Magnetic and Fluorescence Encoded Polystyrene Microparticles for Cell Separation

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ABSTRACT

Materials assisting with the efforts of cell isolation are attractive for numerous biomedical applications including tissue engineering and cell therapy.[1] Here, we have developed surface modification methods on microparticles for the purposes of advanced cell separation. Iron oxide nanoparticles were incorporated into 200 μm polystyrene microparticles for separation of particle-bound cells from non-bound cells in suspension by means of a permanent magnet. The polystyrene microparticles were further encoded with fluorescent quantum dots (QD) as identification tags to distinguish between specific microparticles in a mixture. Cluster of differentiation (CD) antibodies were displayed on the surface of the microparticles through direct adsorption and various methods of covalent attachment. In addition, a protein A coating was used to orientate the antibodies on the microparticle surface and to maximise accessibility of the antigen-binding sites. Microparticles which carried CD antibodies via covalent attachment showed greater cell attachment over those modifications that were only adsorbed to the surface through weak electrostatic interactions. Greatest extent of cell attachment was observed on microparticles modified with protein A – CD antibody conjugates. B and T lymphocytes were successfully isolated from a mixed population using two types of microparticles displaying B and T cell specific CD antibodies, respectively. Our approach will find application in preparative cell separation from tissue isolates and for microcarrier-based cell expansion.

Keywords: Encoded microparticles, magnet microparticles, polystyrene, lymphocytes, CD antibodies, cell separation

1. INTRODUCTION

Intelligent biomaterials that are capable of selectively capturing cells and assisting with the isolation of cells from tissue samples are of invaluable importance for biomedical applications. Currently, harvesting a large number of cells for clinical use in stem cell therapies is generally limited by the efficiency of current cell isolation and expansion techniques. Panning[2] and compliment lysis[3] (removal of unwanted cells) are two separation techniques commonly used. They provide

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large numbers of cells, but can be difficult to control, present poor yields, and require large amounts of reagents.[4] Density gradient centrifugation is another technique that exploits the physical properties of cells such as size and density for separation, but the purity of cells is generally regarded as low.[5] More advanced methods of cell sorting using immuno-separation techniques exploit specific cell surface markers to yield a higher purity of cells. Of these methods, the most commonly used is fluorescence-activated cell sorting (FACS). For this technique, cells are tagged with antibody labelled fluorescent probes and processed by flow cytometry. This method requires extensive equipment infrastructure and is limiting in terms of throughput.[6] Magnetic-activated cell sorting (MACS) is another immuno-separation technique that binds cells to antibody labelled magnetic particles. MACS does not require advanced equipment, but is generally limited by the availability of the size and label of magnetic particles. In addition, MACS beads are usually produced in the < 10 μm diameter size regime for cell sorting applications. While for cell expansion, having larger platforms to support cell growth is desirable. Combing the two aspects of magnetic cell separation and cell expansion would prove to be beneficial in designing particle-based tissue culture platforms.

The use of polymeric microparticles as an isolation platform has many advantageous characteristics, including their large surface area in comparison to 2D tissue cultureware[7] and the possibility to attach a range of different biological factors.[8] Polystyrene is a particularly desirable platform since this material is also used in conventional tissue culture. Polystyrene microparticles can be produced in sizes ranging from a few nanometers to a few hundred microns in diameter and can be surface-modified to fit multitudes of biomedical applications.

Incorporating semiconductor nanocrystals, so-called quantum dots (QDs), into polymer microparticles has been useful in bioanalytical applications such as multiplexed high throughput screening.[9, 10] As QDs can be capped with an inert layer, they are believed to be less toxic than dyes[11], have extremely high photostability, and have intense and narrow symmetrical emission peaks[9] making them a better choice than conventional fluorescent dyes. Techniques for integrating magnetic nanoparticles and QDs have only recently been investigated.[10, 12] A large emphasis has been placed on using these materials for high throughput screening while the use of these materials as magnetic, encoded micro-carriers to support cell growth has been relatively unexplored. The work presented here combines optical encoding methods, magnetic separation, immuno-separation techniques, and large microparticle platforms for design of multi-functional micro-carrier systems for cell expansion.

