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Notes

Synthesis and Characterization of Iron Oxide Nanoparticles Decorated with Carboxymethyl Curdlan

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Introduction

Recently, superparamagnetic iron oxide nanoparticles (SPION) have been extensively studied and used for applications such as magnetic resonance imaging, magnetic drug delivery, magnetofection, and bioseparation.[1] However, bare SPION have many problems to resolve for these biological applications. Their main problems by the hydrophobic surface may be summarized as follows. SPION are readily precipitated and aggregated in an aqueous medium. When they are injected into the body, SPION are rapidly cleaned from bloodstream by reticuloendothelial system (RES).[2] In addition, the SPION show cytotoxic effects on cell viability. Fortunately, the hindrance to biological applications can be removed if SPION have sufficient hydrophilicity through the surface modification. These surface characteristics can be accomplished with formation of a surface layer using hydrophilic polymers such as dextran, pullulan, starch, albumin, silicones, alginate and poly(ethylene glycol) (PEG).[3] In a recent publication, Gupta *et al.* suggested that the modification of SPION with pullulan reduced the cytotoxicity and enhanced the cellular uptake of the nanoparticles.[4] Ma, *et al.* reported that SPION stabilized by alginate were very stable in size and zeta-potential at pH 7.0 solution for 12 months.[5] Like this, SPION stabilized with a polysaccharide show good stability in an aqueous solution and cytotoxicity suppression effect.

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Curdlan is a beta-1,3-glucan polysaccharide derived from *Alcaligenes faecalis*. It has been used for a gelling agent in food, a carrier for drug delivery, and an anti-coagulant.[6] In addition, curdlan has anti-tumorigenicity, anti-infective activities against bacterial, fungal, viral, and wound repair.[7] Even though curdlan has a little solubility in water, carboxymethylated curdlan (CMC) has good water solubility as well as good bioactivity.[8] In this paper, we attempted the synthesis of the SPION decorated with CMC. The modification of SPION using CMC may improve their solubility in water and prevent the aggregation between each particles. In the paper, it is reported that a COO^- of alginate bound to iron ion on the surface of the SPION, resulting in electrostatic repulsion between particles, thus improvement of water stability. We speculated that CMC used in this study is available for coating SPION.

Experimental

Materials. Curdlan (Mw=9×10^4) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Ferric chloride hexahydrate ($FeCl_3\cdot6H_2O$), ferrous chloride tetrahydrate ($FeCl_2\cdot4H_2O$), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and XTT assay kit were purchased from Sigma-Aldrich (St. Luois, MO, USA). All chemicals were used without further purification.

Synthesis of CMC. CMC was synthesized according to the previous method with some modifications.[8] In briefly, 1 g of curdlan was suspended in 40 mL of isopropyl alcohol and was stirred for 30 min at room temperature. Four milliliter of NaOH solution (10 M) was added to the solution. After strongly stirring for 1 h, monochloroacetic acid (1.8 g) was added into the mixture. The final solution was stirred for 3 h at 50 °C and then neutralized with acetic acid. After washing with aqueous methanol solution and dialysis against water for 3 days, the solid product was freeze-dried. The synthesized CMC was characterized by the FT-IR and ^1H NMR.

Preparation of CMC-Coated SPION. SPION were synthesized by a coprecipition method.[9] Ferric chloride hexahydrate ($FeCl_3\cdot6H_2O$, 0.5 g) and ferrous chloride tetrahydrate ($FeCl_2\cdot4H_2O$, 0.184 g) were dissolved in 30 mL of deoxygenated distilled water under N_2 streaming. Ammonium solution (7.5 mL) was added to the solution under stirring vigorously. The pH of the reaction solution changed from strong acid to alkaline about 10. At this time, the color in the solution changed also from orange to dark black. The reaction mixture was heated at 80 °C for 30 min. To obtain the SPION, the final solution was placed on an external magnetic field, using a permanent magnet. After several minutes, the black particles sank down toward the magnet. The supernatant was discarded and the particles were

