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Journal of Chromatography B, 833 (2006) 236–244
JOURNAL OF CHROMATOGRAPHY B
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Preparation and characterization of magnetic microspheres for the purification of interferon α-2b

Yu Cao [a], Gang Bai [a,*], Jiaqi Chen [a], Wang Tian [a], Shenqi Wang [b], Wenbo Yang [a]

[a] *Department of Microbiology, College of Life Sciences, Nankai University, Tianjin 300071, China*
[b] *Key Laboratory of Bioactive Materials, Ministry of Education, Nankai University, Tianjin 300071, China*

Received 27 September 2005; accepted 2 February 2006
Available online 21 February 2006

Abstract

Magnetic agarose microspheres (MAMS), magnetic cellulose microspheres (MCMS), and magnetic poly(vinyl alcohol) microspheres (MPVAMS) were prepared by various different preparation methods. MCMS coupled with anti-IFN α-2b monoclonal antibodies (mAb) were selected for the purification of interferon α-2b (IFN α-2b) after performance characterization among microspheres. Parameters of immunomagnetic separation (IMS), including binding mAb, elution behavior, and sample pretreatment conditions, were optimized to improve the purification efficiency of the separation of IFN α-2b by MCMS. Size-exclusion HPLC (HPSEC) showed that the IFN α-2b was purified from crude cell lysate had an overall purity of 92.9%, while immunological and biological assays showed an activity recovery of 88.5% and specific antiviral activity of 2.7×10^8 IU/mg. Identity and molecular mass of purified IFN α-2b were confirmed by western blot and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) analysis. This study illustrated the favorable separation media which combined desired properties for the development of magnetic separation of biological materials.

Keywords: Agarose microspheres; Cellulose microspheres; PVA microspheres; Immunomagnetic separation; Interferon α-2b

1. Introduction

Magnetic microspheres (MMS), since introduced in the 1970s, have been benefiting a broad variety of applications in bioscience and biotechnology [1,2]. MMS are used as the easily separable support material that can be easily removed from the reaction medium and stabilized in a fluidized bed reactor by applying a magnetic field. The use of MMS reduces both fixed and operational costs of purification procedures [3]. The initial work featured the incorporation of a ferrofluid into a polymer, such as polyacrylates [4], polyacrolein [5], and polyglutaraldehyde [6]. Those microspheres were designed for cell labeling and biological detection. Ugelstad et al proposed an elaborate technology that was commercialized [7], but high cost prevents it from large-scale industrial application. Compared to previously described MMS, preparing MMS synthesized from biomaterials overcomes the cost problem and has good biocompatibility regarding the separation of medicine and biological materials.

Immunomagnetic separation (IMS), which utilizes MMS as the basis for separation, takes advantage of selectivity of affinity chromatography with high availability and efficiency of magnetic response, because MMS can be easily collected when a magnetic field is applied [8,9]. When coupled with appropriate ligands such as antigens or antibodies, they provide an effective tool for achieving rapid, simple and specific target protein separation even in large-scale preparation [10,11]. Immunomagnetic microspheres (IMMS) have also been applied to cell sorting, radio immunoassay, enzyme immunoassay, pathogen detection and nucleic acid purification [12–15].

Current methods for the isolation and purification of interferon (IFN), including gel filtration chromatography, ion-exchange chromatography, hydrophobic chromatography, and affinity chromatography, either provided high purity but low recovery of activity of IFN, or involved troublesome procedures [16–18]. In this study, a rapid and efficient IMS method was established and evaluated for the purification of IFN α-2b from crude cell lysate. We compared the preparation process and performance of magnetic agarose microspheres (MAMS), magnetic cellulose microspheres (MCMS), and magnetic poly(vinyl alcohol) microspheres (MPVAMS) in which Fe_3O_4 colloid was

* Corresponding author. Tel.: +86 22 23508371; fax: +86 22 23508371.
E-mail address: gangbai@nankai.edu.cn (G. Bai).

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doi:10.1016/j.jchromb.2006.02.001

incorporated. Then MCMS coupled with anti-IFN α-2b monoclonal antibodies (mAb) were used for the isolation of IFN α-2b with good characteristic properties.

In previous papers, MCMS were prepared for immobilization with enzymes for the enzymatic oxidation of substrates [19,20] and medical and technical immunoassay for the clinical samples [21,22]. In this study, a new chromatographic matrix is prepared by coupling mAb to the MCMS, which displayed a new use for affinity separation of biological materials with highly favorable biocompatibility and preferred efficiency. We optimized separation parameters and minimized cumbersome steps in order to make MCMS a practical material in protein purification.

2. Experimental

2.1. Materials

Ferrous chloride ($FeCl_2{\cdot}4H_2O$) (Analytical Grade, Merck, Germany), polyethylene glycol (PEG) (M_w 4000), agarose (Analytical Grade, TBD, USA), glutaraldehyde (EM Grade, Merck, Germany, 25% w/w) and PVA-1788 (M_r 75,000–79,000, 88% hydrolyzed), were purchased from Tianjin Chemical Agent Co. Ltd. (Tianjin, China). Purified cotton was obtained from a local store in Tianjin. Gasoline 200# was supplied from Chemical Plant of Nankai University (Tianjin, China). *O*-phenylenediamine (OPD, BR1965), diaminobenzidine (DAB) (Sigma, USA) and bovine serum albumin (BSA) (Electrophoresis Grade, Sigma, USA) were purchased from TBD Bio. Co. Ltd. (Tianjin, China). Mouse IgG antibodies for standards and control were purchased from Sino-American Biotechnology Co. Ltd. (Luoyang, China). All other reagents were of analytical grade. *Pseudomonas* sp. strain VG-84 crude cell lysate, recombinant human IFN α-2b standard reference and commercially available Biotin Avidin System-ELISA (BAS-ELISA) kit were kindly provided by Tianjin Hualida Bioengineering Co. Ltd. (Tianjin, China).

