Proceedings of the ASME 2010 3rd Joint US-European Fluids Engineering Summer Meeting and 8th International Conference on Nanochannels, Microchannels, and Minichannels
FEDSM-ICNMM2010
August 1-5, 2010, Montreal, Canada

FEDSM-ICNMM2010-30168

NANOPARTICLE-COATED MICROTUBES FOR THE MANIPULATION OF CANCER CELLS

Robin Zhou
Cornell University
Ithaca, NY, USA

Andrew Hughes
Cornell University
Ithaca, NY, USA

Jane L. Liesveld
Strong Memorial Hospital
Rochester, NY, USA

Michael R. King
Cornell University
Ithaca, NY, USA

ABSTRACT

The development of novel methods for the isolation of primary stem and progenitor cells is important for the treatment of blood cancers, tissue engineering, and basic research in the biomedical sciences. Our lab has previously shown that microtubes coated with P-selectin protein can be used to capture and enrich hematopoietic stem and progenitor cells from a mixture of cells perfused through the tube at physiologically-relevant shear stresses[1][2], and that using a surface coating of colloidal silica nanoparticles (12 nm diameter, 30% by weight SiO_2) increased cell capture and decreased rolling velocity[3]. Here we show that 50 nm colloidal silica nanoparticle coatings may similarly increase cell capture, and that these protocols are effective for enrichment of human adult CD34-positive HSCs from primary apheresis and bone marrow aspirate samples. Future research may include long-term colony-forming assays to confirm stem cell activity of enriched cells, and transplantation in immune-deficient mice.

INTRODUCTION

Hematopoietic stem and progenitor cells (HSPCs), typically identified by the CD34 antigen[4], are used clinically in transplants for treatment of diseases of the blood and immune system[5][6][7]. Isolation of HSPCs from adults is preferable over use of embryonic stem cells due to a decreased risk of immune response and fewer ethical and political objections.

Most current methods for isolation and enrichment of HSPCs rely on expression of the stem cell marker CD34, while varying the means of separating these cells. Currently available techniques and products employ immunoaffinity columns (Ceprate LC), immunomagnetic beads (Dynabeads, Baxter Isolex 50), and submicroscopic magnetic beads (MiniMACS). A multi-center European study comparing these techniques found that they generally provided efficient enrichment of CD34+ cells and colony-forming cells (CFCs). [8]

However, these methods require multiple conjugation and wash steps, reducing yield with each. [1] Also, the binding of immunological molecules to the CD34 antigen and non-physiological shear stresses experienced by cells in wash steps may have unintended effects on HSPC phenotype.

While CD34-positive (CD34+) cells are a large portion of the HSPC population[4], there is evidence to show that a small but significant portion of HSPCs are CD34-negative and CD34-dim (or CD34-low). Furthermore, these CD34-negative and CD34-dim HSPCs may be the rare, more primitive, pluripotent stem cells. [9][10][11]

P-selectin is a protein of the selectin family of type 1 membrane proteins that is involved in rolling of leukocytes on blood vessel walls during inflammation. HSPCs have been shown to roll on P-selectin via P-selectin glycoprotein ligand-1 (PSGL-1) as well as other unidentified ligands [12].

Previous research has shown that primitive CD34+ HSPCs have stronger rolling adhesion on selectins than mature CD34- mononuclear cells[12], which can be exploited in P-selectin-coated microrenathane (MRE) microtubes to selectively enrich CD34+ HSPCs. Furthermore, capture of HSPCs from flow in a cylindrical tube mimics the physiological process of stem cell homing from peripheral blood to bone marrow.

Recently, we have shown that P-selectin coated microtubes can be used to separate HSPCs from human bone marrow

aspirate [1] and peripheral blood [2], and that nanoparticle coatings can enhance cell capture in similar devices [3].

Here, we show that a nanoparticle coating can improve the yield of a P-selectin based stem cell enrichment process without decreasing purity. We expect a nanoparticle coating to do so by increasing surface area, enhancing protein adsorption, and altering surface roughness to change flow profile in microtubes, enhancing margination effects.

MATERIALS AND METHODS

Cells

KG1a cells were cultured in RPMI media 1640 (GIBCO® Invitrogen, USA) with 10% fetal bovine serum (GIBCO® HI FBS 10082, Invitrogen, USA) and 1% penicillin-streptomycin (Invitrogen, USA).

Primary samples were obtained from the Liesveld Lab in the form of fresh bone marrow aspirate, frozen 1ml vials of bone marrow mononuclear cells or frozen apheresis bags Frozen samples were stored in liquid nitrogen until needed.

