

DEVELOPMENT OF IMMUNOMAGNETIC REAGENTS FOR HUMAN HEMATOPOIETIC CELL SORTING

E. Golenkina¹, P. Ivanov¹, D. Blochin¹, V. Filippov², O. Yershov³

¹ Blokhin Memorial Cancer Research Center, RAMS, Moscow, Russia ² Research Institute of Chemical Physics, RAS, Moscow, Russia, ³ Research Institute of Chemistry and Technology of Hetero-organic Compounds, Moscow, Russia

INTRODUCTION: Immunomagnetic separation is generally recognized as one of the most specific, reliable and fastest techniques to isolate cell subpopulations from complex cell mixtures.

The objective of our work was to develop the immunomagnetic reagent for human haematopoietic cell sorting. To this end monosized, superparamagnetic microspheres were generated and completely characterized. The conditions to conjugate monoclonal antibodies to the particles surface were optimized. The selection technique with the obtained immunomagnetic particles was further worked out.

METHODS: *Preparation and characterization of magnetic polystyrene microspheres (MPM):* Magnetite (Fe₃O₄) prepared by the reaction of Fe(II) and Fe(III) with the ammonia aqueous solution was dispersed at organic solvent enriched with nonionic surfactant to obtain stable colloidal solution. Polystyrene magnetic particles with surface hydroxyl groups were prepared by the method of emulsifying polymerization of copolymers and magnetite ferocolloid. Briefly, styrene (20 g), divinyl-benzene (2g) and magnetite ferocolloids (4,5 g) in styrene were emulsified in 400 ml of distilled water containing 0.25% SDS and agitated well for 10 min at room temperature. The mixture was ultrasonicated for 30 sec (frequency 22 kHz, power 40 W/cm²). To perform polymerization 100 ml of 1% potassium peroxydisulphate in distilled water was added and stirred at 60 °C for 24 h followed by incubation at 70 °C for 1 h. After reaching room temperature they were centrifuged at 500 g for 15 min. The sediment was resuspended in SDS and the magnetic particles concentrated in the magnetic field of a permanent magnet of magnetic intensity 2000 oersted for 30 min. SDS treatment was carried out twice. MPM were filtered with a Millipore 3 µm filter and resuspended in SDS for a final concentration of 10 mg/ml.

Particles were analyzed by transmitted-light microscopy. Size distribution was estimated by laser correlation spectroscopy. Magnetic properties (saturation and residual magnetization) were determined by magnetometry (Bruker, USA).

To increase the protein-binding capacity magnetic support was activated by *p*-toluenesulfonyl chloride. MPM were washed with water, water : dioxane (3:1, v:v), water : dioxane (1:3), dried dioxane. Wet weight treated particles (1g) were put into a round-bottomed flask containing 200 mg of tosyl chloride dissolved in 2 ml dioxane. Pyridine was added slowly under stirring. After 1 hour, the MPM were washed with dioxane and transferred back to water gradually. Activated particles were stored at 4 °C until being used.

Binding of antibodies to the particles: Ammonium sulfate precipitation was used to obtain immunomagnetic

conjugates murine IgG monoclonal antibodies (MoAbs) against human lymphocyte antigens CD3 (clone ICO-90), CD4 (clone ICO-86), CD8 (clone ICO-31) and CD20 (clone ICO-180) raised and purified from ascites fluids. Searching for optimal conjugation conditions we used varying buffer solutions (0.025M borate-buffered saline, pH 8.5; 0.05M carbonate-buffered saline, pH 9.5; 0.05M and 0.015M phosphate-buffered saline, pH 7.4). Then we studied kinetics of immunoglobulin binding by altering the reaction time from 30 min to 24 h. Generally, to prepare conjugates 10 mg of corpuscular magnetic support (approximately 5x10⁸ beads) were incubated with 200 µg MoAbs in a total volume of 1 ml buffer solution at 4 °C under gentle rotation. Microspheres were then collected and washed twice using a magnetic field. Unoccupied protein-binding sites were then blocked with 0.05M PBS containing 2% human serum albumin for 12 h at 4 °C under gentle rotation. Antibody-coated magnetic particles were resuspended to a concentration of 10 mg/ml (approx. 5x10⁸ particles per ml) with 0.01M PBS containing 2% human serum albumin (HSA) and 0.05% NaN₃ and stored at 4 °C until being used.

Separation of cells: Peripheral blood mononuclear cells (PBMC) from normal donors were isolated by density centrifugation on a Ficoll Paque gradient.

