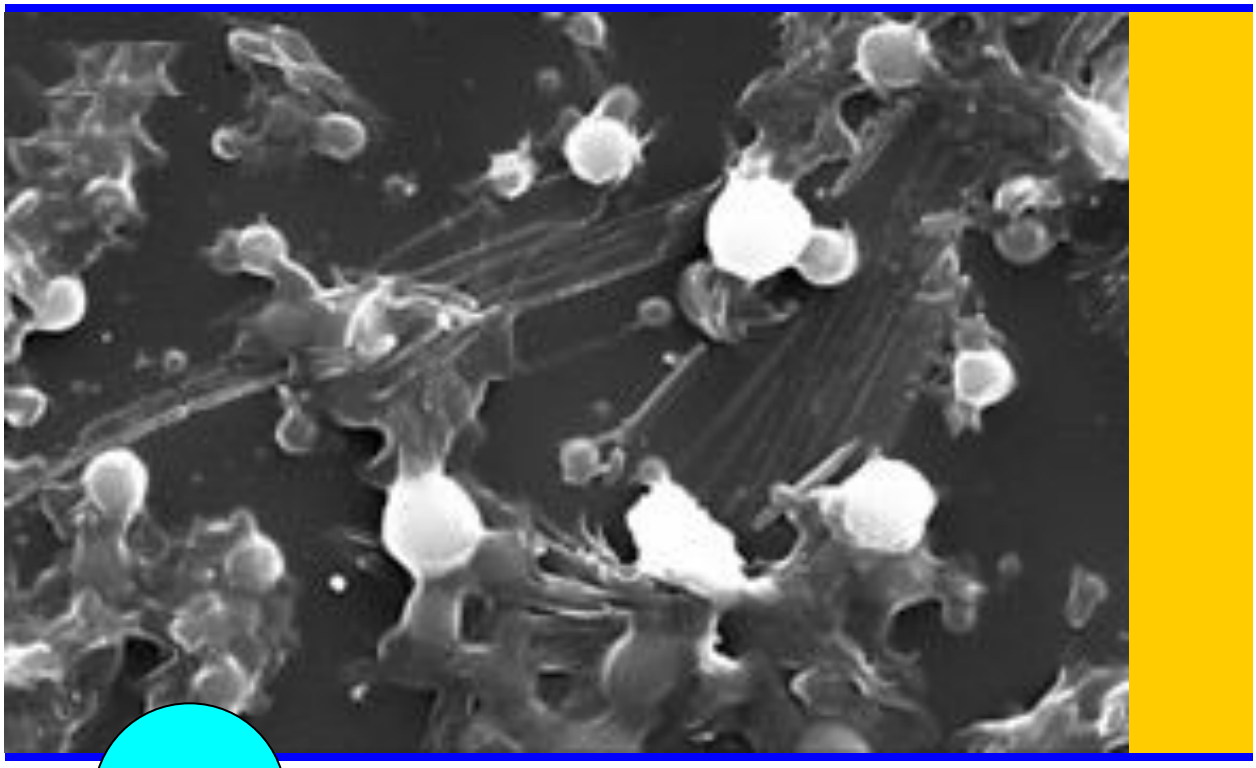

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Bangladesh Journal of Pharmacology

Research Article

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A Journal of the Bangladesh Pharmacological Society (BDPS)
Journal homepage: www.banglajol.info
Abstracted/indexed in Academic Search Complete, Agroforestry Abstracts, Asia Journals Online, Bangladesh Journals Online, Biological Abstracts, BIOSIS Previews, CAB Abstracts, Current Abstracts, Directory of Open Access Journals, EMBASE/Excerpta Medica, Google Scholar, HINARI (WHO), International Pharmaceutical Abstracts, Open J-gate, Science Citation Index Expanded, SCOPUS and Social Sciences Citation Index; **ISSN:** 1991-0088

**Bangladesh J Pharmacol 2016; 11: S116-S122**

# Degradation behavior of theophylline/chitosan/β-cyclodextrin microspheres for pulmonary drug delivery

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## Article Info

Received: 7 November 2015
Accepted: 22 February 2016
Available Online: 7 March 2016

DOI: 10.3329/bjp.v11iS1.25634

Cite this article:
Wang L, Yang T, Ju W, Ren M, Feng Z, Zheng Z, Hu Z, Liu Y Zhang W. Degradation behavior of theophylline/chitosan/β-cyclodextrin microspheres for pulmonary drug delivery. Bangladesh J Pharmacol. 2016; 11: S116-S122.