Bags were thawed in a 37°C incubator (5% CO2), and divided into 5 ml aliquots in 50 ml cylindrical tubes. Extra samples were refrozen at -80°C for later use. For immediate use, 20 ml warm media (described above) and 50 µl DNaseI (RNase Free, Cat. #M0303S, Lot #0070810, New England Biolabs, Ipswic h, MA, USA) were added, and samples were incubated at 37°C for 1-2 hours. To prepare apheresis sample for flow experiments, samples were centrifuged (1100 RPM, 37°C, 8 minutes). The pellet was resuspended in 25 ml of media, and passed through a 40 nm cell strainer to remove clumps. Samples were centrifuged again (1100 RPM, 37°C, 8 minutes) and resuspended in media to a final concentration of 1x106 cells/ml, and incubated overnight (37°C, 5% CO2).

Fresh bone marrow samples were used within 24 hours of sample collection, and any excess sample was separated by a Ficoll density gradient separation. The buffy coat (mononuclear cells, or MNCs) was washed and frozen in fetal bovine serum with 10% DMSO by volume (-80°C overnight, then stored liquid nitrogen until needed).

Frozen bone marrow MNCs were thawed in a 37°C water bath, carefully transferred to 5 ml of warm media (described above), and allowed to rest overnight before flow experiments.

Cells were centrifuged (KG1a at 1200 RPM, 6 min; apheresis sample at 1100 RPM, 8min) to isolate them from media, and resuspended in Dulbecco's Phosphate Buffered Saline (PBS, GIBCO® Invitrogen, USA) at a concentration of $1x10^6$ cells/ml. Fresh bone marrow aspirate was spun at 800 RPM for 5-7 minutes to separate serum, and resuspended to twice the original volume in PBS with Ca^{2+} (PBS+). Bone marrow MNCs were centrifuged (1200 RPM, 8 min) and resuspended to $2x10^6$ cells/ml. All cells used in flow experiments were suspended in PBS with Ca2+ (PBS+), except for the no calcium negative control tube, in which PBS without Ca2+ (PBS-) was used.

Microtube Coatings

Microtubes were coated with SiO_2 nanoparticles (NPs) (IPA-ST-L, colloidal silica in isopropanol, 50nm diameter, Lot #190566, Nissan Chemical Industries, Ltd, Japan) with poly-L-lysine (PLL) as previously described [3]. To explore the effect of various concentrations of nanoparticle incubations, the original suspension of NPs was diluted with methanol prior to incubation in the tube.

After nanoparticle coating, tubes were coated with recombinant human P-Selectin/Fc Chimera (NS0-derived, >95% purity, Cat. #137-PS, R&D Systems, Minneapolis, MN, USA). To coat with P-selectin, NP-coated or clean microtubes were washed with PBS-, and P-selectin diluted to the specified concentration in PBS- was incubated in the microtubes for 2 hours, allowing the protein to adsorb to the surface. After washing with PBS-, 5% non-fat dry milk was incubated inside each microtube as a non-specific blocking agent. After another wash with PBS-, microtubes were filled with PBS+ and incubated 30 minutes to overnight at 4°C to activate the P-selectin. Microtubes used for the no calcium negative control were simultaneously incubated with PBS- at 4°C.

Characterization of surface roughness

Atomic Force Microscopy (AFM) on blank and nanoparticle-coated tubes was used to verify that the nanoparticle coatings resulted in changes in surface roughness.

Characterization of adhesive sites on surface

Microtubes also coated with P-selectin were incubated with 100 µg/ml Functional Grade Purified Anti-human CD62P (P-selectin) (eBioscience, Cat# 16-0628-85) in PBS+ for 2 hours, washed, and incubated with 200 µg/ml Alexa Fluor 546 donkey anti-mouse IgG (Invitrogen, A10036) in PBS+ for 2 hours. After washing twice with PBS+, tubes were imaged on an Olympus IX81 microscope. In ImageJ, images of 3 random areas on each tube were analyzed for average brightness, and normalized by subtracting the average background brightness.

Flow Experiments

Prepared microtubes were placed on the stage of an Olympus IX71 or IX81 (Figure 1) microscope and viewed in bright field. A syringe pump (KD Scientific, Holliston, MA, USA) was used to impose flow rates of 25-100 ul/min, corresponding to a shear stress of 1.5-6.67 dynes/cm2 at the inner surface of the microtube. A HITACHI CCD Camera (Model KP-M1AN) and SONY DVD Recorder (DVO-1000MD) were used to record videos of cells adhered to or rolling on the inner surface of the microtubes for video analysis.