Anti-IFN α-2b monoclonal antibodies (mAb) were purchased from Anhui Anke Biotechnology Co. Ltd. (Hefei, China). The mAb were purified from the ascitus fluid by combined octanoic acid-ammonium sulfate precipitation with an overall purity of 95%. The isotypes of mAbs were all of IgG1 subclass.

2.2. Magnetic separator

A flow-through magnetic separator was prepared by importing and embedding two permanent magnets into a plastic rack. A 20-mL glass syringe with a plug for feeding and a plastic tube on the other end for down-flowing was used as a vessel for IMS. The rack has removable magnetic plate to ease the washing of separated magnetic microspheres.

2.3. Synthesis of ferrofluids

Ferrofluids containing super paramagnetic Fe_3O_4 nanoparticles were prepared by precipitation–oxidation method [23]. A mixture of 150 g of PEG (M_w 4000) and 6 g of $FeCl_2{\cdot}4H_2O$ was dissolved in 400 mL of deionized water. A 20-mL aqueous solution containing 0.3 mL of 30% H_2O_2 was heated at 50 °C for 6 h, and then adjusted to pH 13 with 3 M NaOH with stirring (300 rpm). The product was washed with water under nitrogen, and then transferred into ethanol by centrifugation and decantation.

2.4. Preparation of magnetic microspheres

2.4.1. Preparation of MAMS

MAMS were prepared according to Ref. [24] by a double emulsification procedure. In summary, 40 mL of agarose solution (6%, w/v) containing ferrofluids was prepared by heating a suspension of agarose in water to 95–100 °C. The solution was then transferred to a thermostat (60 °C) glass reactor with stirring and a mixture containing 100 mL toluene, 40 mL tetrachloromethane, and 2 mL Span 80. The mixture was emulsified by stirring for 10 min in a thermostat, and then adjusted to 4 °C immediately. The MAMS were isolated on a sieve and washed with ether and then with water.

2.4.2. Preparation of MCMS

MCMS were prepared by a suspension embedding procedure [25]. Thirty grams of purified cotton was soaked in 19% NaOH solution at room temperature and placed in a flask for 3 days. Fifteen-milliliter carbon disulfide was reacted with the aged cellulose for 5 h to convert it into a viscose solution, which was then diluted to 300 g with 6% NaOH solution to form a viscous solution of cellulose. In a reactor equipped with a stirrer, a mixture of 180 mL chlorobenzene, 40 mL tetrachloromethane, and 0.5 g potassium oleate was stirred for 30 min under room temperature. Then 70 mL of viscose solution containing ferrofluids was added to the mixture and the temperature was slowly raised to 90 °C. The solution was kept stirred for 2 h to solidify the liquid particles into microspheres. After cooled to room temperature, MCMS were isolated on a sieve and washed with ethanol and then with water.

2.4.3. Preparation of MPVAMS

MPVAMS were prepared by applying a suspension cross-linking procedure [26]. Twenty-five percent glutaraldehyde (GA) solution and 3 M HCl were added to a 10% (w/v) aqueous PVA solution (M_r 75,000–79,000) containing ferrofluids. The mixture was sonicated for 1 min in an ultrasonic bath and then added to an organic phase containing 130 mL gasoline 200#, 120 mL tetrachloromethane and 6 mL Span 80. Suspension cross-linking was carried out by stirring at 70 °C for 2 h, after which MPVAMS were isolated on a sieve and washed with petroleum ether and finally with water.

2.5. Characterization of magnetic microspheres

2.5.1. Microsphere yield

Product yield (%): The product ratios of microspheres were calculated by using the equation

$$\text{Product yield}\,(\%) = \frac{W_m}{W_t}$$

where W_m is the weight of 100–300 μm microspheres, and W_t is that of total microspheres.

2.5.2. Microsphere morphology and size analysis

Morphology: The morphology characterization of the microspheres was evaluated by scanning electron microscopy (SEM X-650, Hitachi, Japan) observation. The homogeneity of magnetic materials (Fe_3O_4) in the microspheres was determined from optical micrographs of the microspheres taken with an Olympus IX71 optical microscope (Olympus, Japan). The size and size distribution of microspheres were determined by a laser granulometer (Mastersizer 2000, Malvern, UK).

2.5.3. Magnetic properties

Magnetic content: The weight percentage of ferrofluids from dried microspheres is given as previously added magnetite content, assuming that added ferrofluids is pure magnetite. Final iron contents of microspheres were measured by an inductively coupled plasma-atomic emission spectrometry (ICP-AES) (ICP-9000, Thermo, USA) analysis. The prepared microspheres were dried and weighted and then solubilized in aqua regia to determine the contents of Fe. The following conditions were set for ICP-AES: input RF power 1.1 kW, observation height above load coil 16 mm, argon coolant flow rate 17 L/min, aerosol carrier argon flow rate 0.5 L/min, and wavelength set for iron 259.94 nm.

Magnetic response: The magnetic response was determined by the migration time (second) of magnetic microspheres during a certain distance in the response of a particular magnetic field.

2.5.4. Mechanical strength

Microspheres mixed with equal volume of glass beads (3 mm diameter) were stirred at 150 rpm and the mechanical strength was determined by computing the time (hour) for 50% breakage. Rigidity could also be measured by observing shape change of the beads after adding weights onto the microspheres.