2. MATERIALS AND METHODS

2.1 Magnetizing microparticles

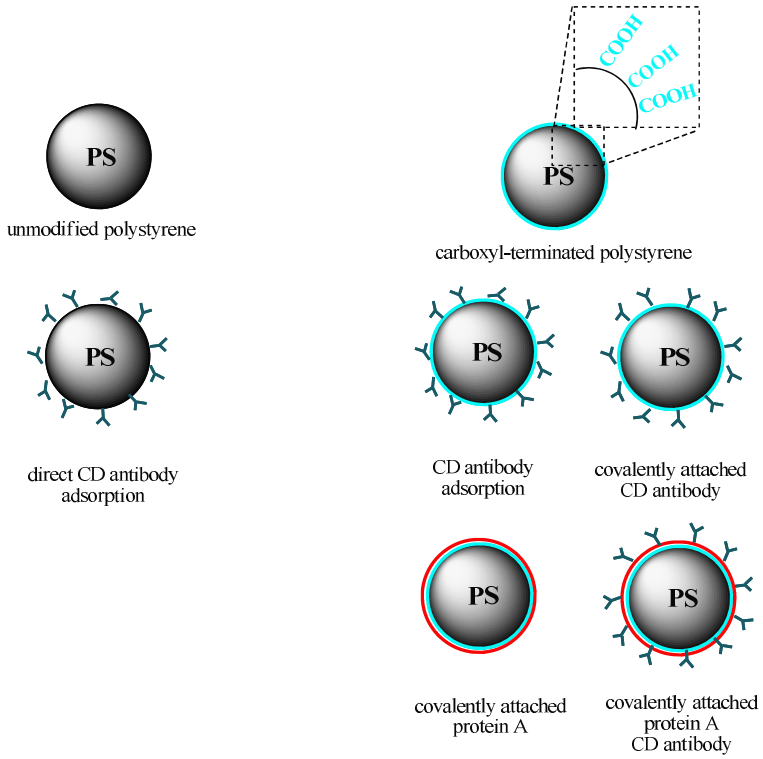
Iron oxide nanoparticles of ~ 14 nm diameter were prepared following the protocol of Berger *et al.*[13] Magnetite was incorporated into 100 μL of polystyrene microparticles (2 x 10^3 particles/mL in water, 200 μm diameter, Sigma) and carboxyl-terminated polystyrene microparticles (2 x 10^3 particles/mL in water, 200 μm diameter, Discovery Scientific). The aqueous solution in the microparticle suspension was removed and 50 μL of 1:1 (v/v) mixture of methanol and magnetite suspension (100 g/mL in MilliQ water) was added. The microparticle solutions were shaken on an orbital mixer (Ratek) for 16 hr. The particle suspension was then filtered using Amicon Ultra–4 centrifugal filter tubes (100kDa, Millipore) for 10 min at room temperature (Sigma 3-18K centrifuge), and the polystyrene particles were resuspended and washed 2x's in MilliQ water to remove excess magnetite.

2.2 Encoding microparticles with CdSe/ZnS quantum dots

After magnetizing, the 200 μm polystyrene and 200 μm carboxyl-terminated microparticles were dried using a CentriVap concentrator for 30 min at 35 °C. Once dry, CdSe/ZnS QDs were incorporated by swelling 50 μL of microparticles in 100 μL mixture of 5 % chloroform and 95 % N-butanol (v/v) prior to addition of 10μL QD suspension (5 mg/mL Lumidot™ in toluene) as adapted from the procedure from Han *et al.*[9] Two different fluorescing QDs were incorporated, red (λ_{ex} = 595 nm, λ_{em} = 610 nm) and green (λ_{ex} = 515 nm, λ_{em} = 530 nm). The microparticles required ~ 2 hr for complete incorporation, although longer incorporation times were evaluated. Afterwards, the swelling solution was removed, and the microparticles were washed with chloroform and N-butanol followed by multiple rinses with MilliQ water to remove any free QDs.