washed with distilled water. To introduce CMC to the surface of the SPION, 50 mg of SPION were suspended in 10 mL of PBS. After adding 25 mg of EDC, the solution was ultrasonicated for 10 min. One milliliter of CMC solution (25 mg/mL in PBS) was added to the reaction mixture and then ultrasonication was carried out for 1 h. The final product was collected with the magnet and then washed with distilled water. The morphology of the CMC-coated SPION was characterized with a transmittance electron microscope (H-7650, Hitachi Ltd., Jeonju, Center for Chonbuk National University-Wide Research Facilities) and the size distribution was determined using a particle size analyzer (UPA-150, Microtrac, Jeonju, KBSI). Thermogravimetric analysis (TGA) was performed using a Perkin-Elmer TGA-7 instrument.

Determination of T_2 Relaxation Rate. T_2 relaxivity of the CMC-coated SPION was measured, using a clinical 1.5 T MRI scanner (GE Signa Exite Twin-speed, GE Health Care, Milwaukee, WI, USA). The T_2-weighted scans were performed as following; 2,400 ms of repetition time (TR), echo time (TE) of range from 20 to 200 ms. The relaxation rate was calculated through the least-squares curve fitting of relaxation time versus iron concentration.

***In Vitro* Cytotoxicity Study.** The cytotoxicity assay of the CMC-coated SPION was performed with a XTT method, using a B16F10 cell line (breast cancer cell line). The cells were seeded in a 96-well plate at density of 1×10^4 cells/well in 100 μL of medium. The plate was incubated overnight at 37 °C. The medium solution containing the CMC-coated SPION with different concentration was added to the wells. After incubation for 24 or 48 h, the medium in the well was removed and the cells were washed twice with fresh phosphate-buffered saline (PBS). After adding XTT solution to the wells, the viability of the cells was determined by measuring UV absorption at 450 nm on a plate reader.

Results and Discussion

To introduce curdlan on the surface of the SPION, carboxymethyl groups were chemically introduced to C-6 of curdlan (Figure 1). The chemical modification of curdlan was confirmed by a FT-IR analysis. Figure 2 shows the FT-IR spectra of curdlan and CMC. In the CMC spectrum, the absorption band at 1589 cm^{-1} is attributed to the asymmetrical COO^- stretching vibration. The peak at 1334 cm^{-1} indicates the symmetrical COO^- stretching vibration. These assignments demonstrate that the carboxylmethyl groups were successfully introduced to curdlan. It was determined that the degree of substitution was 0.68 units of carboxymethyl group per on glucose unit of curdlan by ^1H NMR.

A common magnetic iron oxide nanoparticle can be dispersed into suitable solvent, whereas it is precipitated in water. Thus, it is necessary to modify the surface of the nanoparticles for biomedical applications. As shown in

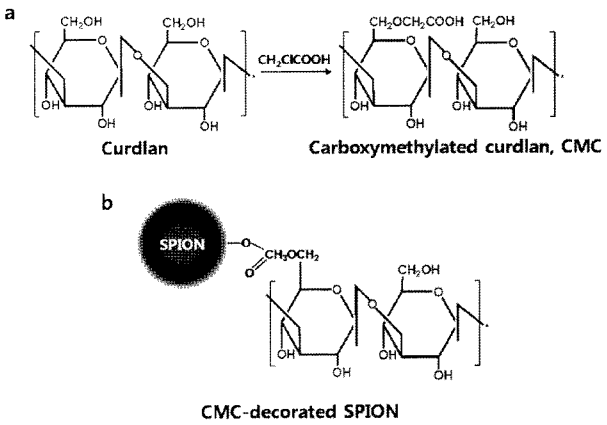

Figure 1. Schematic illustration of the synthesis of carboxymethylated curdlan, CMC (a) and the surface modification of SPION with CMC (b).