2.6. Activation and coupling procedures to magnetic microspheres

MAMS, MCMS, and MPVAMS containing hydroxyl groups were activated by direct method (see Fig. 1A) and indirect method (see Fig. 1B), then coupled with Anti-IFN α-2b mAb.

Direct method: Magnetic microspheres were activated with epichlorohydrin (ECH) in 3 M NaOH solution for 3 h. The coupling with mAb was performed directly to ECH-activated microspheres.

Indirect method: Magnetic microspheres activated with ECH by direct method were treated with 5% ammonia hydroxide in ambient temperature for 6 h. The aminated microspheres were then incubated with 1% glutaraldehyde (GA) for 2 h, washed, and stored at 4 °C. After coupling with mAb, magnetic microspheres underwent an Amadori rearrangement to form a stable linkage with sodium boro-hydride.

Coupling with mAb after direct method or indirect method was performed as follows: 3 mL of 0.01 M Na_2CO_3–$NaHCO_3$ buffer (pH 9.6) containing 15 mg IgG of Anti-IFN α-2b mAb was added to 1 mL ECH- and GA-activated magnetic microspheres, followed by shaking for 12 h at 4 °C. After coupling, 5 mL of 1 M glycine was added to microspheres for 8 h to block the unreacted groups. The antibody concentration in all the samples was determined using Lowry method [27]. Antibody coupling capacities of magnetic microspheres were measured by collecting the samples during loading and washing steps. IMMS were stored at 4 °C for future use.

2.7. Analysis of leaking antibodies by sandwich ELISA

Different immunomagnetic microspheres (IMMS) were quantitatively estimated for the leakage of antibodies by sandwich ELISA: the wells in a microtiter plate (Costar, USA) were coated with 100 μL goat anti-mouse IgG (10 μg/mL) for 2 h at 37 °C in 50 mM $NaHCO_3$–$NaCO_3$ (pH 9.6). After the wells were washed with 0.1% Tween 20 in PBS (PBST), 200 μL 1% BSA in PBS was added in each well to block nonspecific sites. The plate was then incubated overnight at 4 °C. Wells were washed with PBST and incubated with eluates during IMS by three IMMS and pure mouse IgG dilution samples (0.001–10 μg/mL) as control for 1 h at 37 °C. After washing with PBST, the plates were incubated with goat anti-mouse IgG conjugated to horseradish peroxidase at 1:2000 dilution in PBS for 1 h at 37 °C. The plates were washed again and 100 μL of an OPD substrate was added to each well. After stopping the reaction by adding 50 μL 1 M

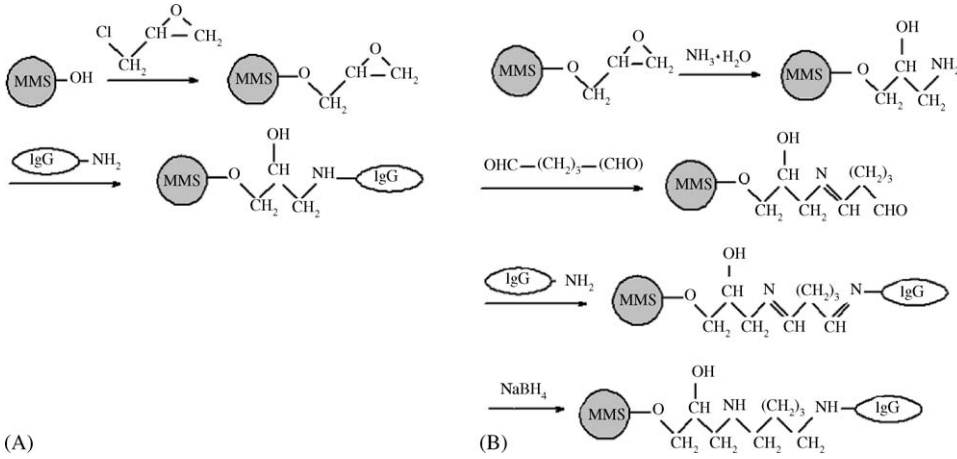

Fig. 1. Scheme of activation and coupling MMS with IgG by direct method (A) and indirect method (B).

H_2SO_4, the optical densities (OD) at 490 nm were determined using a microplate reader (ELX800, Biotech, USA).

2.8. Immunomagnetic separation of IFN α-2b by IMMS

The crude cell lysate was diluted for IMS of IFN α-2b. The optimization of different parameters such as binding mAb, elution behavior, and sample pretreatment conditions were conducted for IMMS. The diluted sample was added to a syringe containing IMMS and incubated on a shaker overnight at 4 °C. After installing the syringe into magnetic separator, washing procedure could be simply performed by importing and removing magnetic plate from the separator. Dissociation of IFN α-2b was facilitated with 0.1 M Gly–HCl buffer (pH 2.5), and the eluate was dialyzed and condensed and stored at 4 °C. Finally, IMMS were washed with PBS containing 1‰ sodium hydrazoate and stored at 4 °C for reuse. All the performances were carried out by flow-through magnetic separator designed and made by our laboratory.

