Preparation of Anti-CD4 monoclonal antibody-conjugated Magnetic Poly (glycidyl methacrylate) Microspheres for CD4+ Lymphocyte Separation

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ABSTRACT

Novel immunomagnetic microspheres have been prepared for separation of $CD4^+$ lymphocytes. The magnetic nanoparticles with a diameter of approximately 7-8 nm were first synthesized by co-precipitation from ferrous and ferric iron solutions and subsequently entrapped within poly (glycidyl methacrylate) (PGMA) microspheres by a swelling and penetration process. Monoclonal antibody specific to CD4 molecules expressed on $CD4^+$ lymphocytes was conjugated to the surface of magnetic PGMA microspheres through covalent bonding between epoxide functional groups on the microspheres surface and primary amine groups of the antibodies. Flow cytometer was used to confirm the presence of antibody on the surface of microspheres. The new immunomagnetic microspheres are promising to be used in a various biomedical applications including diagnosis, and monitoring of human diseases.

Keywords: immunomagnetic microspheres, poly (glycidyl methacrylate), anti-CD4 monoclonal antibody, swelling and penetration process

1 INTRODUCTION

Recently, magnetic separation technique has received much attention since it provides many advantages over other methods as it is less time consumable and cost-effective [1-3]. The two key magnetic components of such systems are the magnetic particles used in the separation of the biological entities, and the magnetic field used to separate them [4]. For magnetic particles, monodispersity, high magnetization, stability in physiological salinity, and affinity targeting of magnetic particles are crucial [5]. Since the naked magnetic particles are sensitive to air and aggregate easily due to their magneto-dipole interparticle interaction, surface-protective layers are required to protect them from oxidation and to magnetically isolate individual particles. In particular, polymer shell provides both steric and electrostatic stabilization in physiological medium and protects the leakage of magnetic particles. Moreover, polymers with abundant functional groups, e.g. amine, carboxylate, and thiol groups, are of great interest as they can covalently conjugate with protein of interest.

In this paper, we report on the preparation of immunomagnetic microspheres for separation of $CD4^+$ lymphocytes without the need for column separation technique.

2 EXPERIMENTAL

2.1 Preparation of magnetic polymer microspheres

Iron oxide nanoparticles (IO) coated with oleic acid were synthesized by a coprecipitation method. PGMA particles were prepared by precipitation polymerization of GMA monomer in acetonitrile medium. To prepare IO-entrapped PGMA microspheres, 0.4 mL of IO (2 mg/mL) was mixed with 30 mL of chloroform. One gram of PGMA microspheres was added to the above solution, mixed, and incubated at room temperature overnight. The resulted particles were purified by decantation and concentrated at $1x10^7$ bead/mL for subsequent experiments.

2.2 Characterization

Morphology and particle size of IO were observed with a transmission electron microscope (TEM, JEM-2010, JEOL). The size and morphology of IO-entrapped PGMA particles were observed with scanning electron microscope (SEM, S-2500, Hitachi, Japan). The magnetic properties were investigated by using room temperature vibrating sample magnetometer (VSM, Lakeshore 7400, Lakeshore). Flow cytometry analysis was performed on a FACSort flow cytometer (Becton Dickinson, San Jose, CA).

2.3 Anti-CD4 monoclonal antibody conjugation

An anti-CD4 monoclonal antibody (mAb), named MT4 (IgM isotype), specific recognized CD4 molecules expressed on $CD4^+$ lymphocytes was used in this study. Production and purification of mAb MT4 were described elsewhere [6]. IO-entrapped PGMA particles were washed 3 times with 0.1 M phosphate buffer pH 7.4 (PB) and decanted. The particles were then adjusted to $1x10^6$ particles with PB. Purified mAb MT4 (20 µg) was added to 500 µL particle suspension. The mixtures were incubated at

for 6 hour. Then, the residual active epoxide functional groups on the surface of particle were blocked with bovine serum albumin. Afterward, the particles were washed 3 times to remove unbound mAb MT4.

2.4 Evaluation of the mAb MT4 conjugated immunomagnetic microspheres

To determine the presence of mAb MT4 on the immunomagnetic PGMA microspheres, the MT4 coated immunomagnetic microspheres (50 µL) were mixed with 50 µL of PE-conjugated goat anti-mouse IgM antibodies (Beckman Coulter Inc., Brea, CA, USA). The mixture was incubated at room temperature for 30 min in dark. Thereafter, the immunomagnetic PGMA microspheres were magnetically washed with the PBS. The fluorescence of the particles was evaluated by a flow cytometer.

3 RESULTS AND DISCUSSION

3.1 Preparation of magnetic polymer microspheres

The iron oxide nanoparticles were synthesized from the coprecipitation of Fe^{2+} and Fe^{3+} ions in an aqueous solution upon the addition of ammonium hydroxide [7]. The size distribution was found to be narrow and an average size of 7-8 nm, as shown in Fig. 1. The synthesized IO revealed a superparamagnetic behavior, as evidenced by zero coercivity and remanence on the magnetization loops. The saturated magnetization (Ms) of IO was about 54 emu/g, which is comparable to IO produced by other methods [8, 9].