Video Analysis

Rolling velocities of captured cells were calculated as previously described. [3]

The number of cells captured was determined by capturing images of cells on the microtube surface at 20 different locations within each microtube. The number of cells in each frame was counted and averaged, and divided by the actual size of each frame to find the average density of captured cells on the microtube. To find the total number of cells captured, the density was multiplied by total internal surface area of the microtube.

Flow Cytometry

Primary samples were analyzed before and after flow experiments for HSPCs with an Accuri C6 Flow Cytometer, by labeling samples with BD Pharmingen™ PE Mouse Anti-Human CD34 Antibody (Cat. #55822) and BD Pharmingen™ PE Mouse IgG1 kappa Isotype Control (Cat. #555749).

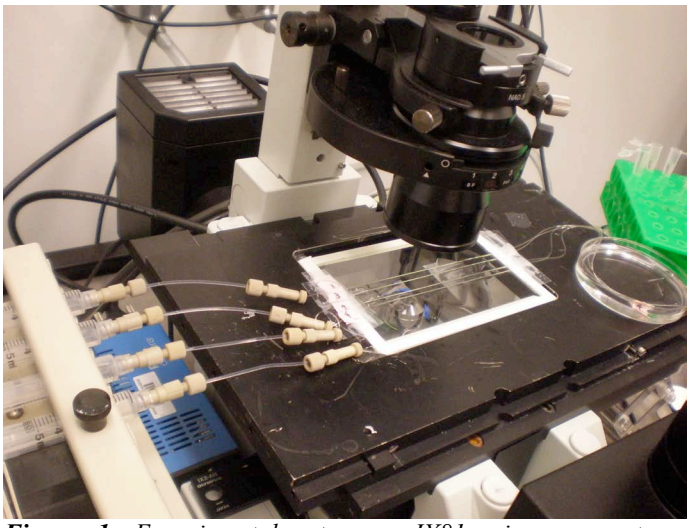

Figure 1. *Experimental setup on IX81 microscope stage. Syringe pump on left controls flow rate, and connectors and larger tubing connect syringes to microtubes on microscope stage. The right-hand ends of the microtubes are placed in samples, to be withdrawn through the tube.*

CD34+ Purity in Captured Bone Marrow MNC Cell Samples

Primary cells captured in flow experiments were labeled in the tube for CD34 with BD Pharmingen™ PE Mouse Anti-Human CD34 Antibody (Cat. #55822) and BD Pharmingen™ PE Mouse IgG1 kappa Isotype Control (Cat. #555749), diluted 1:1 with PBS+. Tubes were washed with 110 µl of PBS+ at 10 µl/min, and flow was allowed to stop completely before fluorescent imaging to determine CD34+ cell purities.

RESULTS

Atomic Force Microscopy of the inner surfaces of blank, no nanoparticle tubes and tubes coated with 50 nm colloidal silica particles showed that nanoparticle-coated tubes were significantly rougher and had large bumps, up to approximately 350 nm in height, protruding from the tube surface (Figure 2).

Labeling nanoparticle-coated tubes with anti-P-selectin IgG showed that the nanoparticle coating resulted in increased availability of active P-selectin binding sites on the tube surface (Figure 3).

KG1a cells were used as a model human cell line for HSPCs, as they are known to be CD34-positive and roll on selectins [5][13][14]. We varied the concentration of nanoparticle suspension used to coat the tubes, and found the greatest increase in number of cells captured with the 100% NP coating (Figure 4). Thus, further experiments were done to compare only the 100% NP and 0% NP (P-selectin only) tube coatings.

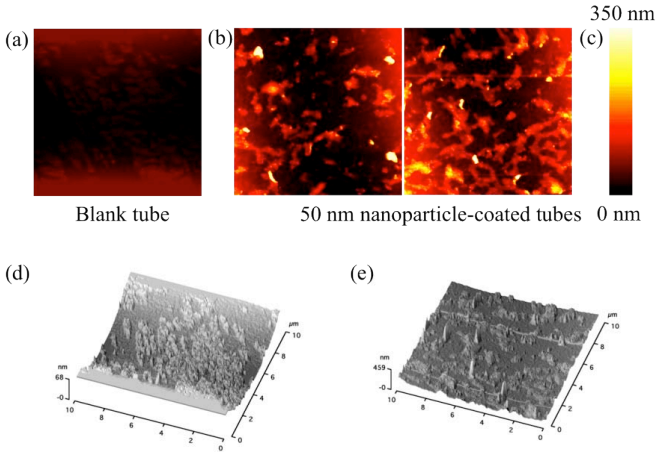

Figure 2. *Atomic Force Microscopy Images of Tube Surface. (a) Blank tube, with no nanoparticles. (b) Two locations of a tube coated with nanoparticles. (c) Thermal scale for images in (a) and (b). Three-dimensional representations of (a) and (b) are shown in (d) and (e).*