2.3 Surface modifications of polystyrene microparticles

Three types of surface modifications were employed: 1.) directly adsorbed CD antibodies on microparticles, 2.) covalently attached CD antibodies on microparticles, and 3.) Protein A – CD antibody conjugated microparticles (Schematic 1). The directly adsorbed antibody coated particles were prepared by incubating 20 µL of CD20 (Roche) or CD2 (Pharmingen) antibodies (200 µg/mL) with 100 µL of unmodified polystyrene microparticle suspension (2 x 10^3 particles/mL) for 2 hr at room temperature. The particles were extensively rinsed with PBS to remove any non-adsorbed antibody. The covalently bound CD antibodies were coupled to the carboxyl-terminated polystyrene microparticles using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) mediated protein coupling[14] as follows: 20 µL of 1 mg/mL EDC (Sigma) in MilliQ water was added to 100 µL of microparticle suspension (2 x 10^3 particles/mL) for 5 min at room temperature. The solution was removed from the microparticles, and 20 µL of CD antibody (200 µg/mL) was added and left for 2 hr followed by repeated washing with PBS. The protein A – CD antibody modified particles were prepared in a similar manner. Protein A was covalently attached through EDC coupling as described above. After protein A was bound to the microparticles, 20 µL of CD antibody (200 µg/mL) was incubated with 50µL of carboxyl-terminated particles and reacted for 2 hr followed by repeated rinses with PBS. Herceptin monoclonal antibody (Roche) was used as a negative control and was attached to the particles in the same manner as the CD antibodies.



Schematic 1. Schematic of various surface modifications applied to polystyrene microparticles.

2.4 Cell culture

Lymphoblastoid B and T cell lines (Ramos and Jurkat, respectively) were cultured in RPMI-1640 medium (Multicel™ without L-glutamine) supplemented with 10 % (v/v) foetal bovine serum (Bovogen) and 1 % (v/v) penicillin/streptomycin with glutamine (Sigma). The cells were incubated at 37 °C under 5 % CO_2 and passaged by a 1:5 split approximately every 2-3 days.

2.5 Cell attachment to microparticles

Microparticles were incubated with B and T lymphocytes. Each lymphocyte cell line was spun down, aspirated, and warm complete medium was used to dilute the cell suspension to 1 x 10^6 cells/mL. Cell counts were performed using a haemocytometer, and cell viability was determined with Trypan Blue (Sigma).

500 μL of cell suspension was added to a 100 μL aliquot microparticle suspension (2 x 10^3 particles/mL). The initial 30 min of incubation was conducted in a gyratory incubator at 37 °C, and then transferred into a CO_2 controlled incubator at 37 °C for a total of 2 hr. After incubation, the microparticle cell suspensions were transferred into sterile Eppendorf tubes. A strong magnet was held to the Eppendorf tube wall to immobilize the magnetic microparticles while the supernatant was aspirated. The microparticles were washed with PBS. Cells were fixed using 3.7 % formaldehyde in PBS for 15 min at room temperature.

2.6 Cell Staining

After fixing, cells were stained in the dark using Hoechst 33342 (Invitrogen). The dye was diluted to a working concentration of 1 μg/mL. 50 μL of dye solution along with 500 μL of serum-free medium was added to each different type of functionalized particle and incubated for 10 min under normal conditions. The microparticles were held by a magnet and gently washed with sterile PBS to remove excess dye.

For mixed cell population experiments, lymphocyte B and T cells were pre-stained with Cell Tracker Blue CMAC (7-amino-4-chloromethylcoumarin; Invitrogen) and Cell Tracker Orange CMRA (Invitrogen) respectively. Cell Tracker stock solutions of 10 mM were prepared by dissolving the solid in dimethyl sulfoxide (DMSO) and diluting to a 10 μM working solution in serum-free medium. Cells were spun down, and the supernatant aspirated. Cells were re-suspended in Cell Tracker dye working solutions, B cells stained with Cell Tracker Blue and T cells stained with Cell Tracker Orange separately. The cells were incubated for 45 min under normal growth conditions, centrifuged again, and the dye solution replaced with complete medium. A further 30 min of incubation was carried out, and cells were washed three times with sterile PBS. Fresh complete medium was added to reach the desired cell seeding density (1 x 10^6 cells/mL).

2.7 Cell separation in a mixed population of lymphocytes with microparticles

Pre-stained B and T cells were combined (30:70 ratio) to form a cell suspension of a total cell seeding density of 1 x 10^6 cells/mL. 1 mL of mixed cell suspension was incubated with magnetized and fluorescently encoded microparticles functionalized with protein A and CD antibodies (red fluorescing particles were functionalized with anti-CD20 and mixed with non-fluorescent anti-CD2 particles in a 1:1 mixture). 100 μL total volume of microparticle suspension (2 x 10^3 particles/mL) was used. After 2hr incubation at 37 °C in 5.0 % CO_2, the particles were held in place by a magnet and gently rinsed with PBS. The particle-bound cells were fixed in 3.7 % formaldehyde in PBS for 15 min.