## Abstract

To evaluate the degradation behavior of theophylline/chitosan/β-cyclodextrin microspheres, we performed both *in vitro* study by putting the microspheres in phosphate buffered saline or in phosphate buffer saline with enzyme and *in vivo* study by implanting the microspheres into the back of male Sprague-Dawley rats. The results showed that microspheres were degraded in enzymatic hydrolysis and phosphate buffer saline, which were degraded faster in 0.2 mg/mL lysozyme than in phosphate buffer saline. The morphology of microspheres in phosphate buffer saline and enzyme solution developed rough surfaces, and showed irregular shape and pores after 8 weeks. The microspheres were degraded *in vivo* within 8 weeks with irregular, sheet, porous morphology, and the diameters were smaller than 5 μm. These results indicated that the theophylline/chitosan/β-cyclodextrin microspheres had a good degradation both *in vitro* and *in vivo* which can be used as a pulmonary drug delivery carrier.

## Introduction

Chitosan (CTS) is cationic natural biomaterials, and obtained from the deacetylation of chitin, which has been widely proposed as an inhalation drug carrier (Okamoto et al., 2003), with many unique properties such as low toxicity, high biocompatibility, biodegradability. CTS can bind mucosal surfaces because of its cationic nature, which leads to bioadhesion, and reduces mucociliary clearance, thereby prolonging the contact of drugs with mucosal surfaces (Davis, 1999). CTS can improve the drug absorption by opening the intercellular tight junctions of the lung epithelium and enhancing the dissolution rate of low water soluble drugs (Yamomoto et al., 2005; Maestrelli et al., 2004). Therefore, CTS was used in many studies for preparing sustained release form of pulmonary delivery microspheres, which is an excipient to improve the pharmaceutical and biopharmaceutical properties of drugs (Kinnarinen et al., 2003; Yang et al., 2007). In addition, the degraded products of chitosan are also non-toxic, non-imunogenic, and non-carcinogenic because they can be hydrolyzed by lysozyme (Muzzarelli, 1993).

Evaluation of the degradation of CTS as biomaterials was reported in previous studies (Ren et al, 2005; Yang et al., 2007). In recent years, biodegradable microspheres are becoming increasingly popular in the design of pulmonary drug delivery systems. Furthermore, a number of biodegradable microspheres have been

This paper was presented in the 3rd International Conference on Biomedicine and Pharmaceutics in Zhuhai, China, on December 11-13, 2015.

proven to be non-toxic, and non-immunogenic. However, questions still exist such as the safety of the polymer form, microspheres and other excipient because of the specialty of the pulmonary drug delivery system. Therefore, an investigation of the degradation and biocompatibility of microspheres is important for their practical application.

The degradation rate of CTS can be controlled by changing its polymer composition (i.e. the co-polymerization ratio of glucosamine to nacetylglucosamine or the length of acyl side-chain on nacetylglucosamine) and/or its molecular weight (David et al., 1992; Tomihata and Ikada, 1997). Mi et al., (2002) investigated the *in vivo* biocompatibility of the genipin-cross-linked injectable CTS microspheres, and found that the microspheres degraded into a loose and porous structure at the end of 12 week after administration. Yang et al., (2007) suggested that acetylated CTS fiber is more biodegradable than CTS, and the biodegradation rate of chitin fiber can be controlled to desirable extent by modification of acetylation degree. Although CTS is regarded as a biocompatible and degradable polymer, its biodegradation properties cannot be easily tailored for specific application by simply proper choice of the chemistry composite. Being a drug carrier of pulmonary delivery system and reducing the frequency of dosing, the microspheres with good safety profile are thought to be a prerequisite. Therefore, it is important to study systematically their *in vitro* and *in vivo* degradability. In our previous studies (Zhang et al., 2007; Zhang et al., 2008; Zhang et al., 2009) the pulmonary sustained release microspheres of TH/CTS/β-CD (1:3:1) were considered to be an effective sustained release carrier for pulmonary delivery and had a good biocompatibility. Therefore, we considered it important to study the degradability of the microsphere.

