Gelatin Microspheres Containing Multi-Walled Carbon Nanotubes: Preparation, Characterization, and Use as a Drug Carrier

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ABSTRACT

This work investigated how the incorporation of multi-walled carbon nanotubes (MWCNTs) into gelatin microspheres influences the effectiveness of microspheres as drug delivery systems by studying the adsorption of asiaticoside (AC) on the microspheres. We treated MWCNTs with a H₂SO₄/HNO₃ solution. The treated MWCNTs were dispersed in deionized water and sonicated in an ultrasonic bath. The dispersion of treated MWCNTs was then mixed with the gelatin stock solution, and microsphere swelling and AC encapsulation efficiency were determined. Untreated gelatin microspheres were used for comparison. The experimental results indicated that the addition of the treated MWCNTs enhanced the mechanical stability of the gelatin microspheres. Furthermore, the addition of the treated MWCNTs to the gelatin microspheres increased AC encapsulation efficiency and slowed AC release. The maximum AC sorption capacities of the gelatin and the treated MWCNT-filled gelatin microspheres were 115.22 and 230.11 mg/g, respectively. In addition, cytotoxicity was investigated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which revealed that the treated MWCNT-filled gelatin microspheres did not affect murine fibroblast (L929) viability. Thus, the synthesized microspheres were biocompatible with the fibroblasts.

Keywords: carbon nanotubes, gelatin, asiaticoside, microsphere, controlled release

1. INTRODUCTION

Carbon nanotubes (CNTs) have attracted interest in the biomedical field because of the applicability of their unique structure and excellent mechanical properties (high surface area and high strength) to drug delivery systems by studying the adsorption of asiaticoside (AC) on the microspheres. Researcher have also studied the in vivo
effects of carbon nanotubes. Meng et al. [6] demonstrated that carbon nanotubes were able to enter fibroblast cells and potentially accumulate in the cytoplasm, affecting the growth behavior of the fibroblast cells.

The use of CNTs has been limited by their poor solubility in water and the presence of residual synthetic metal catalysts, which prevent the use of CNTs as carriers in biological applications and cause cytotoxic reactions [7, 8]. Therefore, groups of charged nanotubes have been introduced to CNT surfaces to increase binding to other molecules, such as proteins [9], drugs [10], and siRNA or DNA [11-14]. Recently, oxidation of MWCNTs with strong acids has been demonstrated to remove residual metal impurities and improve CNT dispersibility [15]. These developments have enabled the use of carbon nanotubes in biomedical applications.

Biocompatible and biodegradable gelatin microspheres have been extensively used as drug carriers [16] for food applications [17], tissue engineering [18], and gene therapy [19] because gelatin is a naturally occurring polymer. Gelatin is obtained by the partial hydrolysis of collagen, the chemical structure of which comprises heterogeneous polypeptide chains that are composed of unique amino acid sequences [20]. The gelatin isoelectric point (IEP) can be changed by treating gelatin with a base or an acid. Different IEP values are created by the complex electrostatic interactions between the oppositely charged molecules [21].

Gelatin presents some challenges to the encapsulation process because of its low mechanical strength and rapid swelling [22]. To solve this problem, CNTs have been incorporated into the gelatin microsphere to improve mechanical stability and reduce drug leakage.

Asiaticoside is a type of triterpene glycoside from the medicinal plant Centella asiatica (L.) Urban, which is also known as Buabok (in Thai). This extract is commonly associated with wound healing [23]. An increase in antioxidant levels at the initial stage of restoration [24] was observed when asiaticoside was applied to excision-type cutaneous wounds in rats, and up-regulation of the mRNA and pro-collagen and glycosaminogly that can synthesis were observed.

In this study, we prepared carboxylated multi-walled carbon nanotubes (MWCNTs) by treatment with H$_2$SO$_4$ and HNO$_3$ and prepared Gelatin microspheres filled with treated MWCNTs by an emulsion technique. The effects of treated MWCNTs on morphology, swelling behavior of the microspheres, In vitro release were investigated, and evaluated the sorption of AC on the control crosslinked gelatin microspheres and the crosslinked gelatin microspheres filled with the treated MWCNTs.