2.9. Quantification of IFN α-2b by BAS-ELISA kit

BAS-ELISA kit was used for the analysis of purified IFN α-2b. All assays were performed according to the manufacturer's instructions described below: 96-well plates were coated with 100 μL of rabbit anti-IFN α-2b IgG at a 1:100 dilution in coupling buffer per well for 2 h. Then the plates were washed twice with PBST and the wells blocked overnight at 4 °C with 200 μL blocking solution. After two washes with PBST, the plates were incubated with 100 μL per well of IFN α-2b standard dilution samples (0.01–100 ng/mL) and serially diluted test samples for 1 h at 37 °C. The plates were washed and incubated with 100 μL biotinylated anti-IFN α-2b IgG at a 1:200 dilution for 1 h at 37 °C. After six washes with PBST, the plates were finally incubated at room temperature for 1 h with 100 μL horseradish peroxidase streptavidin at a 1:2000 dilution. The plates were finally washed again and 100 μL of an OPD substrate solution was added to each well. The reaction was stopped after 10 min by the addition of stop solution and quantification was based on the optical density of the wells at 490 nm.

2.10. Assay of IFN α-2b biological activities

The antiviral activity of IFN α-2b was determined in vitro by protection of human amnion WISH cells against VSV-induced cytopathic effects as described by traditional method [28]. In brief, 0.5×10^5 cells were seeded into each well of 96 well plates and incubated with two-fold serial dilutions of IFN α-2b samples for 18 h at 37 °C. After incubation, the cells were challenged with VSV and the plates were incubated at 37 °C for 18 h. Virus-induced cytopathic effects were evaluated by microscopic examination and IFN α-2b concentration was expressed as the inverse dilution that provided 50% protection of cells from viral induced cytopathic effects. The IFN α-2b concentrations in IU/mL were derived from the human IFN α-2b standard reference. The detection limit of the assay was 2 IU IFN α-2b/mL.

2.11. Gel electrophoresis and densitometry

The qualitative measurement of purified IFN α-2b was conducted by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE), using 12% resolving gel and 4% stacking gel under denaturing and reducing conditions, according to the method of Laemmli [29]. The gel was fixed and silver stained as described earlier [30]. Densitomertic analysis of SDS-PAGE gel was performed using Image Master VDS (Pharmacia Biotech, Sweden) for data acquisition and Image Master software for integration and analysis.

2.12. Western blot

Following the separation by SDS-PAGE, proteins were transferred onto nitrocellulose membrane for immunoblot analysis as previously described [31]. The membranes were blocked with 5% dried skim milk in PBS overnight at 4 °C, washed three times with PBST, and then probed with anti-IFN α-2b mAb (1 μg/mL) in PBS. The membranes were washed for three times and incubated with horseradish peroxidase, which was conjugated second antibodies, at a dilution of 1:1000 in PBS. Finally, the membranes were washed again with PBST and then developed with DAB as the substrate.

2.13. High-performance size-exclusion chromatography (HPSEC) analysis

HPSEC was carried out for the chromatographic studies of IFN α-2b with a Shimadzu Model LC-10ATVP HPLC apparatus (Shimadzu, Japan) using a SPD-10AV UV detector, processing the samples on a TSK-GEL G2000SWXL column (30 cm × 7.8 mm i.d.) (Tosoh, Japan). The column temperature was maintained at 25 °C. The detection was performed by measuring the UV absorbance at 280 nm and purity was calculated by analyzing of peak area of IFN α-2b. The mobile phase was 0.15 M NaCl, 0.02 M sodium phosphate, pH 7.0, with a flow rate of 0.8 mL/min.

2.14. Mass spectrometry

Matrix-assisted laser desorption ionization time of flight mass spectrometric (MALDI-TOF-MS) analysis was performed with a Reflex-III MALDI-TOF instrument (Bruker-Franzen Analytik, Germany). Samples were prepared by a 1:1 dilution with the UV-absorbing matrix sinapinic acid (SA). Acceleration voltages were 20 kV and Spectra were generated by pulsed irradiation from a 337 nm wavelength N_2 laser and internally calibrated. The instrument control and data processing were conducted with software supplied by Bruker.

3. Results and discussions

3.1. Characteristic analysis of magnetic microspheres

In order to find the most suitable magnetic microspheres (MMS) for long-standing and repetitious application of affin-

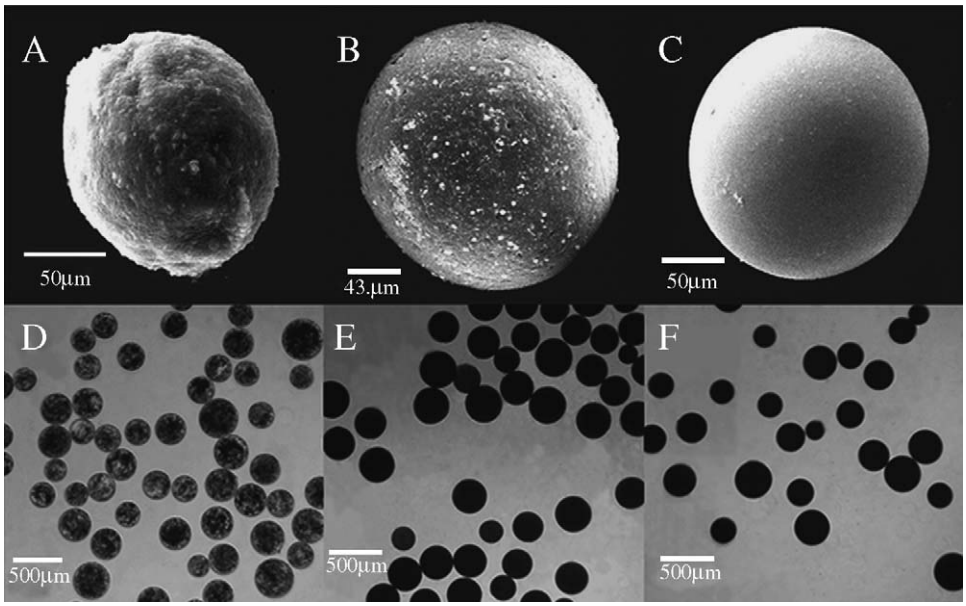

Fig. 2. Representative SEM micrographs and optical micrographs of magnetic microspheres ((A and D) MAMS, (B and E) MCMS, (C and F) MPVAMS) with an average diameter from 100 to 300 μm.

ity separation, three MMS, namely MAMS, MCMS, and MPVAMS, were employed to characterize and analyze the performance parameters of magnetic carriers including morphology, size distribution, magnetic properties, mechanical strength, and the leakage of antibodies, etc.