In the present study, we investigated the degradability of the sustained microspheres serving as pulmonary sustained drug delivery carriers. Series experiments were conducted for the evaluation of *in vitro* degradation of the microspheres in enzyme solution or phosphate buffer saline (PBS), and *in vivo* degradation by using a rat model. The formulation of 1:3:1 of TH/CTS /ß-CD was used as a reference throughout this study unless otherwise indicated. The formulation of 1:3:1 of TH/CTS /β-CD (Sashiwa et al., 1990) was used as a reference throughout this study unless otherwise indicated.

## Materials and Methods

### *Materials*

Microspheres were prepared using spray drying method (Sashiwa et al., 1990; Suh and Matthew, 2000) and the ratios of MS A, B, C were 1/1/0.33, 1/3/1, 1/5/1.67 (TH/CTS/β-CD, w/w/w), respectively, while the blank microspheres was MS K (CTS/β-CD, 3/1). In brief, a predetermined amounts of TH and β-CD were dissolved in 200 mL of 1% CTS in acetic acid solution according to the above formulations. The solutions were spray-dried, using a spray-dryer (Büchi® Mini Spray Dryer, B-191, Switzerland), co-current flow type with a two fluid nozzle (diameter 0.7 mm). The operating parameters were as follows: Airflow rate 600 L/h, aspiration 90%, feed rate 6 mL/min and inlet temperatures 150 ± 2°C, resulting in outlet temperatures 81 ± 2°C. The spray-dried microspheres were collected and stored in a desiccator (with anhydrous $CaCl_2$) at room temperature. The morphology was evaluated by scanning electron microscopy (KYKY2800B, KYKY Technology Development LTD, China). The microspheres were sputter-coated with a thin layer of Au/Pd and photographed.

All chemicals and reagents used in this study were of analytical grade, and were purchased from Shanghai Chemical Reagent Company (Sigma Co. ST. Louis, USA). Male Sprague-Dawley (SD) rats weighting 260～360 g were purchased from Qingdao Municipal Institute for evaluation of the biocompatibility of microspheres *in vivo*. The animal protocol was approved by Shandong Medical Laboratorial Animal Administration Committee. The animals were housed in a room with controlled temperature and humidity. All animal studies were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23) revised in 1996. All efforts were made to minimize the number of animals used and their suffering.

### *In vitro hydrolysis degradation*

The *in vitro* hydrolysis degradation was determined by measuring the weight of microspheres remaining in the PBS (0.2 mol/L, pH 7.4). Microspheres A, B and C were determined in an individual test tube containing 3 mL PBS. The tubes were kept in a shaking water bath (37°C, 100 rpm), and the PBS solution was replaced every week with the fresh PBS. In predetermined intervals, the specimens were collected by filtration, rinsed with distilled water and dried to constant weight in a vacuum desiccator. Then the specimens were weighed, and the degree of degradation was estimated from degradation rate of microspheres as following equation:

$$\text{Weight remaining}(\%) = \frac{M_t}{M_o} \times 100\%$$

Where $M_0$ is the initial weight of the microspheres and $M_t$ is the weight of the microspheres after the incubation

The morphologies before degradation in distilled water and after 12 weeks in PBS were evaluated by scanning electron microscopy (KYKY2800B, KYKY Technology

Development Ltd., China). The microspheres were saputter coated with a thin layer of Au/Pd and photographed.

### *In vitro enzymatic degradation*

The *in vitro* lysozyme degradations of microsphere A, B, C, and K were carried out in 3.0 mL PBS containing 0.2 mg/mL lysozyme (hen egg-white, Sigma Co. ST. Louis, USA). The tubes were kept in a shaking water bath (37° C, 100 rpm) for 12 weeks. The solution containing lysozyme was refreshed daily to ensure the continuous enzyme activity. The specimens, with predetermined intervals of degradation, were removed from the medium, rinsed thoroughly with doubly distilled water to remove the buffer and lysozyme remaining on the surface and dried to constant weight in a vacuum desiccator. Then the specimens were weighed, and the degree of degradation was estimated from the degradation rate of microspheres as the equation above which was used to determine the *in vitro* hydrolysis degradation.

