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# Investigation of acetylated chitosan microspheres as potential chemoembolic agents

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ABSTRACT

The aim was to investigate the potential of chitosan microspheres (CMs) with different acetylation using as a chemoembolic agent. Chitosan microspheres (CMs) were prepared via water-in-oil (W/O) emulsification cross-linking method, and acetylated chitosan microspheres (ACMs) were obtained by acetylation of CMs. Next, we characterized the morphology, size, composition and degrees of deacetylation using scanning electron microscopy (TEM), dynamic laser light scattering (DLS), and Fourier transform infrared spectrometer (FTIR). All microspheres had smooth surfaces and good mechanical flexibility, and all could pass through a 5F catheter. The swelling rate (SR) of CMs decreased significantly with the increase of pH (4.0–10.0) but ACMs did not change under the same conditions. Protein absorption assays suggested that albumin was more greatly adsorbed on CMs than on ACMs. Furthermore, CMs caused more blood clots than ACMs. ACMs caused hemolysis less than CMs (<5% of the time). Data indicated that ACMs had more hemocompatibility. Cytotoxicity tests indicated that ACMs initially had less cell attached proliferation but increased with incubation. In contrast, the relative growth rate of mouse embryo fibroblasts (MEFs) on CMs decreased gradually. The results suggested that ACMs could stimulate the growth of MEFs, and CMs were not cytotoxic to MEFs. Thus, ACMs were more biocompatible with greater potential to be used as chemoembolic material.



## 1. Introduction

Transcatheter arterial embolization (TAE) is known to block blood flow of tissue or organ during the treatment of tumors, vascular lesions, and hemorrhages [1–3]. Embolic agents such as coils, liquid, gelfoams and particles have been applied for this purpose [4,5]. Some embolic agents cannot be widely used due to their irregular shapes which cannot be calibrated and tend to aggregate [6,7]. At this time, microspheres are commercially available such as tris-acryl microspheres (Embosphere; Biosphere Medical, Roissy, France), Contour SE (CSE; Target Therapeutics, Boston Scientific Corp., Fremont CA), and Bead Block (DC-bead, Biocompatible Ltd., UK) [7,8]. This suggests that embolic agents with spherical shells, smooth shapes and an accurately calibrated size range may be viable options for these procedures. Chitosan microspheres have such morphological advantages as embolic biomaterials which are also biodegradable, and can load chemotherapeutic drugs.

Chitosan, composed of β-(1.4)-2-acetamido-2-deoxyglucopyranose and 2-amino-2-deoxyglucopyranose units, is a high molecular weight biodegradable cationic polysaccharide [9,10]. Chitosan has excellent biocompatibility, low toxicity and good biodegradability [11]. In aqueous solutions, chitosan's amino groups are protonated in the form of $-NH^{3+}$ [12]. Most characteristics of chitosan are related to the $-NH^{3+}$ charge. Also, chitosan with different degrees of deacetylation offers different levels of biocompatibility and has various physicochemical characteristics due to various amounts of amino groups [13,14]. Chitosan microspheres (CMs) have useful qualities that enable them to be used in drug delivery system [15,16], immune enhancement [17], antibacterial activity [18], and tissue engineering [19]. Li's group [20] reported that chitosan–alginate microspheres, as embolic agents, had excellent effects in both short and long term on renal arterial embolization.

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Chitosan microcapsules and microspheres have shown promise in clinical applications and experimental studies [8]. Also, CMs can carry chemotherapeutic drugs to treat tumor via chemoembolization [19,21].

In this paper we evaluated morphologic characteristics and embolic properties of CMs that had different degrees of deacetylation. CMs were prepared by water-in-oil (W/O) emulsification cross-linking method, and acetylated chitosan microspheres (ACMs) were made by acetylating CMs with anhydride acetic. Characteristics of CMs/ACMs, including morphology, particle size, FTIR spectra, mechanical flexibility, protein absorption, hemocompatibility and cytotoxicity were investigated to evaluate their potential as embolic agents.

## 2. Materials and methods

### 2.1. Materials

Chitosan (MW 136 kDa; degree of deacetylation 90.6%) was obtained from biochemical medicine plant of Qingdao (Qingdao, China). BSA, DMEM and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were supplied by Amresco (Solon, OH). Acetic anhydride and other chemical agents were analytical grade. Pregnant mice (ICR) were supplied by SFDA of Qingdao.