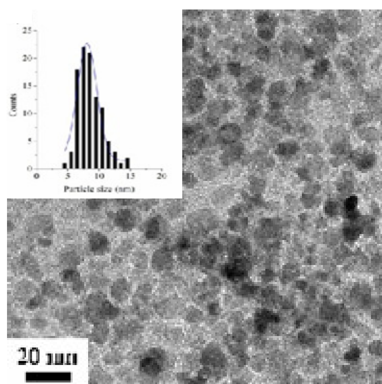

Figure 1: TEM analysis of iron oxide nanoparticles.

The magnetic polymer microspheres were first synthesized by a swelling and penetration process. Fig. 2. shows the scanning electron microscope (SEM) micrographs of of the original PGMA and IO-entrapped PGMA microspheres. Apparently, the original PGMA microspheres had spherical shape with uniform size distribution (Fig. 2a). The average diameter was around 3 µm. Entrapment of IO resulted in the increase of surface roughness since large amount of IO were buried within and coated on the polymer matrix. In comparison with the polymer microspheres, the magnetic polymer microspheres had the same sherical shape and good monodispersity (Fig. 2b).

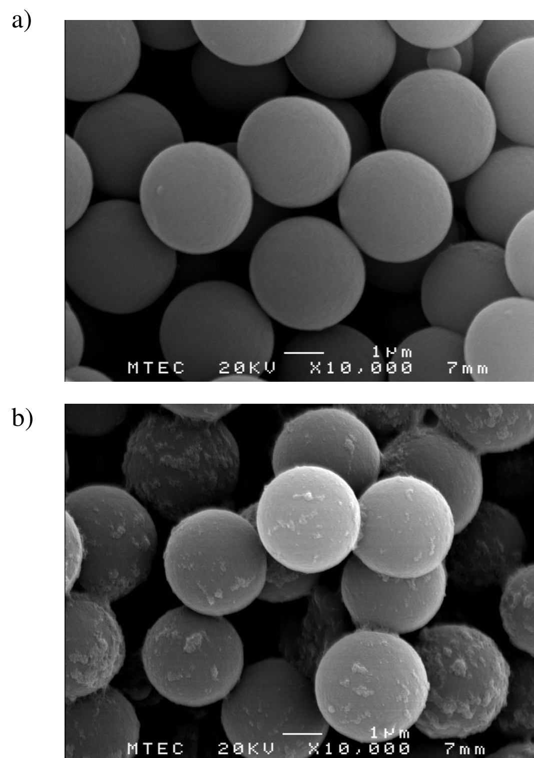

Figure 2: SEM images showing morphology and size distribution of (a) PGMA microspheres, and (b) IO-entrapped PGMA microspheres.

These IO-entrapped PGMA microparticles possessed excellent magnetic responsivity and redispersibility (Fig. 3), which was an important property needed for bioseparation.

Figure 3: Separation of IO-entrapped PGMA microparticles using permanent magnet.

3.2 Anti-CD4 monoclonal antibody conjugation

The mAb MT4 was attached to the surface of IO-entrapped PGMA microspheres through covalent bonding between epoxide functional groups on the surface of particle and primary amine groups of antibody.

To monitor the presence of mAb MT4 on the surface of immunomagnetic PGMA microspheres, the as-prepared microspheres were stained with PE-labeled anti-mouse IgM antibody (PE-αIgM) and analyzed by flow cytometry as shown in Fig. 4. For comparison, unmodified IO-entrapped PGMA microspheres were investigated as negative control. The immunomagnetic PGMA microspheres showed strong fluorescent signal, while that of unmodified particles had very low fluorescence signal. The fluorescence signal attributes to specific reaction between anti-IgM conjugate and the IgM molecules bound on PGMA microspheres surface. These data indicated that mAb MT4 was successfully functionalized on the surface of the magnetic PGMA particles, without the signs of denaturation or degradation.

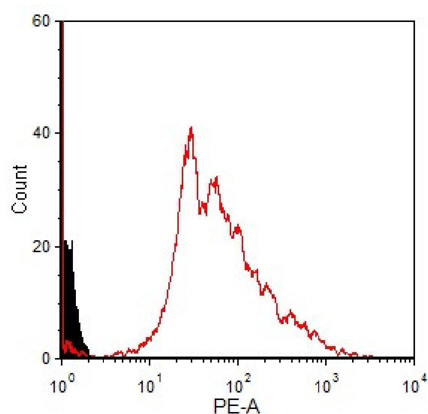

Figure 4: Evaluation of the conjugation of anti-CD4 monoclonl antibody (mAb MT4) to the magnetic microspheres by immunofluorescent staining and flow cytometry.

4 CONCLUSIONS

Magnetic PGMA microspheres were successfully prepared through a two-stage reaction: (1) preparation of iron oxide nanoparticles, and (2) synthesis of the iron oxide-encapsulated PGMA microspheres via the swelling and penetration process. Monoclonal antibody specific with CD4 molecules expressed on $CD4^+$ lymphocytes was conjugated on magnetic PGMA microspheres surface through covalent bonding between epoxide functional groups on the surface of particle and primary amine groups of antibody. Therefore, the generated immunomagnetic PGMA microspheres can be useful for $CD4^+$ lymphocytes separation which may be applied to various biomedical applications including treatment, diagnosis, and monitoring of diseases.

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