The morphologies before degradation in distilled water and 12 weeks after degradation in lysozyme solutions were evaluated by scanning electron microscopy (KYKY2800B, KYKY Technology Development LTD., China). The microspheres were sputter-coated with a thin layer of Au/Pd and photographed.

### *In vivo degradation*

The *in vivo* degradation was examined by implanting the microspheres in the skeletal muscle of SD rats. The test microspheres MS B was sterilized using UV. Subsequently, the sterilized microspheres weighing 60 mg were implanted into the skeletal muscle with the rat under anesthesia. Each animal received two implantations in the back just lateral to the midline. Rats were killed after 2, 4, and 8 weeks, the implanted microspheres with portions of the excised surrounding tissues were fixed with osmium tetroxide, and then were prepared to coat gold and observed using SEM (KYKY2800B, KYKY Technology Development Ltd, China).

### *Statistical analysis*

All the data were presented as mean ± SD. Statistical analysis was carried out with one-way ANOVA using SPSS19.0 and differences were considered to be significant at a level of $p<0.05$.

## Results

### *In vitro hydrolysis degradation*

The results of *in vitro* hydrolysis degradation are shown in Figure 1-3. The weights of MS A, B, C and K were maintained relatively constant throughout degradation (Figure 1). The average weight of MS A was significantly decreased compared with the weight of the other three microspheres, and 53.9% of the original weight remained within the first week, after 12 weeks, only 26.3% of the original weight was left. The microspheres MS B, C, and K showed the same tendency with MS A and the degradation rate was slower with the time lasting. The average weights of MS B, C and K were decreased to 36.3, 48.6, and 40.1% of their respective initial weights after 12 weeks, therefore no significant differences were observed between any of the three formulations. The morphology of the microspheres degraded in PBS was examined in SEM images at the end of 12-week-hydrolysis. It was shown in Figure 2. that the appearances of MS A, MS B and MS C were fabricated with spherical shape and smooth surfaces in distilled water, while in PBS, after 12 weeks *in vitro* degradation, the appearance of MS B became compact and aggregated, there was little granule at the surface of the microsphere aggregate (Figure 3).

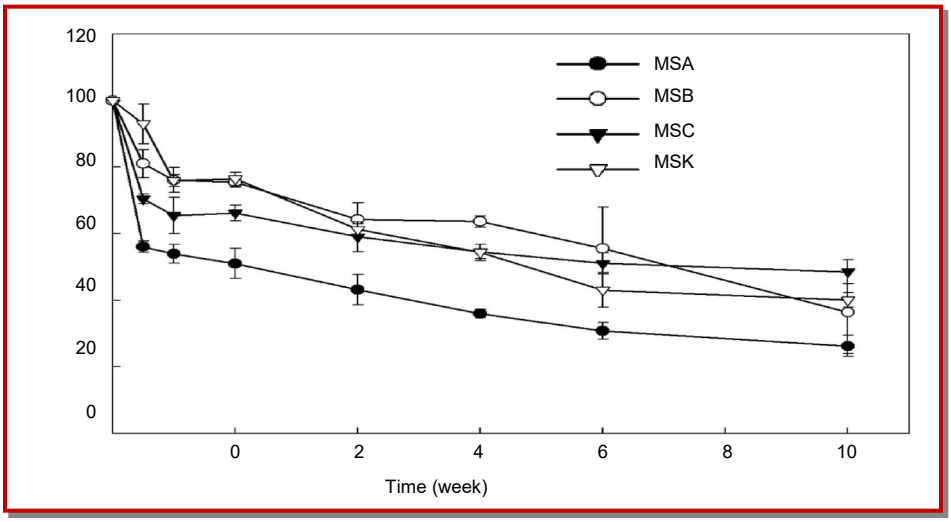


Figure 1: *In vitro* degradation curves of microspheres system in PBS solutions (mean ± SD, n=5