2. MATERIALS AND METHODS

2.1 Materials

Gelatin from bovine skin (type B, Bloom no. 175-225) was purchased from Sigma-Aldrich (USA). Multi-walled carbon nanotubes (MWCNTs) were obtained from Nanocyl from USA. Saturated glutaraldehyde aqueous solution (5.6 M) and nitric acid (70 wt%) were purchased from Fluka (Switzerland). Acetone (AR grade), chloroform (AR grade), and sulfuric acid (98 wt%) were purchased from Lab-Scan (Thailand). All other chemical agents were analytical grade and used without further purification.
2.2 Chemical Modification of MWCNTs

MWCNTs (1 g) were dispersed in 150 mL of a mixed acid solution of H₂SO₄ and HNO₃. The suspension was sonicated in an ultrasonic bath for 1 h at room temperature, followed by refluxing with magnetic stirring at 90 °C for 3 h. After cooling to room temperature, the suspension was diluted with deionized water until a neutral pH was obtained. The MWCNTs were isolated by vacuum filtration using a PTFE membrane. Finally, the treated MWCNTs were dried at 120 °C for 24 h in an oven and stored in desiccator until use.

2.3 Characterization of MWCNTs

2.3.1 Transmission electron microscopy (TEM)

TEM images of the unaltered and treated MWCNTs were obtained with a JEM-2100 TEM. All of the samples were sonicated in ethanol for 30 min, and one drop of each ethanol suspension was then transferred to a carbon-coated copper grid.

2.3.2 Surface acidic group determination

The Boehm titration was used for this study. The deionized water was prepared to disperse the carboxylated CNTs (100 mg) and place the solution into the sonicated bath for about 1 h. Then, the Na₂HCO₃ (0.01 M, 50 mL) was prepared and was added into the solution and being stirred for 24 h. Thus, the solution was filtered through 0.2 μm PTFE syringe filter and washed by water. The 0.01 M HCl was prepared to back titrate with the filtrate in triplicate to obtain the amount of carboxylated functional group in CNTs which were being calculated by analyzing that Na₂HCO₃ neutralizes carboxylated functional group. Neutralisation point was realized using pH indicator of methyl red solution [25].

2.3.3 IR analysis

The functional group contents of the unaltered and treated MWCNTs were determined using a Nicolet/Nexus 670 FT-IR spectrophotometer. All spectra were obtained from 400 to 4000 cm⁻¹ at a 4-cm⁻¹ resolution with 64 scans.

2.3.4 Thermal stability

The thermal stability of the pristine MWCNTs and treated MWCNTs were examined with a Perkin-Elmer TG-DTA. The system was operated under an O₂ atmosphere at a heating rate of 10 °C/min over a temperature range of 50 °C to 900 °C.

2.4 Microsphere Preparation

Gelatin microspheres filled with treated MWCNTs were prepared using a thermal gelation method. A 15% (w/v) gelatin stock solution was prepared by dissolving gelatin powder in distilled water at 40 °C. The treated MWCNTs (10 mg) were dispersed in 10 mL of deionized water and sonicated in an ultrasonic bath for 10 min. The dispersion of treated MWCNTs was then mixed with the gelatin stock solution. Asiaticoside (AC) was added to the solution, and the solution was stirred at 25±1 °C for 30 min. The solution was added to 200 mL of soya oil and stirred at 1,000 rpm for 10 min at 40 °C with a homogenizer to obtain a water-in-oil emulsion. The emulsion was cooled to 4 °C in an ice bath and stirred for 30 min to induce gelation of the gelatin. Then, 200 mL of acetone chilled at 4 °C was added to the emulsion, and the emulsion was stirred for 60 min. The microspheres were collected by filtration under vacuum, washed 3 times with chilled acetone to remove any residual oil, and freeze dried at room temperature overnight.
Microspheres were crosslinked by stirring at 500 rpm for 1 h at 4 °C in 10 mL of acetone-water (2:1, v/v) containing 1% (w/v) glutaraldehyde solution. The crosslinked microspheres were subsequently collected by vacuum filtration and washed with chilled acetone. To block the activity of unreacted glutaraldehyde, the crosslinked microspheres were agitated at 50 rpm for 1 h in 20 mL of 10 mM aqueous glycine solution containing 0.1 wt% Tween 80 in a 37 °C water bath. The crosslinked microspheres were again collected by filtration and washed with chilled acetone. The crosslinked microspheres were freeze dried at room temperature for 24 h and stored at 4 °C until use [26].