3.1.1. Morphology of magnetic microspheres

The morphology and intensity of three MMS were studied by SEM analysis (see Fig. 2A–C). As could be seen, all the microspheres showed a good spherical geometry. This particular size distribution (100–300 μm) was chosen because it enabled good magnetic response and suitable antibody coupling. MCMS (Fig. 2B) and MPVAMS (Fig. 2C) were well shaped spheres with a rather smooth surface and good intensity. MAMS showed (Fig. 2A) a wavy surface by vacuum drying in SEM performance resulting from weak intensity.

The Fe_3O_4 particle distributions with the microspheres were evaluated from the optical micrographs (see Fig. 2D–F). It was clearly shown that a homogenous distribution of Fe_3O_4 particles was achieved in MCMS (Fig. 2E) and MPVAMS (Fig. 2F), but relatively heterogeneous distribution of Fe_3O_4 was observed in MAMS (Fig. 2D).

3.1.2. Size and size distribution

The size distributions of three MMS were studied by laser sizing. Fig. 3 showed a standard normal distribution of all MMS. When average particle sizes were employed for three MMS, a reduction of the mean diameter of the MAMS (A) can be observed compared with MCMS (B) and MPVAMS (C). The mean diameters were 183.9, 228.5, and 265.7 μm for MAMS, MCMS, and MPVAMS, respectively.

3.1.3. Magnetic properties

The magnetic properties of MMS were evaluated from the respects of magnetic contents and magnetic response. The previously added magnetite contents of MMS were 5.41, 3.83, and 4.72% for MAMS, MCMS, and MPVAMS, respectively, which were close to the final iron contents measured by ICP-AES analysis: 5.54, 3.80, and 5.2% for MAMS, MCMS, and MPVAMS respectively (see Table 1). In addition, better performance in the magnetic response displayed from MCMS and MPVAMS than MAMS can be caused by the Fe_3O_4 particle distribution in MMS.

3.1.4. Mechanical strength

The mechanical strength of MMS is important for many applications, especially for IMS in long-lasting and repeated use [32]. Therefore, the mechanical strength was evaluated mainly from the respects of pressure and impact resistance. About 50% MAMS were broken and deformed after adding 500 g weights onto MMS for 1 h, while no apparent shape changes occurred to MCMS and MPVAMS. Impact resistance results showed that it took about fifteen days for MCMS and MPAVMS to reach 50%

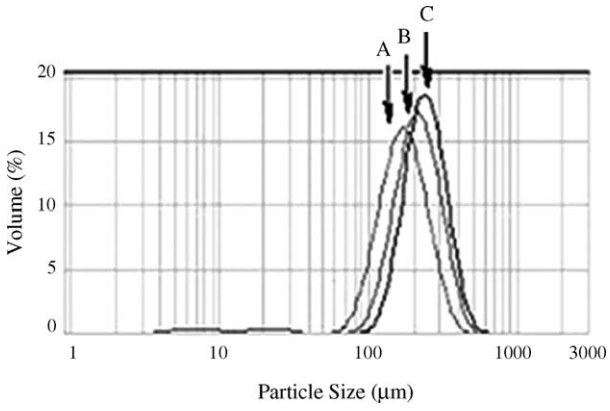

Fig. 3. Frequency and cumulative size distribution plots of three MMS ((A) MAMS, (B) MCMS, (C) MPAVMS).

Table 1
Parameter comparison of MAMS, MCMS, and MPVAMS with an average diameter from 100 to 300 μm

Performance parameters[a]	MAMS	MCMS	MPVAMS
Product yield (%)	70.8 ± 2.5	84.4 ± 1.8	50.2 ± 3.3
Mean diameter (μm)	183.9 ± 1.5	228.5 ± 2.7	265.7 ± 4.2
Magnetic content (%)[b]	5.54 ± 3.2	3.80 ± 2.8	5.20 ± 5.3
Magnetic response (s)[c]	107 ± 4.5	62 ± 2.7	48 ± 2.2
Mechanical strength (h)[d]	92 ± 2.3	338 ± 7.2	360 ± 4.3
Direct Coupling of Antibody (mg/mL)	0.86 ± 0.04	0.75 ± 0.05	0.76 ± 0.04
Indirect Coupling of Antibody (mg/mL)	11.82 ± 0.55	8.27 ± 0.73	3.66 ± 0.14
Leakage of Antibodies (ng/mL)[e]	214 ± 12.31	71 ± 6.35	55 ± 5.27

[a] The value of all parameters were measured for at least three times (mean value ± S.D.).
[b] Magnetic content was determined by ICP-AES analysis.
[c] Magnetic response was determined by response time.
[d] Mechanical strength was determined by calculation the time for 50% of breakage percentage.
[e] Leakage of antibodies was determined with the IMMS from three times of preparation.

of breakage percentage under vigorous stirring, but it took only 4 days for MAMS.

