histocompatibility was observed. The results of blood analysis in each group in different time (0, 3, 7, 14 d) were shown in Table A.1. The test items included aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), serum creatinine (Cr) and white blood cell (WBC). In the pre-operation (0 d) and post-operation (3 d, 7 d, 14 d), there were no significant differences in the levels of AST, ALT, BUN and Cr among the CMs group, the ACMs group and the sham-operated group. The results implied that the microspheres had a good histocompatibility and harmless for the liver function and kidney function. Compared to pre-operation (0 d), the WBC count increased significantly on the 3rd and 7th day after operation in three groups. There was an extremely significant differences on 3rd day ($p < 0.01$) and significant differences on 7th day ($p < 0.05$). However, there was a non-significant difference ($p > 0.05$) in the WBC count on 14th day compared to pre-operation. The phenomenon of the sham-operated group was similar to the other two groups (the CMs group, the ACMs group), which could explain that the inflammation reaction was not caused by microspheres implanting. These results suggested that inflammation reaction caused by surgery lead to WBC count increasing, and then the WBC count decrease with inflammation disappears slowly with time. AST and ALT were used as a diagnostic marker for liver function in clinical detection, and BUN and Cr as for renal function. The results of blood analysis implied that the microspheres had a good histocompatibility and might be safe for implanting.

The histological sections of muscle implantation in vivo were shown in Fig. 7. The muscle was implanted with microspheres (ACMs/CMs) in 1 week, 8 weeks, 16 weeks and 24 weeks. All animals survived without serious adverse effects in whole operation indicated that the model was successfully established (Lee, Ha, & Park, 1995). In the1st week, preliminary animal experiment showed the wounds healed well without infection or fester, and the microspheres were wrapped by connective tissue. In the 8th week, cell infiltration was observed for both time points. The cellular response was moderate, the fibrocytes and a few macrophages encircling the microspheres. In the 16th week, some cavities on microspheres were formed because of partial degradation in vivo. The chamber in microspheres was filled with mature granulation tissue (the details are indicated by arrows in Fig. 7). The shapes of microspheres with a smooth surface and spherical shell have changed, e.g. the size reduction, rupture and distortion were clearly observed. In the 24th week, the microspheres were also surrounded by connective tissue and most of them were degraded in vivo. The matrix of microspheres seems to be collapsed and the residual fragment was gradually phagocytosed by macrophage. Finally, the microspheres will disappear and replaced by connective tissue. The above results of muscle implantation indicated that chitosan microspheres have good biocompatibility as a potential embolic material and could be biodegraded by body.

3.8. Embolization performed in vivo

In order to investigate the embolization efficiency of microspheres in vivo, rabbit ear model in our study was used for evaluating the medical embolic materials due to its easy establishment and observed by macrography as well as its low cost and efficacy. Therefore, rabbit ear model was chosen to test the embolization behavior and the photography was shown in Fig. 8. The acetylated chitosan microspheres were smoothly injected into microcatheters without any blockage and embolization was successfully performed in all rabbits' ear. The central auricular artery and branch of ear artery was visible before embolization (Fig. 8 – 0 d). However, after the 3rd day embolization, many symptoms had begun to appear, including the arterial blood flow clogged, scabbed and ear edema accompanied by a moderate inflammatory reaction, due to the vascular occlusion caused by ACMs and the branches of the artery were no longer visible (Fig. 8 – 3 d). In the 7th day, these symptoms got worst, e.g. ischemic necrosis with blackened and scabbed on the tip of ear tissue because of embolization, angioplerosis on ear was visible, due to its residual inflammatory reaction (Fig. 8 – 7 d). In the 15th day, the arterioles on the tip of ear were atrophy and disappear. Some neointimal thickening in embolism part could be observed (He, Davis, & Illum, 1998). The tissue around embolism position was completely ischemic necrosis and detachment because of vascular occlusion caused by the microspheres (Fig. 8 – 15 d). Therefore, these results of embolization behavior indicated that chitosan microspheres with proper acetylation could achieve embolization efficacy and could be used as a potential chemoembolization agent (Haipeng et al., 2000).

4. Conclusions

The aim of work was to investigate chitosan microspheres as a biodegradable embolic agent for arterial embolization applications. In this study, biodegradable chitosan microspheres with different deacetylation degree were successfully prepared using W/O emulsification cross-linking method (CMs) and acetylated method (ACMs). The microspheres with 132 nm mean size particle showed a quite smooth surface and a spherical shell. CMs swelling were more influenced by pH than that of ACMs, and both swelling ratios of CMs/ACMs were affected by temperature. The microspheres with excellent thermal stability could remain stable structure under extreme conditions (121 °C, 150 kPa for 1 h) and subsequently placed in room temperature for 3 months. The degradation process of ACMs was faster than that of CMs in lysozyme solution. Doxorubicin, as a model drug, was successfully incorporated into these microspheres for drug release study in vitro and exhibited excellent control release profile. The results of hemolysis assay in vitro showed that ACMs exhibited better haemocompatibility than CMs (even in 50 mg/mL, hemolysis rates < 5%). Cytotoxicity analysis showed that ACMs could stimulate the growth of MEFs, and CMs had no cytotoxicity effect on MEFs. Rat muscular implant test indicated that the microspheres were biodegradable and no rejection. In vivo evaluation of ACMs in rabbit ear embolization model revealed that the auricular arteries could be embolized by ACMs in 15 day and the ischemic necrosis on ear was visible due to the vascular occlusion. All these results indicated that the acetylated chitosan microspheres can be used as potential biocompatible and biodegradable embolic agents for transarterial embolization. More experiments about in vivo evaluation of the chitosan microspheres in animal model will be further investigated.

Acknowledgements

The authors are indebted to the financial support from the National Natural Science Foundation of China (Grant No. 81271727) and the International S&T Cooperation Program of China (2013DFG32880).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.06.080.

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