Preparation and characterization of lectin-conjugated chitosan fluorescent nanoparticles

Jingping Liu [a], Lanlan Zhang [a], Chen Wang [b], Hongyan Xu *[c] and Xiaojun Zhao *[ad]

[a] Nanomedicine Laboratory, Center for Regenerative Medicine, West China Hospital, Sichuan University, Chengdu, 610041, P. R. China. E-mail: xiaojunz@mit.edu; Fax: +86-28-85164073; Tel: +86-28-85164069

[b] National Center of Nanoscience and Technology, Beijing 100080, P. R. China

[c] GL Biochem (Shanghai) Ltd., 519 Ziyue Rd., Shanghai 200241, P. R. China. E-mail: glsyn@mail.online.sh.cn; Fax: +86-21-61263300; Tel: +86-21–61263333/61263398. Web: www.glbiochem.com

[d] Center for Biomedical Engineering, NE47-379, Massachusetts Institute of Technology, Cambridge, MA 02139-4307, USA

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1.0 Experimental procedure

Preparation of biotinylated chitosan

Chitosan (Low molecular weight, Sigma, USA) was biotinylated using NHS-biotin (Merck, USA). In brief, the chitosan solution (3 mg/ml) was prepared by adding chitosan to 1% (w/v) acetic acid, mixed overnight, and then centrifugated (10000 rpm × 10 min) with a high-speed refrigerated centrifuge (Beckman, USA) to remove undissolved particles. The pH of the chitosan solution was adjusted to 6.0 with 1 M NaOH and the biotinylated reaction was initiated by adding 10 mM Biotin-NHS of DMSO (Sigma, USA) solution. The biotinylation reaction was proceeding overnight at 4℃ under magnetic stir. After complete reaction, the pH of biotinylated chitosan solution was adjusted to 8.0, and then sediments of biotinylated chitosan were collected by centrifugation. The biotinylated chitosan were excessively rinsed with PBS (PH=7.2), and then dissolved to the original volume with dilute acetic acid. The pH of the purified biotinylated chitosan solution was adjusted to 6.0 and stored at 4 ℃ for use.

Preparation of biotinylated PHA

Biotinylated lectin of phytohemagglutinin (PHA, Sigma, USA) was also prepared by NHS-biotin. Appropriate volume of NHS-biotin DMSO solution was added to PHA solution in PBS with a concentration of 1 mg/ml, and the biotinylation reaction was allowed to proceed overnight at 4 ℃. Thereafter, the biotinylated solution was transferred to an ultrafiltration centrifuge tube (Microncon YM-3, Millipore, USA), washed repeatedly with PBS and centrifuged to remove the excess biotin molecules. The purified biotinylated PHA was collected and stored at 4□.

Evaluation of biotinylated reaction

The evaluation of biotinylated reaction was carried out with biotin quantization kit (Pierce, USA) according to the manufacture's instructions. 100 μl of ultrapure water was added to one microtube of HABA/avidin premix and mixed; in a microplate well, 20 μl of HABA/avidin premix solution was added to 160 μl of PBS and mixed with a shaker. The absorbance value at 500 nm (A_{500}) was measured and recorded, and after 20 μl of biotinylated sample was added, the A_{500} was measured and recorded again. Each sample was measured 3 times to average.

Preparation of PHA–conjugated chitosan

PHA–conjugated chitosan was prepared in biotin-avidin method. In brief, the biotinylated chitosan solution, biotinylated PHA solution and avidin (Merck, USA) solution were mixed with a mole ratio of 1:1:1, the pH of the reaction solution was adjusted to 6.0, and the reaction proceeded overnight at 4 ℃ under magnetic stir. After reaction, the pH of the solution was adjusted to 8.0. PHA–conjugated chitosan was collected by centrifugation and washed repeatedly with PBS to remove excess lectin and avidin. Purified PHA–conjugated chitosan was dissolved with dilute acetic acid, of which the pH was adjusted to 6.0, and stored at 4 ℃.

Preparation of FITC-labeled PHA-conjugated chitosan

The FITC-labeled reaction was initiated by adding 10 mg/ml FITC solution in DMSO to lectin-conjugated chitosan solution, and the volume ratio of FITC solution and PHA–conjugated chitosan solution was 1:10. The reaction tube was fully wrapped with tinfoil; the reaction was allowed to proceed overnight at 4℃. After reaction, the pH of reaction solution was adjusted to 8.0. FITC-labeled PHA–conjugated chitosan was collected by centrifugation, and excessively washed with PBS. The purified FITC-labeled PHA–conjugated chitosan was dissolved in dilute acetic acid solution to the original volume, of which the pH was adjusted to 6.0, and stored at 4 ℃ for use.