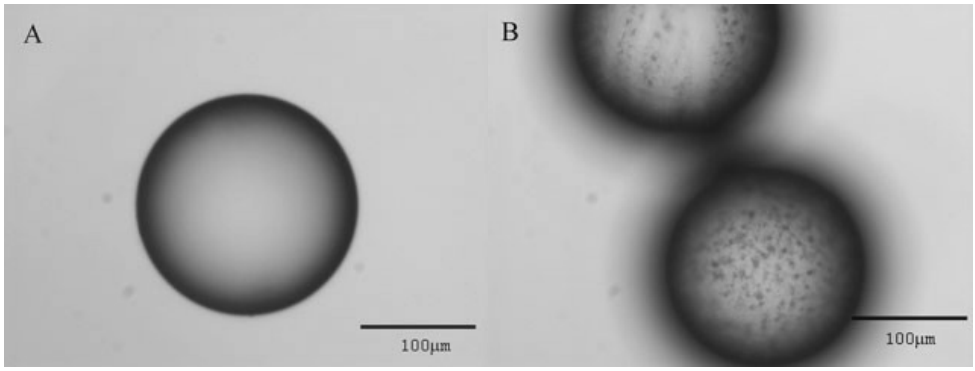
2.8 Microscopy

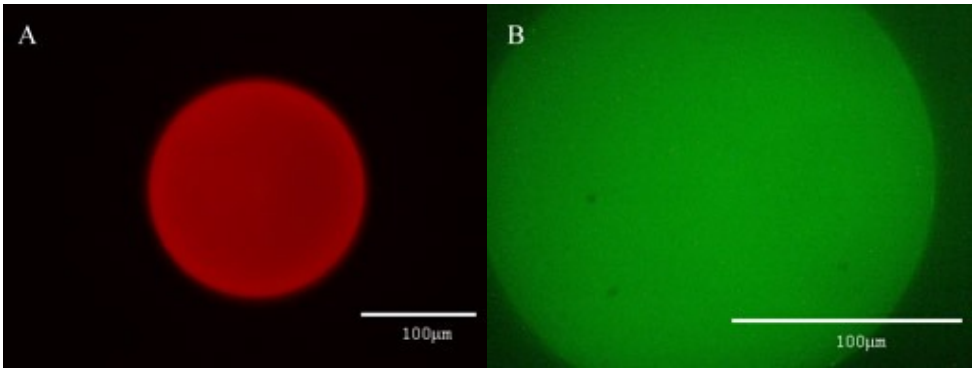
Microparticles were mounted onto clean glass slides (pathology grade; Livingstone) using Biomedia gel mount (Fisher Scientific) and Prolong gold antifade reagent (Invitrogen). Samples were imaged using a Laborlux D fluorescence microscope using 20x and 40x objectives in air. Microparticles were also imaged using a confocal microscope (TCP SP5, Leica) with an oil immersion 20x objective.

3. RESULTS AND DISCUSSION

3.1 Incorporation of magnetite nanoparticles into polystyrene microparticles

Iron oxide nanoparticles of ~ 14 nm diameter were incorporated into polystyrene microparticles using a 1:1 (v/v) mixture of methanol and magnetite suspension (100 g/mL in MilliQ water). The methanol acts as a mild swelling agent that allows the nanoparticles to infuse into the swollen polystyrene particles. Magnetized microparticles were examined by means of optical and confocal microscopy. Aggressive shaking during incorporation resulted in a more uniform distribution of magnetite in the microparticles (Figure 1).