2.5 Characterization of the Microspheres
2.5.1 Morphological observations
The morphologies of the MWCNT-filled gelatin microspheres were determined using a JEOL JSM-5200 scanning electron microscopy (SEM) at 15 kV. The samples were placed onto a carbon-coated copper stub and then coated with gold using a JEOL JFC-1100 sputtering device.

2.5.2 Microsphere swelling
The swelling behavior of the control crosslinked gelatin microspheres and the crosslinked gelatin microspheres filled with the treated MWCNTs were examined by immersion in 10 mL of 10 mM aqueous glycine solution containing 0.15 M NaCl at 37 °C. After 24 h, the swollen microspheres were examined under a polarizing optical microscope (DMRXP, Leica) at 20x magnification equipped with UTHSCSA Image Tool software (version 3.0).

2.6 Sorption Experiments and Actual Loading of Asiaticoside in the Microspheres
Sorption was studied by adding 2.5 mg of the control crosslinked gelatin microspheres or crosslinked gelatin microspheres filled with the treated MWCNTs to a 21 mL AC solution in a 22-mL glass vial sealed with a stainless screw cap and incubated at 30 °C. The initial AC concentrations ranged from 0.001 to 0.500 mg/L. The pH of the solutions was maintained at a neutral level. After shaking for 24 h, UV-Vis spectroscopy was used to determine the time required to reach AC concentration equilibrium in the aqueous solutions. The sorption study of AC on the control crosslinked gelatin microspheres and the crosslinked gelatin microspheres filled with the treated MWCNTs were calculated by the following equation:

\[
\text{Sorption of AC on the microspheres (mg/g) at equilibrium} = (A_0 - A) \times \frac{V}{m} \quad (1)
\]

in which \(A_0\) is the initial AC concentration (mg/L); \(A\) is the AC equilibrium concentration (mg/L); \(V\) is the solution volume (L); and \(m\) is the mass of the microsphere dosage.

The maximum amount of AC captured in the control crosslinked gelatin microspheres and crosslinked gelatin microspheres filled with the treated MWCNTs was determined by sorption experiments because the sorption study was carried out, since, at the equilibrium state, that could determine the maximum amount of loaded AC in the microspheres. The AC encapsulating efficiency (EE) was calculated using the following equation:
Encapsulating Efficiency (%) = total mg AC encapsulated at equilibrium / initial mg AC loaded \( \times 100 \) (2)

2.7 **In vitro Release**

The release characteristics of AC from the control crosslinked gelatin microspheres and the crosslinked gelatin microspheres filled with the treated MWCNTs were measured by monitoring the concentration of AC in the supernatant. Each of the samples (1 mg) was incubated at 37 °C in 2 mL of phosphate-buffered saline solution (PBS) with 150 mM NaCl in a shaking water bath. At various time points between 0 and 24 h (1440 min), 500-μL aliquots of the sample solution supernatant were withdrawn, and an equal volume of fresh medium was added to maintain a constant medium volume. The AC amounts in the sample solutions were analyzed by high-performance liquid chromatography (HPLC) (Shimadzu LC-10 AD) on an Inertsil ODS-3 C18 column (particle size = 5 μm; column dimensions = 4.6 × 250 mm) with an Inertsil ODS-3 guard column (particle size = 5 μm; column dimensions = 4.0 × 10 mm) and a 1 mL min\(^{-1}\) flow rate. The absorbance of the samples was measured at 204 nm with a UV-Visible detector. The mobile phase was 26:24:50 v/v/v acetonitrile/methanol/distilled water. Prior to HPLC analysis, each of the samples (1 mg) was dissolved in 2 mL of 10% (v/v) phosphate-buffered saline solution (PBS). After 24 h, the suspensions were centrifuged for 5 min at 5,000 rpm, and the sample solutions were filtered through 0.45 μm nylon membrane filters; 50-μL aliquots of the filtered solutions were injected into the HPLC system. The retention time of AC was 7.5-7.7 min. The AC concentration was calculated from an AC calibration curve (0.0001 to 0.0010 mg/mL).