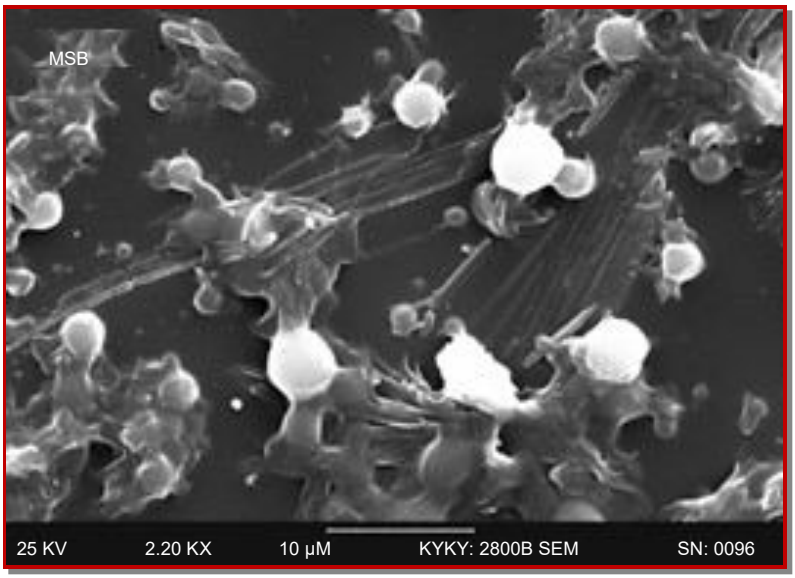


Figure 2: SEM of spray-dried MS B. (before degradation in distilled water)