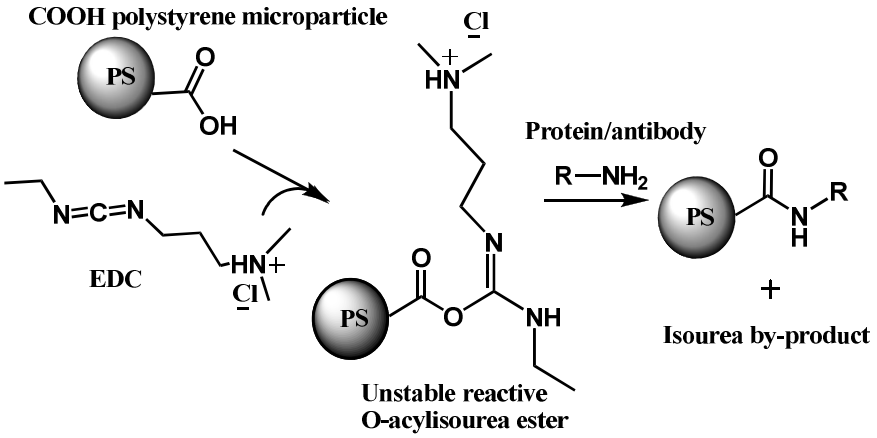
diameter. In order to determine the extent of cell attachment for the various surface modifications, anti-CD20 was immobilized on the particles, incubated with B cells and evaluated. The three types of surface modifications were 1.) directly adsorbed anti-CD20 on microparticles, 2.) covalently bound anti-CD20 on microparticles, and 3.) Protein A – CD20 antibody conjugated microparticles. Scoring was carried out on ~100 microparticles of each type to determine general trends in cell attachment and is summarized in Table 1.

The direct adsorption of the antibody occurs through weak electrostatic interactions with the microparticles. Cell attachment was observed to be very low to moderate ranging between 1 and 15 cells/particle for anti-CD20 adsorbed on plain polystyrene (Figure 3A - B) and carboxyl-terminated polystyrene particles.

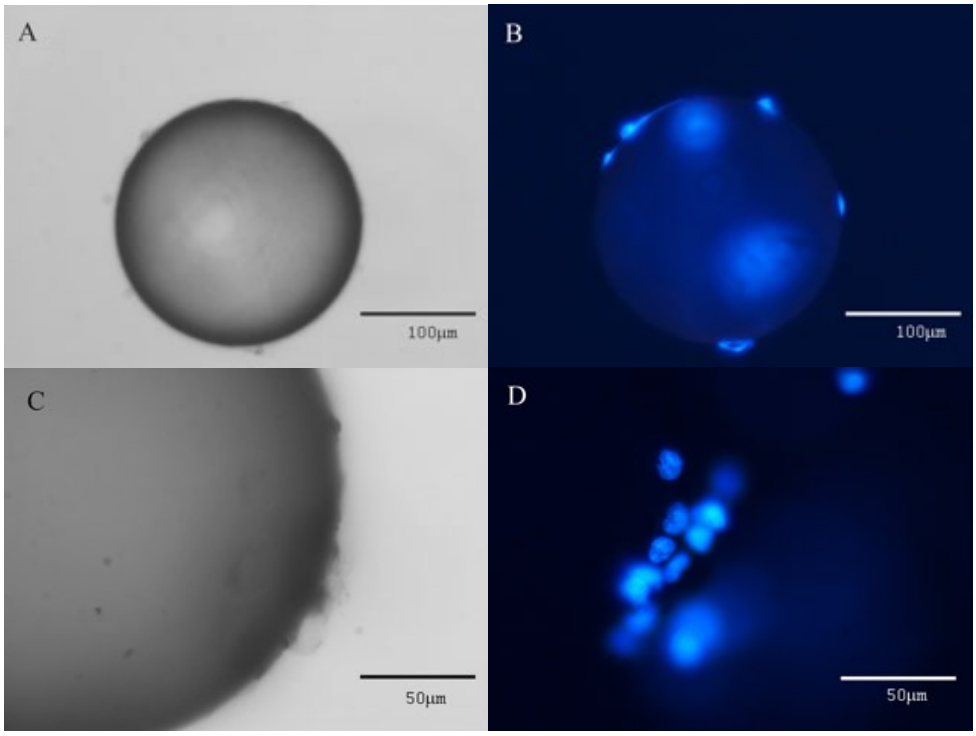
Anti-CD20 covalently bound to the carboxyl-terminated particles through an EDC coupling reaction (Schematic 2) showed moderate cell attachment displaying ~ 10-15 cells/particle.