Releasing with time (%) = the value of releasing at any time point / encapsulation efficiency \( \times 100 \) (3)

2.8 **Cell Culture**

Murine fibroblasts (L929) were used to study the microsphere cytotoxicity. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, USA) supplemented with 10 vol% fetal bovine serum (FBS; Biochrom AG, Germany), 1 vol% L-glutamine (Invitrogen, USA), and 1 vol% antibiotic and antimycotic formulation (containing penicillin G sodium, streptomycin sulfate, and amphotericin B; Invitrogen, USA). The cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO\(_2\). The medium was replaced once every 2 d.

2.9 **Indirect Cytotoxicity Studies**

Cell cytotoxicity was analyzed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay is a colorimetric assay based on the ability of viable (i.e., metabolically active) cells to convert a yellow tetrazolium salt into purple formazan crystals. For this assay, the microspheres were sterilized by UV irradiation for 20 min and were then incubated in serum-free medium. During this incubation, the cells were plated at a density of 40,000 cells/well in 24-well culture plates containing DMEM with serum. After 24 h, incubation at 37 °C and 5% CO\(_2\), the cell medium was replaced with extraction medium for 24, 48, or 72 h. The extraction medium was then removed and replaced with 250 μL/well MTT solution (0.5 mg mL\(^{-1}\)). After 10 min of incubation at 37 °C and 5% CO\(_2\), the MTT solutions were replaced with 1 mL/well dimethylsulfoxide (DMSO). To determine cell
viability, the absorbance of each sample solution was measured at 570 nm in a Thermospectronic Genesis10 UV-visible spectrophotometer [27].

2.10 Statistical Analysis

One-way analysis of variance (ANOVA) and Scheffe’s post hoc test in SPSS (SPSS, USA) were used to analyze the data, which are expressed as the mean ± standard deviation. The 0.05 confidence level was used to determine statistical significance.

3. RESULTS AND DISCUSSION

3.1 Dispersion Stability, Surface Acidic Group Determination and Morphological Analysis of the MWCNTs

A dispersion of MWCNTs in deionized water was obtained by sonication. Untreated MWCNTs were insoluble and completely sedimented to the bottom in 1 h. After 5 d, most of the untreated MWCNTs had precipitated from the dispersion. By contrast, the dispersions of the treated MWCNTs exhibited improved stability in solution, as evidenced by the black and homogeneous dispersion shown in Figure 1. These results demonstrated that the introduction of polar, modified groups by treatment of the MWCNTs with a mixed acid solution of $\text{H}_2\text{SO}_4$ and $\text{HNO}_3$ increased the chemical affinity of the MWCNTs for water, improving and stabilizing its dispersion in water.

The surface acidic group of the treated MWCNTs was determined by titration. This study found that the mol of $\text{Na}_2\text{HCO}_3$ which added to treated MWCNTs dispersion and the mol of $\text{HCl}$ which used for the titration of filtrate were $5.0 \times 10^{-4}$ and, $1.5 \times 10^{-4}$ respectively. This result showed the end point at pH 8. The concentration of carboxylated functional group on the treated MWCNTs was $1.5 \times 10^{-3}$.

The morphologies of the untreated and treated MWCNTs were investigated by TEM. Figure 1 shows the TEM images of the untreated and treated MWCNTs. The untreated MWCNTs were entangled and aggregated because of intermolecular attractions, whereas treatment of the MWCNTs created defect sites that resulted in shorter tubes. The MWCNT treatment also linked the nanotubes and resulted in the removal of both residual metal catalyst and amorphous carbon. Based on the morphologies of the untreated and treated MWCNTs, we concluded that the $\text{H}_2\text{SO}_4$ and $\text{HNO}_3$ oxidative treatment led to a large number of carbon-containing defect sites where functionalization with oxygen-containing groups easily occurred.
3.2 IR Analysis of the MWCNTs