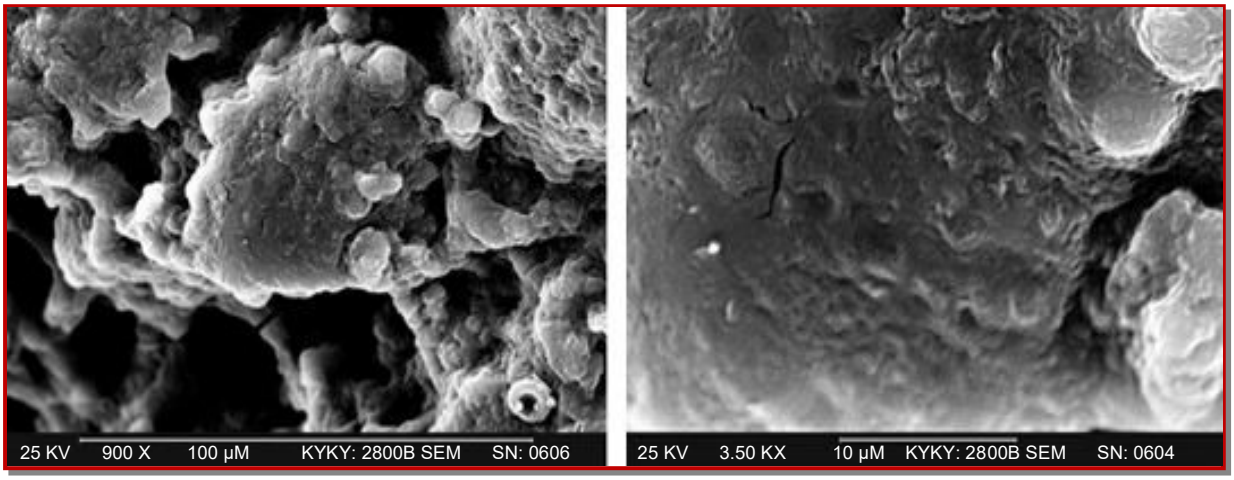


Figure 3: SEM of MS B degradation in PBS (pH 7.4) after 12 weeks

***In vitro enzymatic degradation***

The results of *in vitro* enzymatic degradation were shown in Figure 4-5. The weights of MS A, B, C and K decreased relatively moderate respectively in vitro enzymatic degradation (Figure 4). This was in accordance with the result of hydrolysis. The remaining weight of chitosan microspheres MS A, B, C and K degraded in 0.2 mg/mL lysozyme solutions was shown in Figure 4. The average weight of MS A decreased more quickly than that of the other three microspheres, and the average weight of MS A was decreased to 36.8% of the original weight within the first week, after 12 weeks, only 19.9% of the original weight remained, and the degradation in lysozyme solution was quicker than that in PBS solution. The remaining weights of MS B, C and K were 27.3, 27.3, and 45.6% after 12 weeks, and the degradation rate became slow with the time lasting. There was no significant difference of the weight remaining in different drug/polymer ratio; this result was in accordance with the degradation rate in hydrolysis. As shown in Figure 5, morphology of MS B degraded in lysozyme solution 12 weeks after incubation had the same appearance as that in PBS solution, which became compact and aggregation with little granule in the surface.

***In vivo degradation***

The *in vivo* degradation of TH/CTS/β-CD microspheres was shown in Figure 6. The appearance of microspheres changed remarkably after implantation with time prolonged. The SEM pictures of MS B before implantation were showed, with a regular spherical shape and a smooth or slightly wrinkled surface (Figure 6a). Two weeks after implantation, Figure 6b showed the surface of MS B became clearly rough and MS K had lots of microspores distributed on their surface, while

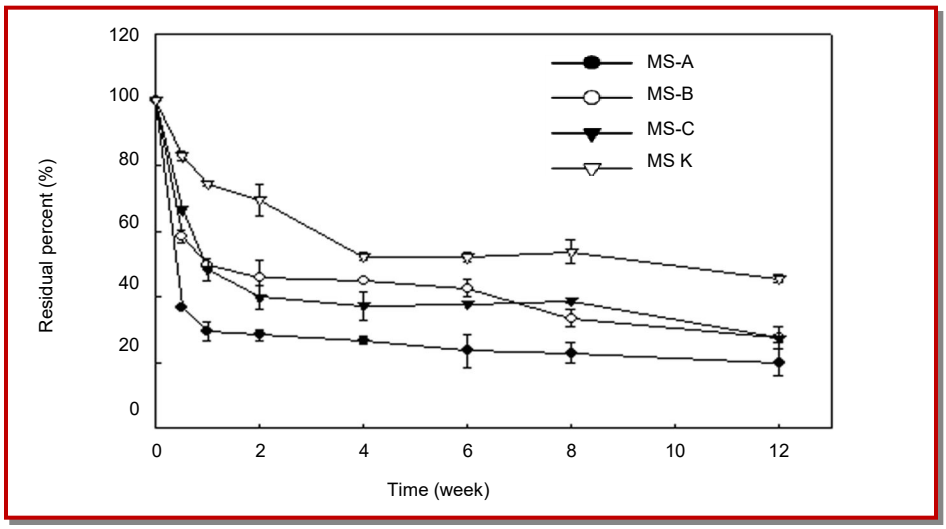


Figure 4: *In vitro* degradation curves of microspheres system in 0.2 mg/mL lysozyme solutions (mean ± SD, n=5)