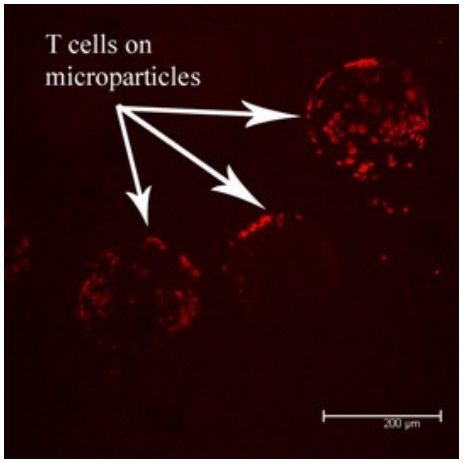
Protein A immobilisation on the microparticles prior to antibody attachment was also carried out in order to control the orientation of the antibody on the particle, leading to enhanced binding efficiency. Protein A binds with the fragment crystallisable (Fc) region of antibodies. This would leave the fragment antigen binding (Fab) region available to bind to cell surface antigens. The carboxyl-terminated particles were modified covalently with protein A via the EDC coupling reaction and subsequently reacted with anti-CD20. Protein A – CD20 conjugated particles showed the greatest cell attachment displaying ~ 30 cells/particle (Figure 3C-D). High cell attachment is presumably due to the stability of the covalent linkage and increased accessibility of bound antibody for cells.

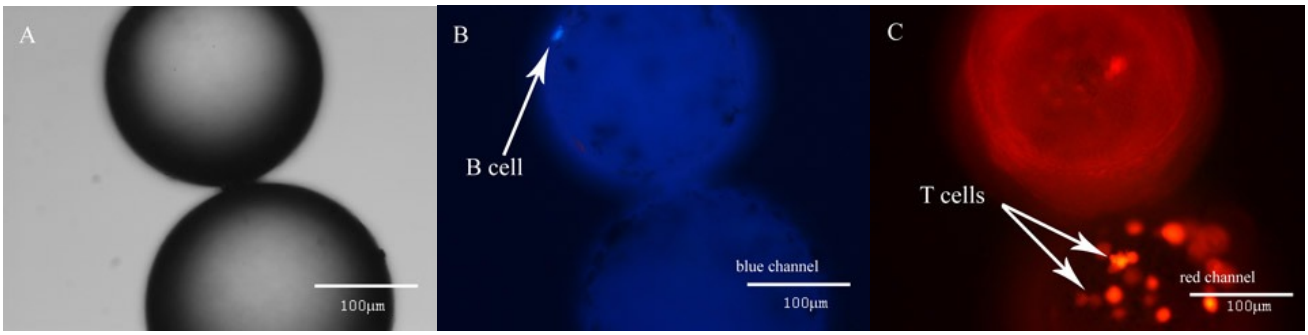
Appropriate controls were run to confirm that cell attachment was occurring due to the antibody modifications. No cell attachment was observed on unmodified polystyrene microparticles, carboxyl-terminated particles, and herceptin-modified microparticles. Herceptin (HER) is a humanized monoclonal antibody which does not bind to any surface antigens of B cells. Separate experiments were also carried out to confirm the lack of any non-specific cell attachment occurring due to the protein A coating. Carboxyl-terminated microparticles required EDC coupling of protein A to the surface before CD antibody binding; otherwise, no cell attachment was observed.



Schematic 2. Carboxyl-terminated polystyrene microparticles react with EDC to form an unstable reactive O-acylisourea ester intermediate which reacts further with proteins or antibodies to form a covalent peptide bond.







4. CONCLUSION

We have developed an optically encoded and magnetic polystyrene microparticle-based platform for the purposes of preparative cell separation. We have developed protocols for entrapping both magnetic nanoparticles and quantum dots within polystyrene microparticles. Fluorescently encoded and magnetic microparticles were modified with CD antibodies for selective cell attachment though various surface modifications. CD antibodies were directly adsorbed or covalently anchored to the magnetic particles while another method to orientate the antibodies through a coating of protein A was also studied. The latter approach gave more extensive cell attachment, demonstrating the effect of presentation of antigen-binding sites. B lymphocyte cells were shown to attach to CD20 antibody modified microparticles. Similarly, T cells were seen to attach to CD2 antibody coated microparticles. No cross-reactivity was observed. The CD20 and CD2 antibody coated particles were successfully used to separate both cell types from a mixture of B and T cells. The methods developed here can potentially be amenable to microcarrier-based techniques for cell expansion while also providing platforms for selective cell isolation from tissue isolates containing multiple cell types.

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