3.1.5. Coupling of antibodies

The presence of antibodies on the MMS following covalent immobilization was determined by Lowry method in the mobile phase. The results in Table 1 showed maximal coupling capacities of MAMS, MCMS, and MPVAMS activated by direct and indirect method. There was little difference on the coupling capacities among three MMS activated by direct method, while much better antibody coupling capacities were observed for MAMS and MCMS: 11.82 mg/mL and 8.27 mg/mL compared with that of MPVAMS: 3.66 mg/mL by indirect activation.

When comparing the different activation/coupling methods to attach Anti-IFN α-2b mAb to microspheres (see Table 1), the results revealed that the indirect method which imported spacer arm was the only method giving satisfactory results. The coupling capacity with Anti-IFN α-2b mAb was found to be 11.82, 8.27, and 3.66 mg/mL on MAMS, MCMS, and MPVAMS, respectively. In contrast, the poor coupling results obtained with three MMS by direct method (0.7–0.8 mg/mL) were caused by insufficient surface area and apparent spatial steric effect, which prevented the coupling with antibodies.

3.1.6. Leakage of antibodies

Leakage of antibodies coupled to MMS is important because leaking antibodies prevents repeated use of IMMS, and reduces the purity of products [33]. The leakage of antibodies, which should primarily due to nonspecific adsorption of mAb, was studied with the MMS coupled with maximal amount of antibodies by indirect method (see Table 1). It was shown that the leakage of antibodies was 214 ng/mL of MAMS for each time during IMS, which was three to four times higher than that of MCMS and MPVAMS, which were 71 and 55 ng/mL, respectively.

As shown by performance parameters of three MMS summarized in Table 1, the yield of MCMS was the highest. The MCMS and MPVAMS showed better performances in magnetic properties, mechanical strength, and leakage of antibodies than MAMS. Therefore, the MAMS are not suitable for repeated and long-lasting use because of the bad mechanical strength and serious leakage of antibodies. In addition, the antibody coupling capacity of MPVAMS was inferior to MAMS and MCMS because of the low swelling property of MPVAMS, which results in small coupling quantity with macromolecules such as antibodies [34]. From the results above, we can conclude that MCMS are the most suitable carriers for the purification of IFN α-2b than other microspheres because of their good performance in long-lasting and repeated use.

3.2. Optimization of different parameters of IMS

MCMS were used for the purification of IFN α-2b and a number of parameters were optimized to improve the efficiencies of matrices [35]. The binding mAb, elution behavior and sample pretreatment conditions were optimized to give the maximum purity and activity recovery of IFN α-2b. All experiments were carried out using 1 mL MCMS, and then the samples collected after IMS were examined with a BAS-ELISA kit to determine the recovery and adsorbed amount of IFN α-2b.

3.2.1. Optimization of binding efficiency of mAb

It is necessary to distinguish the total immobilized mAb (8.27 mg/mL) from the binding capacity of IMMS. To optimize the binding efficiency of mAb, MCMS coupled with different amount of mAb were analyzed with respect to the absorbance of IFN α-2b in IMS. The binding efficiency of mAb showed optima at 1.0 mg mAb bound to 1 mL of MCMS, which corresponded to purifying 15.5 μg of IFN α-2b. With an increasing amount of the antibodies bound to MCMS (≥1.0 mg/mL), no obvious differences in the absorbance of IFN α-2b were observed. Saturated binding capacity of antibodies is likely to result from spatial hindrance of excess antibodies coupled to the surface of MCMS. Thus, optimal coupling quantity of antibodies to MCMS was determined to be 1.0 mg/mL of MCMS, which exhibited 15% binding capacity of MCMS. The following optimization of parameters in IMS and the purification of IFN α-2b were performed with 1 mL MCMS coupled with 1.0 mg mAb.

3.2.2. Effect of elution behavior

The binding of IFN α-2b to affinity matrix was due to the specificity of molecular conformation and ionic interaction. The

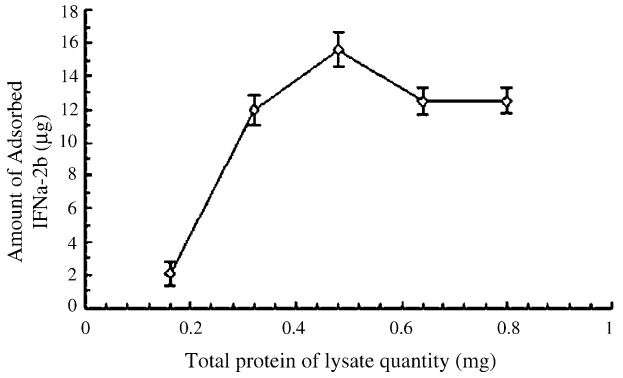

Fig. 4. Effect of lysate quantity on the absorbance of IFN α-2b onto MCMS. Set conditions: pretreatment of centrifugation, elution: 0.1 M Gly–HCl buffer (pH 2.5) for 1 h. Dilution fold: 30.