Generally, immunomagnetic selection (depletion) of antigen-positive lymphocytes was carried out as follows: immunomagnetic particles were washed twice with 0.01M PBS (pH 7.4) using a magnetic field. 10x10⁶ PBMC in 1 ml of 2mM EDTA in 0.01M PBS containing 0.5% HSA were added to the collected particles to obtain an estimated 3:1 bead : target cell ratio. The resuspended mixture was incubated for 30 min at room temperature under gentle rotation. The bead/cell complexes were retained by the permanent magnet whereas unbound cells were removed by four washes with 0.01M PBS. To evaluate depletion effectiveness flow cytometric analysis was carried out by direct immunofluorescence on samples from PBMS both before and after selection and on unbound cells (negative fraction). Data acquisition and analysis were assessed on a FACScan using FACScan research software (Becton Dickinson & Co.).

To determine an optimal quantity of immobilized MoAb magnetic beads conjugated with anti-CD3 and anti-CD20-antibodies that were known to have bound 4, 7, 10 and 12 µg of antibody per 1 mg of support were used, the results obtained were analyzed for different processing variables.

RESULTS: *MPM preparation and analysis:* Our preparation method for the magnetic polystyrene microspheres formed spherical particles (Fig. 1). Laser correlative spectroscopy showed that the particles had a mean diameter of 1.0 µm (var. from 0.3 to 2.0 µm) with size homogeneity of more than 90%.

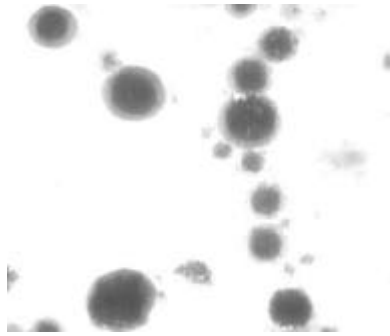


Fig. 1: Transmitted-light microscopy of synthesized magnetic polystyrene particles.

Estimated basic magnetic properties are given in Table 1. Due to the insignificant residual magnetization, MPM can be repeatedly collected in a magnetic field and thereafter easily redispersed.

Table 1. Measured magnetic characteristics of MPM synthesized.

Remanent magnetization, Gs cm ³ /g	Saturation magnetization, Gs cm ³ /g
1.2	25

The saturation magnetization of 25 Gs cm³/g enabled the rapid concentration of the MPM using a permanent magnetic field of 2000 Oe.

Coupling of antibodies to the particles: MPM did not bind MoAbs when incubated in 0.05M carbonate-buffered saline at pH 9.5. The maximum protein-binding capacity was less than 2 μ g MoAbs per mg particles when 0.025M borate-buffered saline at pH 8.5 or 0.05M phosphate-buffered saline at pH 7.4 were used. We elicited 0.015M PBS at pH 7.4 to be best for immunoglobulin coupling to the particles. In this way, 10-15 μ g MoAbs per mg particles could be immobilized after incubation of the MoAbs/MPM mixture for 24 h.

When studying the kinetics of immunoglobulin coupling we discovered that the binding rate was not the same for different MoAbs (Fig. 2).

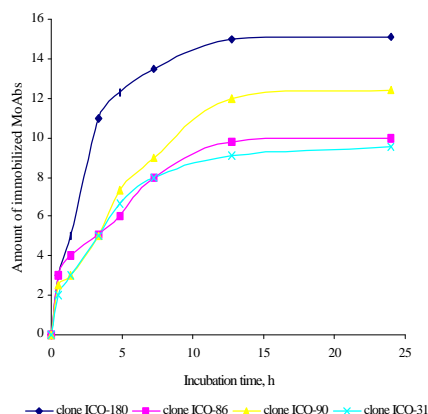


Fig. 2: Rates of IgG immobilization on MPM surface. Amounts of immobilized immuno-globulines (μ g per 1 mg support) (Y) were measured as a function of interaction time (h) (X) for different MoAb clones.

The optimal antibody-binding capacity as determined by subsequent immunomagnetic selection was found to be 7-10 μ g MoAb per mg particles. The required incubation time was averaged to be 10 hours for conjugating with clones ICO-90, ICO-86, ICO-31, and not more than 6 hours for clone ICO-180.