Preparation of PHA-conjugated fluorescent nanoparticles

The PHA-conjugated fluorescent nanoparticles were prepared by ionic crosslinking with sodium tripolyphosphate (TPP, Sigma, USA). In brief, 10 μl of Tween-20 (Sigma, USA) was evenly mixed with 5ml FITC-labeled PHA–conjugated chitosan solution (equivalent to 3 mg/ml chitosan solution), and then 2 ml of 1.5 mg/ml TPP solution was added dropwise to chitosan solution under magnetic stir. Then, nanoparticles were collected by centrifugation, washed repeatedly with acetate buffer, and finally suspended in acetate buffer.

Atomic force microscope (AFM) observation

AFM samples were prepared as follows: 10 μl of diluted nanoparticles suspension was evenly placed on a freshly cleaved mica substrate, the surface was repeatedly rinsed with Milli-Q water (Millipore, USA) and air-dried at room temperature. AFM measurement was performed on a SPI4000 Probe Station (Seiko Instruments Inc., Chiba, Japan) with tapping mode at room temperature. The typical scan parameters were set as: scan speed 1.0 Hz, amplitude 1-1.2 V, integral and proportional gains 0.3–0.4 and 0.03–0.04, respectively. The results were recorded with a resolution of 512 × 512 pixels and analyzed by SPI3800 software.

Haemagglutination test

The bioactivity of PHA coupled to chitosan nanoparticles was assessed by haemagglutination test. Two-fold serial dilution of 25 μl sample solution was made in a 96-well V-bottom microplate, and each sample was finally diluted to 12 wells. The same volume of 2% (v/v) suspension of fresh rat erythrocytes was then added to each well and incubated at room temperature for 2 hours. The result of the test was evaluated by visual inspection. If erythrocytes agglutination was induced by lectin, a shape of diffuse red color layer would be seen, whereas in the case of a negative result, a bright red spot would form because of the sinking of non-agglutinated erythrocytes in the central V-bottom of the well. The procedure was conducted in triplicate. The bioactivity of PHA was evaluated by comparing the agglutination extent of erythrocytes, while PHA solution was set as positive control, non-conjugated nanoparticles was set as negative control.

Fluorescence microscope imaging

Fluorescent imaging was performed on an inverted fluorescence microscope (Olympus, Japan). 200 μl of diluted FITC-labeled PHA–conjugated chitosan nanoparticle suspension was added in a microplate well. The images was observed

and captured under excitation of mercury lamp.

Fluorescence stability analysis

The fluorescence analysis was performed on a fluorescencespectrophotometer (F-7000, Hitachi, Japan). Exposed under light for different preset time , the fluorescence intensity of pure FITC solution and diluted fluorescent nanoparticle suspension was measured at 520 nm emission with 490 nm excitation. The maximal excitation and emission wavelength for pure FITC and PHA-conjugated fluorescent nanoparticles was maintained the same. The variation of fluorescence intensity was calculated from the ratio of fluorescence intensity at different time and the initial fluorescence intensity.

2.0 HABA test of a serial concentration of biotin

In this test, biotinylated HRP (1.0 moles biotin/mole HRP, Pierce, USA) was used as a positive control. The biotinylated HRP was dissolved in Milli-Q water and diluted to 0, 5, 10, 15, 20, and 25 nmol/mL, and the HABA test was conducted as described above. The results showed the A_{500} of biotinylated chitosan and PHA was in the range of positive data, which could help to illustrate the significant difference between non-biotinylated and biotinylated groups.

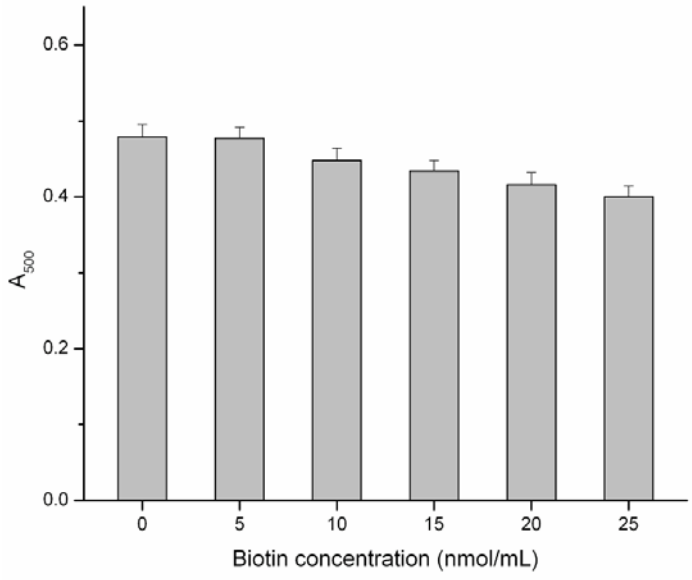

Figure 1. The A_{500} of the avidin-HABA complex, which decreased as the biotin concentration was changed from 0 to 25 nM.