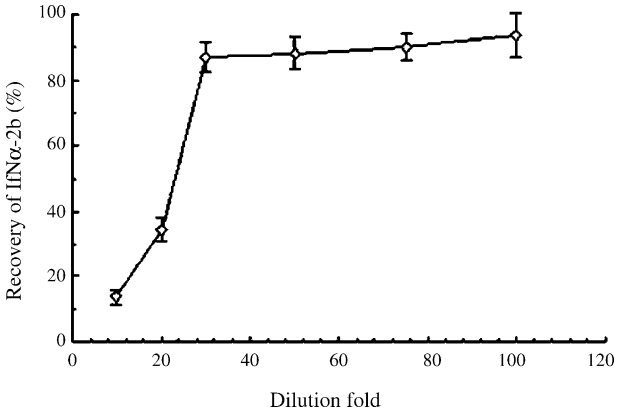

Fig. 5. Effect of dilution folds on the recovery of IFN α-2b onto MCMS. Set conditions: pretreatment of centrifugation, elution: 0.1 M Gly–HCl buffer (pH 2.5) for 1 h, lysate quantity: 0.48 mg of total protein.

elution of bound protein was optimized to give a maximum amount of IFN α-2b. After the loading of IFN α-2b, 0.1 M Gly–HCl buffer (pH 2.5) was used to elute the protein. The elution was performed three times at an interval of 1 h. About 89.5% of the adsorbed IFN α-2b was eluted for the first time, with 8.9 and 2.2% of IFN α-2b in the second and third elution. Thus, all the IMS experiments were carried out with the elution behavior of 0.1 M Gly–HCl buffer (pH 2.5) for 1 h.

3.2.3. Effect of pretreatment

A number of pretreatment methods for crude cell lysate were investigated to find out the optimum condition for maximum recovery by IMS. An 89.5% recovery of IFN α-2b by IMS was achieved with pretreatment of centrifugation, which removed cellular debris and insoluble proteins. In contrast, ammonium sulfate precipitation, and direct separation without any pretreatment showed 84.9 and 41.7% recovery, respectively, which is suboptimal. Therefore, centrifugation, which reduced separation influencing factors such as cellular debris and tanglesome process, was likely the optimal pretreatment in this assay.

3.2.4. Effect of lysate quantity

A comparative study of adsorbed amount of IFN α-2b at different lysate quantities from 200 to 1 mL (corresponding total protein of 0.16–0.8 mg) using 1 mL MCMS was shown in Fig. 4. Dilution fold of 30 was set in the experiments. When the total protein in the lysate was less than 0.48 mg, the adsorption of IFN α-2b could be directly raised by raising the amount of lysate. However, the trend was reversed when the total protein in the lysate exceeded 0.48 mg, indicating that the large amount of lysate interfered with IFN α-2b binding onto MCMS. The maximum adsorbed amount of IFN α-2b was 15.5 μg at 0.48 mg total protein of lysate quantity for 1 mL MCMS in IMS.

3.2.5. Effect of dilution fold

Dilution influenced the binding of IFN α-2b onto MCMS as shown in Fig. 5. Appropriately increasing the dilution fold could reduce the influences of foreign proteins on separation. At low dilution fold of IFN α-2b crude cell lysate, the recovery of IFN α-2b was low due to small amount of binding of foreign proteins. At moderate dilution fold, IFN α-2b bound adequately and strongly with MCMS, and a lower hindrance was observed during IMS resulting in a good recovery. The 30 fold dilution led to the 88.5% of recovery from the total protein (0.48 mg) used.

3.2.6. Validation of optimum parameters

There is no doubt that the optimum parameters should be further confirmed by analyzing every fraction of IMS, after that the quality of immunospecific carriers could be really evaluated. As demonstrated in Fig. 6, after loading the crude cell lysate onto the MCMS, some proteins other than IFN α-2b also bound to the magnetic carriers as the non-specific sorption and binding, which could be overcame by washing with PBS for three times. The proteins in the washing fractions may also be due to the left proteins in the excess lysate. The elution was carried out with 0.1 M Gly–HCl buffer (pH 2.5) for three times. Almost all of the bound IFN α-2b was eluted in the first fraction, and no trace of IFN α-2b was identified from the second and third elution. Maybe the non-specific binding underestimated immunospecific quality of MCMS, but after three washings, the high purity of IFN α-2b, as evident by a single, strong band in the first elution confirmed the optimum and applicable operation parameters.

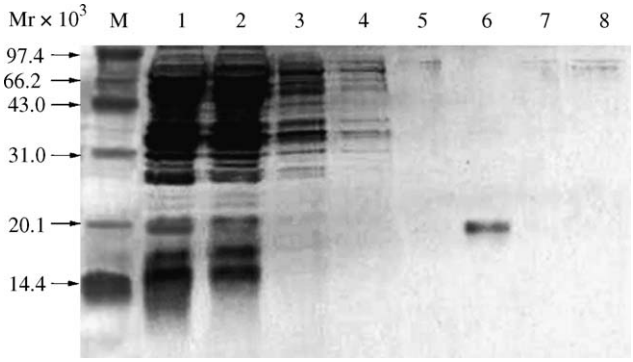

Fig. 6. SDS-PAGE validation of optimum parameters. Low range protein marker (Lane M); crude cell lysate before and after IMS (Lanes 1 and 2); washing with PBS for three times (Lanes 3–5); elution with 0.1 M Gly–HCl buffer (pH 2.5) for three times (Lanes 6–8). (All the fractions were resolved without condensation.)

Table 2
Purification of IFN α-2b by IMS from crude cell lysate[a]

	Total protein (mg)	Interferon activity (IU)	Specific activity (IU/mg)	Recovery (%)
Crude lysate	0.48[b]	4.7×10^6	9.8×10^6	100
Purified product	5.5×10^{-3}[c]	4.2×10^6	2.7×10^8	88.5

[a] All determinations were repeated three times by once prepared MCMS.
[b] Total protein was determined by Lowry method.
[c] Protein of purified IFN α-2b was estimated by BAS-ELISA.