Immunomagnetic cell selection (depletion): Our primary concern was to determine an optimal amount of MoAbs that should be immobilized on the particles' surface. For this purpose, a series of CD3⁺-lymphocytes depletions were carried out. As noted before, immunomagnetic anti-CD3 conjugates with 4 to 12 μ g IgG per mg support were used. There proved depletion grade to be estimably influenced by the level of coupled MoAbs, the other conditions (i.e. source PBMC, beads per cell ratio, incubation time and temperature) being equal. The antibody binding capacity of 4 μ g/mg was apparently deficient because in this case immunomagnetic selection failed to eliminate CD3⁺ target-cells. The maximum antibody binding capacity of 12 μ g per mg support for clone ICO-90 was also not optimal, because excess IgG split out easily and blocked CD3 receptors on target cells interfering with bead/cell complexes formation. The latter was detected by staining of unbound cells after selection with FITC labeling sheep anti-mouse F(ab)₂. The most efficient was the depletion with MPM covered with MoAbs in concentration 7 to 10 μ g. Negative selection in those cases resulted in depletion of 97% CD3⁺-lymphocytes MoAbs (Fig. 3). Data were verified by CD20⁺-lymphocyte depletion. We used MPM conjugated with anti-CD20 MoAbs (clone ICO-180) at concentrations 4, 10 and 15 μ g per mg of support. IgG ICO-180 concentrations of 10 μ g/mg were optimal, providing a depletion rate of 96% (Fig. 4).

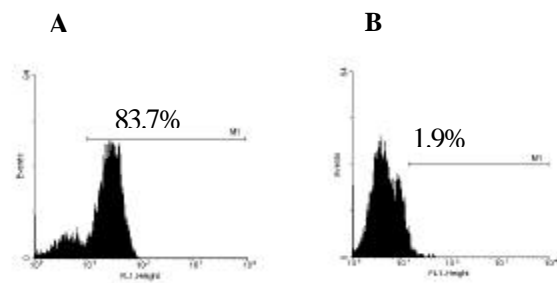


Fig. 3: Negative selection of CD3⁺-lymphocytes from human PBMC using MPM conjugated with MoAbs anti-CD3 (clone ICO-90) (8 μ g IgG per mg support). Cells are stained with CD3 FITC. A: PBMS before selection. B: negative fraction after immunomagnetic depletion.

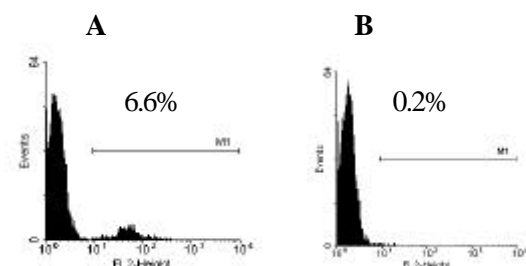


Fig. 4: Negative selection of CD20⁺-lymphocytes from human PBMC using MPM conjugated with MoAbs anti-

CD20 (clone ICO-180) (10 μ g IgG per mg support). Cells are stained with CD20 PE. A: PBMCs before selection. B: negative fraction after immunomagnetic depletion.

For further experiments immunomagnetic particles covered with MoAbs anti-CD4 (clone ICO-86) and anti-CD8 (clone ICO-31) were used at a concentration of 8 μ g/mg. Immunomagnetic separations with these conjugates eliminated 95-97% of antigen positive cells from an initial PBMC suspension (data not shown).

DISCUSSION & CONCLUSIONS: Polystyrene magnetic microspheres were prepared from copolymers and magnetic iron oxide employing the method of emulsifying polymerization. The resulting particles were 1 μ m in diameter, the homogeneity level was better than 90%. Microspheres possessed insignificant magnetic remanence and showed superparamagnetic behavior. To provide active surface groups MPM were activated by *p*-toluenesulfonyl chloride.

Optimal immunoglobulin coupling for the particles was performed in 0.015M PBS at pH 7.4 at 4 °C with gentle rotation. The interaction time should be determined for each MoAb clone because the immobilization rate is not constant. To achieve the optimal amount of surface-bound IgG, which was estimated to be 7-10 μ g MoAbs per mg support, conjugation times had to be 6 h for clone ICO-180, and 10 h for clones ICO-90, ICO-86 and ICO-31. Both deficient and excessive immunoglobulin coupling was proven to be inadvisable. Immunospecific conjugates against human lymphocyte antigens CD3, CD4, CD8 and CD20 were consequently generated. Immunomagnetic separation with these conjugates is able to eliminate about 97% of antigen positive cells from an initial PBMC suspension.

We believe that the developed immunomagnetic reagents will be useful in laboratory and clinical practice. They might be especially useful for the removal of tumor cells from bone marrow before autologous transplantation (in patients with B- or T-cell lymphomas). They also could be applied for T-lymphocyte purging of bone marrow before allogeneic transplantation to avoid development of graft versus host disease

REFERENCES: K. Nilsson, K. Mosbach (1980) *Eur. J. Biochem.* **112**:397-402. A. Rembaum, S. Margel (1979) *Br. Polym. J.* **10**:275-280. E.A. Golenkina, P.K. Ivanov, E.R. Polosukhina, O.N. Donskaya (2000) *High Medical Technologies, Materials of the V Russian Oncological Congress* 243-244