The animal protocol was approved by Shandong Medical Laboratorial Animal Administration Committee. All the 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

CMs were prepared using the W/O emulsion technique [22]. Briefly, 2 g chitosan was dissolved in 100 mL acetic acid (2%), and then the solution was added drop-wise into 500 mL liquid paraffin containing 7 mL Span-80 and 2 mL Tween-80. The mixture was mechanically stirred at 1100 rpm for 60 min (IKA RW 20 stirrer, IKA Co., Ltd., Germany). Next, 10 mL formaldehyde solution (37%) was added drop-wise to the mixture with continuous stirring at the same speed for another 60 min. The suspension was then filtered through a Sub-sieve (120-mesh, 60-mesh and 30-mesh; USP Standard Sieves) and fully rinsed with isopropyl alcohol. CMs were resuspended in $NaBH_4$ solution (1 g/L) for 12 h. Then, CMs with different particle sizes were collected and successively dehydrated in a series of graded ethanol (30%, 50%, 80%, 95% and 100%). Finally microspheres were dried and stored in sealed bags.

ACMs were prepared by acetylation of CMs with acetic anhydride. In brief, CMs were dispersed in methanol, followed by the addition of excessive 98% acetic anhydride; the mixture was stirred for 16 h. Microspheres were immersed in KOH–alcohol solution (0.5 mol/L) for 1 h, next, washed and dried to acquire ACMs.

### 2.3. Characteristic 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., Chinese Academy of Sciences, China). Particle size distribution was measured using a Mastersizer 2000 (Malvern Instruments Ltd., UK).

The infrared spectra of sample were recorded on a Nicolet FTIR 5700 spectrophotometer (Madison, WI) at room temperature (25 °C). Chitosan, CM and ACM samples were mixed in a mortar with KBr and compressed into discs.

Microsphere mechanical flexibility was tested using a microcatheter. Swollen microspheres were mixed in iohexol and injected into a 5F microcatheter with a disposable plastic syringe. The injection process and microspheres shapes were observed with optical microscopy.

### 2.4. Deacetylation degree (DD) of microspheres (CMs/ACMs)

Samples DD were measured using potentiometric titration according to the method of Wang and colleagues [23]. First, a 0.1 g sample (chitosan/CMs/ACMs) was dispersed in 20 mL 0.1 M HCl with stirring and titrated with 0.1 M NaOH. Changes in pH were recorded with a pH meter (DELTA320, China) and the consumed volume of NaOH was also recorded. DD was calculated as follows:

$$\mathrm{DD} = \frac{\Delta V \times C_{\mathrm{NaOH}} \times 10^{-3} \times 16}{W \times 0.0994} \quad (1)$$

where $\Delta V$ is the consumed volume of NaOH between the first and second neutralization point and $W$ is the weight of microsphere samples.

### 2.5. Measuring the swelling ratio (SR)

Microspheres SR were measured by quantifying sample average particle size changes before and after the experiment. Samples with different particle sizes were immersed in buffer solution (pH 4.0, pH 7.2 and pH 10.0) at 37 °C with gentle shaking. 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. (2):

$$SR\% = \frac{D_t - D_0}{D_0} \times 100\% \quad (2)$$

where $D_0$ is the initial average particle size of microspheres and $D_t$ is the average particle size of microspheres after the processing period.

### 2.6. Protein absorption experiment

Dried samples of both microsphere types (30 mg) were soaked in 1 mL PBS (pH 7.2) at 37 °C for 4 h to equilibrate moisture uptake and then PBS was removed. Next, 1 mL BSA solution (100 μg/mL) was added into the samples and incubated at 37 °C with gentle shaking for 0.5, 1, 3, 6, 9, 12 and 15 h. At predetermined time intervals, the supernatant was removed and samples were washed twice by PBS to remove proteins which were not adsorbed onto the sphere surface. Adsorbed proteins were desorbed from the sphere surface by 1 mL PBS (contained 1% SDS) and spectrophotometrically measured ($\lambda$ = 595 nm) with Coomassie brilliant blue. Finally, washed-off proteins were withdrawn for SDS-PAGE (10% separating; 4% stacking gel).

### 2.7. Thrombogenicity assay

Samples (CMs/ACMs) thrombogenicity was measured using fresh rabbit blood and the clotting time was quantified [24]. Blood was drawn from a healthy rabbit and the first portion of blood was discarded to eliminate needle puncture contamination. Blood was anticoagulated by adding 1 mL of acid citrate dextrose (ACD) solution into 9 mL of fresh rabbit blood. Prior to testing, CMs/ACMs were soaked to equilibrium in normal saline at 37 °C. Then, anticoagulated blood (0.8 mL) was added to the samples. Blood clotting was activated by adding 80 μL of 0.1 M $CaCl_2$ solution and mixing it. All the samples were incubated at 37 °C for 15, 30, 45 and 60 min. Clotting process was stopped by incubating the sample with distilled