3.3. Purification of IFN α-2b from crude cell lysate using MCMS

Purification of IFN α-2b from crude cell lysate after optimization of different parameters was extensively studied. The activity recovery of IFN α-2b by IMS was determined with a quantitatively BAS-ELISA kit and the antiviral activity on WISH cells was challenged with VSV virus. Activity assays were performed following the standard protocol of the preparation of IFN α-2b. The results shown in Table 2 suggested that activity recovery of the IFNα-2b by IMS reached 88.5% (c.v. = 4.6%), which was about 30 mg of purified IFN α-2b from 1 L of cell culture in a fermenter. Specific antiviral activity of the biologically active IFN α-2b attained 2.7×10^8 IU/mg (c.v. = 6.3%), which coincided with typical values obtained from the standard purification of IFN α-2b.

The identification of the purified IFN α-2b by IMS was based on the pattern of SDS-PAGE. A major band about 19.0 kDa was visualized by silver staining as shown in Fig. 7A. Gel analysis by densitometry in an Image Master VDS using the software Image Master VDS indicated uniform results with a purity of IFN α-2b reaching 97.2% by IMS. Further purity analysis of IFN α-2b by HPSEC (Fig. 8A) showed an overall purity of 92.9%. Favorable improvement in the purification of IFN α-2b could be achieved by a second separation step such as HPLC after IMS. Western blot (Fig. 7B) confirmed the identity of the purified IFN α-2b and the reduction of altered forms. The molecular mass of purified IFN α-2b analyzed by MALDI-TOF-MS showed a single component of 19,288 Da (Fig. 8B), which was the predicted standard molecular weight of IFN α-2b.

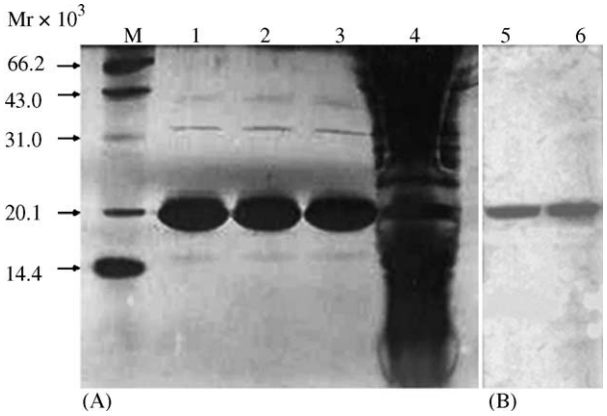

Fig. 7. (A) Silver stained SDS-PAGE analysis of purified IFN α-2b by IMS. Low range protein marker (Lane M); IFN α-2b purified with once prepared MCMS for three times (Lanes 1–3); crude cell lysate (Lane 4). (B) Western blot analysis of IFN α-2b. Purified IFN α-2b by IMS (Lane 5); crude cell lysate (Lane 6). (The lysate quantity loaded for SDS-PAGE and western blot was set to 0.04 mg.).

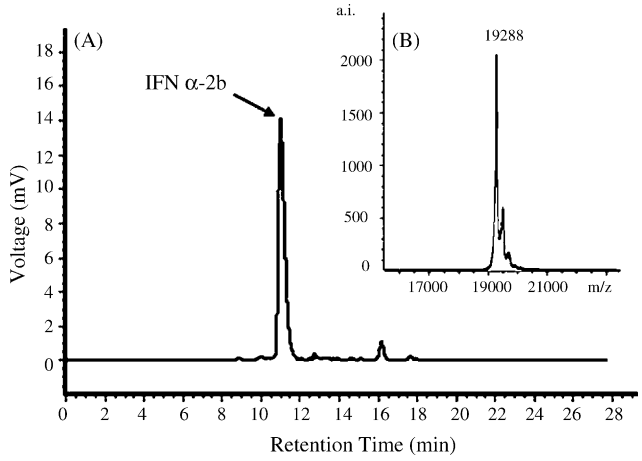

Fig. 8. (A) HPSEC and (B) MALDI-TOF-MS analysis of purified IFN α-2b.

After washing MCMS with 0.1 M Gly–HCl buffer (pH 2.5) and 0.02 M PBS for three and six times, respectively, the MCMS recovered their original capacities. MCMS were reused for three purification cycles, during which no obvious change of product purity: 97.2, 96.8, and 97.3% (Fig. 7A) and activity recovery: 88.5, 85.0, and 89.5% were observed. In this experiment, the prepared MCMS were used for the purification of IFN α-2b for more than ten times, without showing any performance decrease during IMS. Further analysis indicated that the absolute amounts of leaking antibodies from MCMS plateau around 10 ng after applied in the IMS for six times. Low leakage of antibodies from MCMS (about 0.01‰ w/w) predicted long-lasting and repeated use of MCMS in the purification of IFN α-2b.

4. Conclusions

We prepared and formulated a separation media that combines the selectivity of affinity chromatography with the use of magnetic properties during IMS. In our study, MCMS efficiently bound and separated IFN α-2b from crude cell lysate if coupled with anti-IFN α-2b mAb by indirect method. MCMS performed better in the separation of IFN α-2b than MAMS and MPVAMS according to the characteristic analysis. Using MCMS allows simple and convenient procedures to purify recombinant proteins from crude cell lysate. In addition, the separating capacities remained high after repetitive use of MCMS. Therefore, the purification of IFN α-2b using MCMS can be regarded as an efficient and convenient alternative to current methods and held

promises for large-scale application after appropriate standardization.

Acknowledgements

The authors acknowledge the collaboration of Lei Zhang and Xuelin Cao (Tianjin Hualida Bioengineering Co. Ltd.) for providing the methods to carry out parts of this immunological work.

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