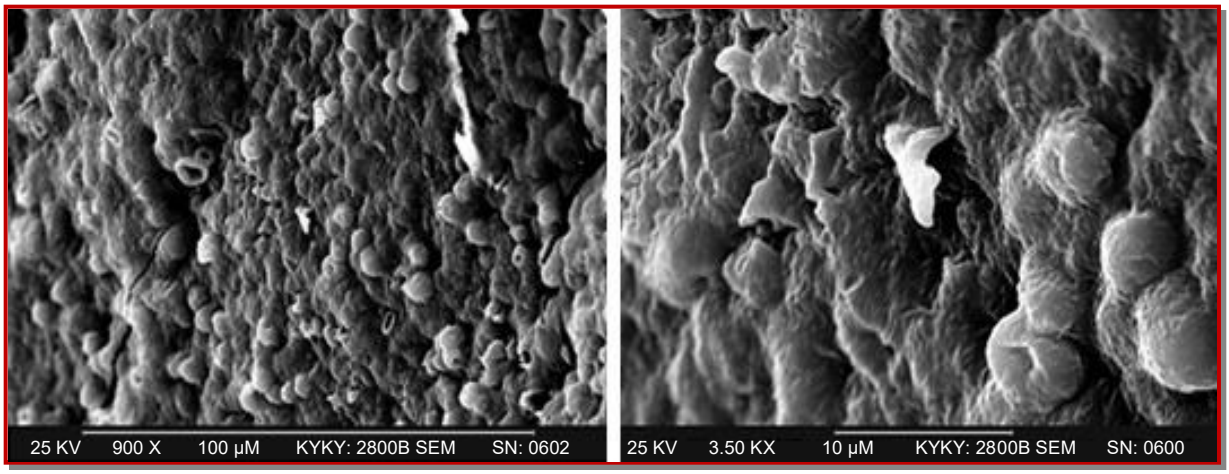


Figure 5: SEM of MS B degradation in 0.2 mg/mL lysozyme solutions after 12 weeks

the shape was also spherical. This result indicated that the degradation of microspheres started from the surface of the two microspheres. The MS B (Figure 6c) developed porous structure, rough surfaces, disappeared round structure after 4 weeks, and became irregular or sheet structure. In 8 weeks, the MS B became oval or irregular, flake porous structure (Figure 6d). The diameters of the largest microspheres were only about 5 µm, and the MS B turned into a 1-2 µm network structure.

## Discussion

*In vitro* hydrolysis degradation, the average weight of MS A was significantly decreased compared with the weight of the other three microspheres. This could be explained by MS A having high drug/polymer ratio and part of the drug staying nearly or under the surface of microspheres being released. CTS derive from the deacetylation of chitin, which is considered to be a good candidate being used in pulmonary delivery because of its biodegradable, mucoadhesive and biocompatible properties (Sashiwa et al., 1990) which is the main advantage to be used in controlled drug delivery systems. Studies showed that CTS were gradually degraded by lysozyme whose target is an acetyl group in vivo (Sashiwa et al., 1990; Suh and Matthew, 2000). Lysozyme is abundant in human milk and human serum, the serum concentration is about 4-13 µg/mL. Lysozyme is one of the secretion enzymes of respiratory epithelial cell and macrophage, the secretion mount is about 10-20 mg per day (Suh and Matthew, 2000).

In our experiment, the concentration of lysozyme was applied 0.2 mg/mL, which based on the Konstan's method (Konstan et al., 1981) and the maximum concentration secreted by the trachea. The lysozyme was dissolved in PBS (pH 7.4), and the pH of solution was 6.8-7, which was the optimum pH and lysozyme, closed to the lung pH (Calvo et al.,1997).