The FTIR spectra of the untreated and treated MWCNTs are presented in Figure 2. The ~3750 cm\(^{-1}\) peak corresponded to alcohol -OH stretching. The ~3450 cm\(^{-1}\) peak was attributed to carboxylic acid (-COOH) -OH stretching. The ~2380 cm\(^{-1}\) peak resulted from -OH stretching within strongly hydrogen bonded -COOH groups. The ~1620 cm\(^{-1}\) peak corresponded to COO\(^{-}\) asymmetric stretching, whereas the ~1150 cm\(^{-1}\) peak was assigned to alcohol C-O stretching. The results demonstrated that the treated MWCNTs contained more oxygen-containing functional groups than the untreated MWCNTs, indicating the presence of carboxylic groups in the treated MWCNTs.

Figure 1. Stability of untreated and treated MWCNT dispersions in deionized water (a); TEM image showing the morphology of the pristine (b); and TEM image showing the morphology of the treated MWCNTs (c).

Figure 2. FTIR spectra of pristine and treated MWCNTs.
3.3 Thermal Stability

The TGA curves of the pristine MWCNTs and treated MWCNTs are shown in Figure 3. The pristine MWCNTs are observed to be quite stable up to 500 °C and show little weight loss below 500 °C. A significant gasification of the MWCNTs begins at 500 °C and ends at 610 °C. The weight remaining after the process is 12 wt% for the pristine MWCNTs, which could be attributed to the remaining materials in the pristine MWCNTs, i.e., primarily the substrate and metal catalyst. The thermal stability of the treated MWCNTs is better than that of the pristine MWCNTs. The weight loss slightly decreased for the treated MWCNTs until the temperature was between 440 °C and 550 °C. Above 550 °C, a rapid decrease in weight started, and after the process was completed, the remaining weight was 1.7 wt% for the treated MWCNTs. This behavior for the treated MWCNTs indicates that the most of the metal catalyst from the synthesis of the MWCNTs was removed.

![Figure 3: TGA curves of the pristine MWCNTs and treated MWCNTs. The weight loss slightly decreased for the treated MWCNTs. This behavior for the treated MWCNTs indicates that the most of the metal catalyst from the synthesis of the MWCNTs was removed.](image)

3.4 Morphological Analysis of the Microspheres

SEM was used to investigate the morphologies of the treated MWCNT-filled gelatin microspheres containing AC. The morphologies of these microspheres are displayed in Figure 4. The selected images show that the microspheres are spherical in shape and possess smooth surfaces.
3.5 Microsphere Swelling Studies
The swelling behavior of the gelatin microspheres filled with treated MWCNTs was determined by measuring the microspheres before and after swelling in PBS at 37°C for 24 h to encapsulate the AC into the microspheres. The diameters of at least 100 freeze-dried and wet microspheres are apparent in Figure 5. Figure 5 shows that, the diameter of microspheres before and after swelling was $15 \pm 5.7$ and $22 \pm 6.5 \mu m$, respectively. The microspheres exhibited an ability to absorb water, and they increased in diameter as they became hydrated.

3.6 Sorption Experiments and Loading of Asiaticoside into the Microspheres
The sorption study of AC on the control gelatin and treated MWCNT-filled gelatin microspheres are shown in Figure 6. The results revealed that the AC adsorption capacities on both the gelatin and treated MWCNT-filled gelatin microspheres increased with increasing equilibrium concentration. The sorption capacities of AC on the control gelatin and treated MWCNT-filled gelatin microspheres were 115.22 and 230.11 mg/g, respectively, at 30°C (Table 1). Therefore, the sorption of the treated MWCNT-filled gelatin microspheres to AC was stronger than the
sorption of the control gelatin microspheres to AC. Due to the similarity in the chemical structures between CNT (benzene rings) and AC (triterpene), AC could readily be adsorbed onto the MWCNTs surfaces [28]. The incorporation of AC within the gelatin microspheres was enhanced by the presence of MWCNTs.