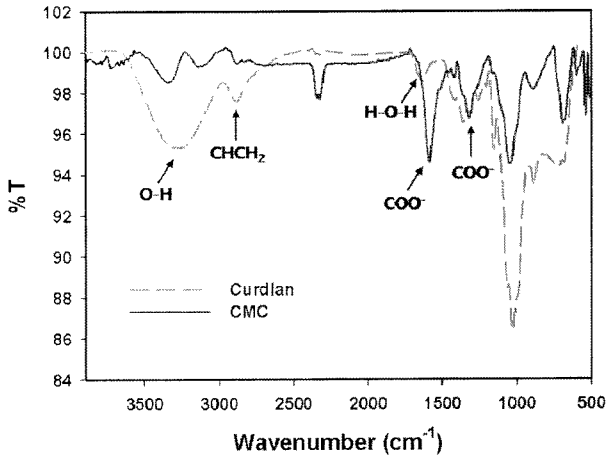

Figure 2. FT-IR spectra for curdlan (dot line) and carboxymethylated curdlan, CMC (solid line).

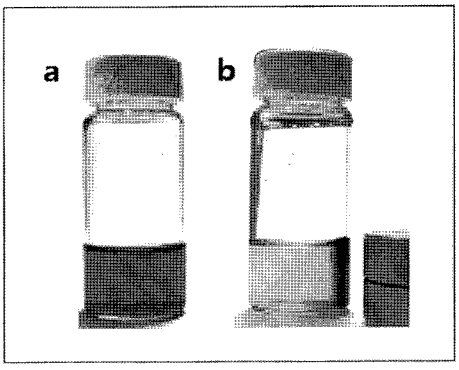

Figure 3. Photographs of the CMC-coated SPION dispersed in water (a) and the CMC-coated SPION dragged by magnetic force (b).

Figure 3, the CMC-coated SPION were well dispersed into water without any aggregation and precipitation.

The size distribution and the TEM image of the CMC-coated SPION were shown in Figure 4. The mean diameter

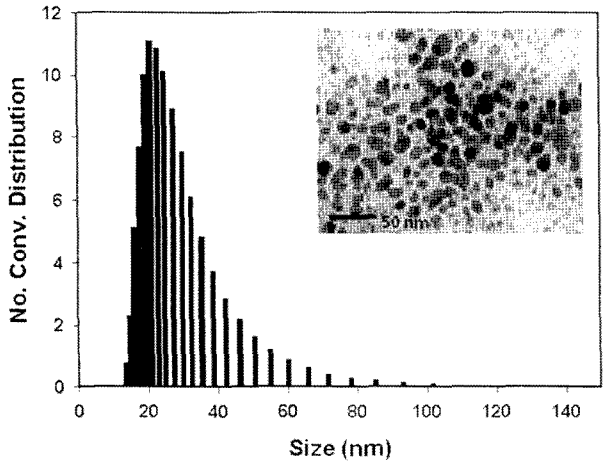

Figure 4. Size distribution of CMC-coated SPION. The inserted photograph is a TEM image for CMC-coated SPION. The scale bar is 50 nm.

of the CMC-coated SPION, determined by a dynamic light scattering analysis was approximately 23.8 nm. The shape of the CMC-coated SPION was relatively spherical and uniform. Furthermore, TGA revealed that the CMC account for approximately 13% (w/w) of the CMC-coated SPION.

Magnetic resonance sensitivity of the CMC-coated SPION was measured using a clinical 1.5 T magnetic resonance imaging (MRI) scanner (GE Signa Exite Twin-speed, GE Health Care, Milwaukee, WI). The T_2 relaxivites of the CMC-coated SPION were linearly increased as the concentration of Fe was increased (Figure 5). The T_2 weighted images of the CMC-coated SPION showed dark intensity with dependent manner to Fe concentration. Dextran-coated SPION in clinical trials have the T_2 relaxivity value of 30-50 $mM^{-1}S^{-1}$. In contrast, the CMC-coated SPION showed a T_2