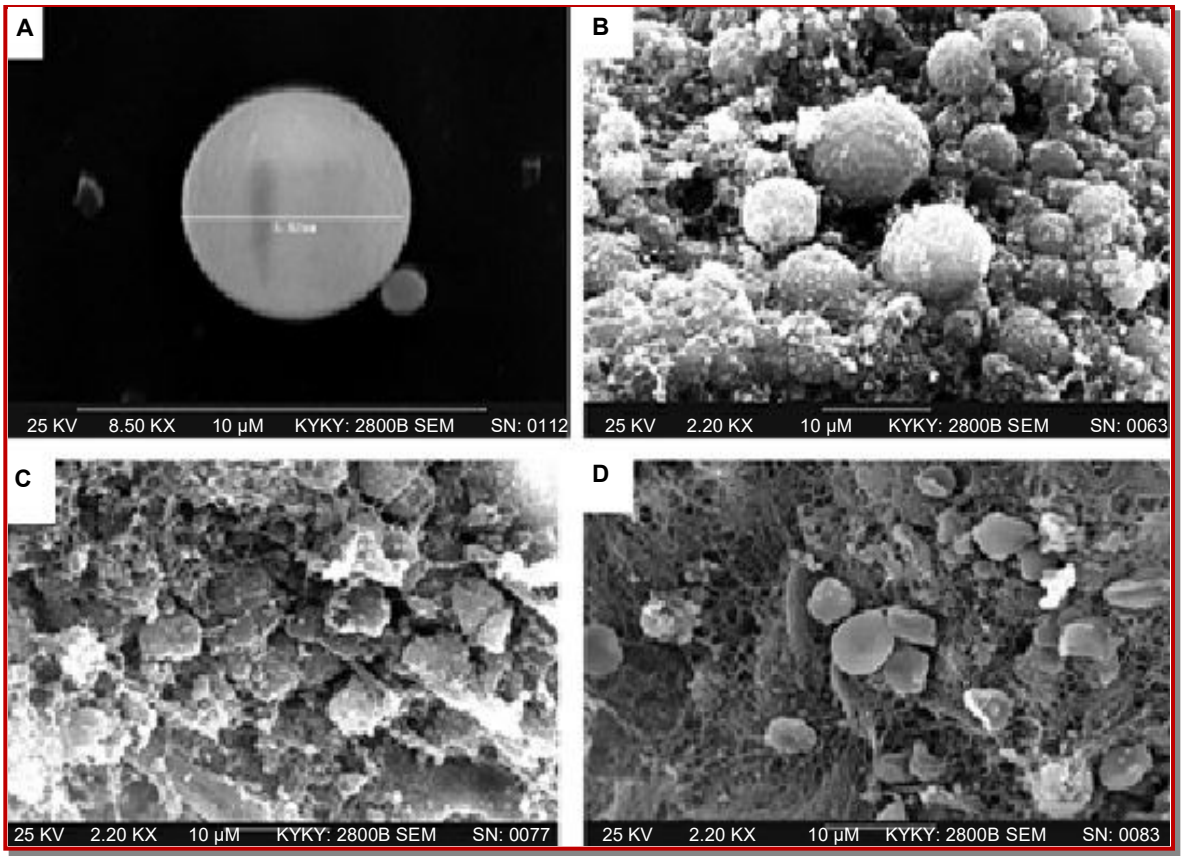


Figure 6: SEM pictures of the change of MS B before implantation (A) and after implantation 2 week (B) 4 week (C) and 8 week (D)

*In vitro* experiment, the degradation rate in enzyme solution of microspheres is faster than that in PBS solution (Figure 1 and Figure 4). The enzymolysis and hydrolysis rates of MSA were higher than those of MS B, C and K, which may be caused by the low molecular weight of small molecules (such as TH gradually released) in solution in the early beginning stages, while in the late stage, the degradation was attributed to the polymer hydrolyzation. The crystalline structure of the CTS is also an important factor affecting the degradation. The crystallized CTS can prevent lysozyme into the N-acetyl-β-glucosaminidase (NAG) areas, and therefore reduce the degradation rate, while the amorphous structure allows the enzyme to penetrate into the NAG areas. The low crystallinity of the spray dried microspheres can increase the hydrolysis rate, which have good degradability (Mi et al., 2002). Previous studies demonstrated that the spray dried TH/CTS microshperes were in crystal or amorphous forms (Asada et al., 2004), which made water molecules easily penetrate inside the CTS and increased the degradation rate of CTS. As reported (Mi et al., 2002) the CTS microspheres showed excellent biodegradability *in vivo*, which had been degraded into sheets in 12 weeks. From the results of this experiment, the TH/CTS/β-CD microsphere (MS B) became irregular, flaky, porous structure 8 weeks after incubation, and the majority of microspheres were degraded to a diameter of less than 5 μm. The experimental results showed that TH/CTS/β-CD microspheres (MS B) have excellent degradability, which can be used as an excellent carrier for pulmonary drug delivery.

## Conclusion

The TH/CTS/β-CD microspheres could be degraded in vitro by enzymatic hydrolysis and PBS, and the rate of enzymatic hydrolysis was faster than that of PBS. The MS B microspheres *in vivo* were degraded into irregular, sheet, porous shape, and the diameters were less than 5 μm.

## Financial Support

National Key Technology R and D Program of the Ministry of Science and Technology (No. 2013GA740103); National Natural Science Foundation of China (No.81573717); Shandong Provincial Natural Science Foundation, China (ZR2012CM025) for grant support; Shandong Province Higher Educational Science, Technology Program (J11LF67). and Department of Science and Technology of Weifang City(2014WS045).

## Conflict of Interest

Authors declare no conflict of interest

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