The amounts of asiaticoside loaded in the control gelatin microspheres and gelatin microspheres filled with treated MWCNTs is reported as a percentage (Table 1). The AC encapsulation efficiencies of the gelatin and treated MWCNT-filled gelatin microspheres were 30 and 65%, respectively. The treated MWCNT-filled gelatin microspheres exhibited a higher drug encapsulation percentage due to the unique properties of the treated MWCNTs, including their large surface areas, exible configurations, specic surface functionalization, and sorption of biological substances [29]. The treated MWCNTs were expected to promote encapsulation efficiency.

**Table 1.** Microsphere encapsulation efficiency of asiaticoside. In each group, the data are significantly different at $p < 0.05$. 

<table>
<thead>
<tr>
<th>Sample</th>
<th>Encapsulation efficiency of AC-loaded microspheres (%)</th>
<th>The sorption capacities of AC on the microspheres (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin Microspheres</td>
<td>30±3.9</td>
<td>115.22±7.1</td>
</tr>
<tr>
<td>Gelatin / Treated MWCNTs microspheres</td>
<td>65±4.7</td>
<td>230.11±9.5</td>
</tr>
</tbody>
</table>
3.7 *In vitro* Asiaticoside Release

The release of AC from the control gelatin and treated MWCNT-filled gelatin microspheres was evaluated by examining the cumulative amount of AC release relative to the loaded amounts. For the experiment, the loaded samples were immersed in PBS and incubated at 37 °C in a water bath, with constant shaking at 40 rpm. The AC release profiles of the control gelatin and treated MWCNT-filled gelatin microspheres exhibited two stages (Figure 7). The first stage was characterized by rapid AC release. This period was followed by a second stage of slower AC release. Notably, the treated MWCNT-filled gelatin microspheres released AC more slowly than the control gelatin microspheres. In addition, the total AC released from treated MWCNT-filled gelatin microspheres was approximately 96%, whereas only approximately 72% of the drug was released from the gelatin microspheres. Therefore, the MWCNTs were incorporated into the gelatin microsphere.

3.8 Cytotoxicity

The cytotoxicity of the control gelatin and treated MWCNT-filled gelatin microspheres was assessed by the cell viability of murine fibroblasts (L929). The MTT assay was used to measure the metabolic activity of the mouse fibroblasts after culturing for 24, 48, and 48 h in extraction media from the control gelatin and treated MWCNT-filled gelatin microspheres. The results in Figure 8 demonstrate that the microspheres and gelatin microspheres filled with the treated MWCNTs only reduced cell viability slightly compared with the control for 24 and 48 h. For 72 h, the cell viability was greater than the control. The non-toxicity of the materials was found when the cells viability was higher than 80%. These results can be demonstrated that the microspheres are biocompatible with the mouse fibroblasts.

![Figure 7](image.png)

*Figure 7.* Sorption experiments of different samples (n = 5) (gelatin dose: 2.5 mg/22.0 mL; gelatin/treated MWCNTs dose: 2.5 mg/22.0 mL; temperature: 30 °C; pH: 7.0; equilibrium time: 24 h.)
4. CONCLUSIONS

Gelatin microspheres filled with treated MWCNTs were successfully prepared using a thermal gelation method. The MWCNTs were chemically modified with mixed H₂SO₄/HNO₃. The chemical treatment removed residual metal catalysts and the amorphous carbon, and the treated MWCNTs exhibited an ability to disperse well in water. FTIR studies revealed that the treated MWCNTs possessed more oxygen-containing functional groups than the unaltered MWCNTs. The treated MWCNT-filled microspheres were able to encapsulate AC, and in vitro studies demonstrated that the treated MWCNT-filled gelatin microspheres released AC more slowly than the control gelatin microspheres. The sorption of the treated MWCNT-filled gelatin microspheres to AC was stronger than the sorption of the control gelatin microspheres to AC because the CNT and AC show similar chemical structures, i.e., benzene rings of CNT versus triterpene of AC. Consequently, AC could then be easily adsorbed onto the benzene rings of CNT. The control gelatin and treated MWCNT-filled gelatin microspheres prepared using the thermal gelation technique were non-toxic to murine fibroblasts.

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