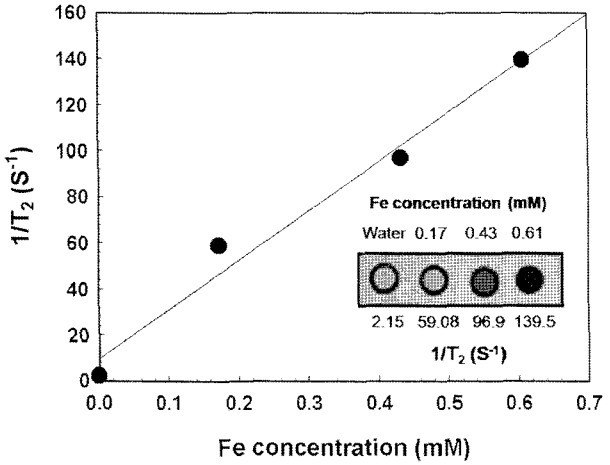

Figure 5. T_2 relaxation rate ($1/T_2$, S^{-1}) versus iron concentration (mM) in the CMC-coated SPION on a 1.5 T clinical MR scanner. The inserted photographs are T_2-weighted MR images of the CMC-coated SPION solution in water.

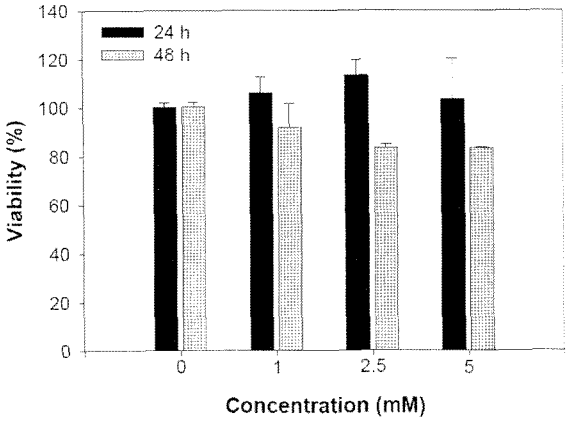

Figure 6. Cytotoxicity of the CMC-coated SPION to B16F10 cells during 48 h.

relaxivity coefficient of 223.5 $mM^{-1}S^{-1}$. From MR studies, it was confirmed that the CMC-coated SPION have superparamagnetic behaviors. T_2 relaxation times of iron oxide nanoparticles are changed by synthesis conditions such as heating temperature and reaction time. Ma *et al.* reported that T_2 relaxation time of the SPION stabilized by alginate was 281.2 $mM^{-1}S^{-1}$.[5]

To examine the cytotoxicity of the CMC-coated SPION, we investigated the affect of the CMC-coated SPION on the cell viability, using the B16F10 cell line. Figure 6 shows the cell viability after incubation with different concentration of the CMC-coated SPION. After incubation for 24 h, the CMC-coated SPION show no toxicity at high concentration of the SPION. On the other hand, after 48 h, when the concentration of the CMC-coated SPION was higher than 1 mM, the cell viability was reduced about 81.03%. Previous reports demonstrate that the presence of iron oxide nanoparticles in cells can results in significant changes behavior and viability of cells.[4] In the paper, Gupta, *et al.* reported that bare SPION caused a significant reduction of 80% against control cells in cell viability at the lowest concentration of 0.05 mg/mL. Consequently, it is concluded that the coating of SPION with CMC suppressed their cytotoxicity to cell viability.

Conclusions

We have synthesized the SPION coated with CMC and investigated the stability in water, T_2 relaxation rate and cytotoxicity. The coating with CMC improved the stability in water and reduced the cytotoxicity to cell viability. Furthermore, the CMC-coated SPION showed superparamagnetic behaviors. The CMC-coated SPION may be used for cellular and *in vivo* imaging. When CMC are chemically modified for conjugation of specific ligand, the CMC-coated SPION may have a great potential for biomedical applications.

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