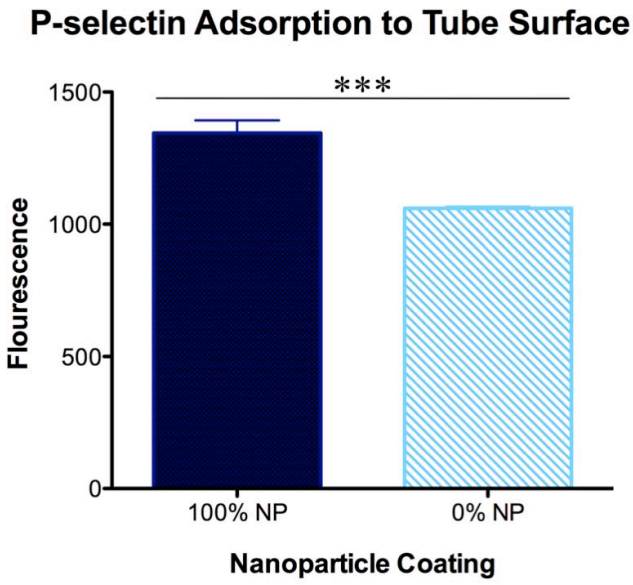

Figure 3. *P-selectin Adsorption to Tube Surface. Microtubes were coated with 20 µg/ml P-selectin. *** Student t test, 2 tailed. $p < 0.005$*

Since the strength of a selectin bond can be inferred from the rolling velocities of cells on a selectin-coated surface[15], we compared rolling velocities of cells on these surfaces. We found that the average rolling velocity of KG1a cells on 0% NP surfaces was 2-fold greater than that of cells on the nanoparticle-coated surface (Figure 5), indicating stronger attachment.

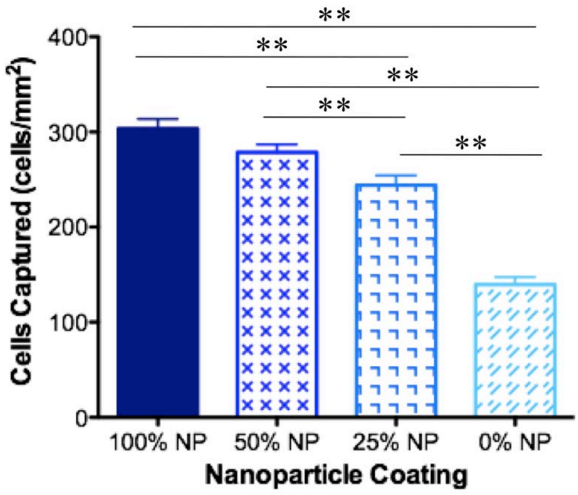

Figure 4. *KG1a Cells Captured at Various Nanoparticle Coating Concentrations. Microtubes were coated with 5 μg/ml P-selectin. ** Student t test, 2 tailed. $p < 0.05$.*

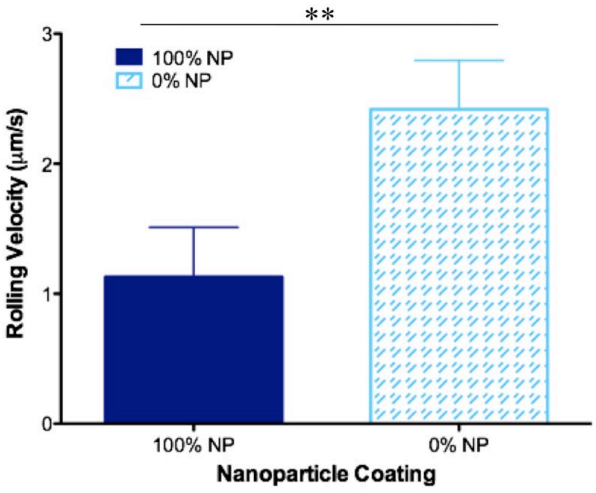

Figure 5. *Rolling Velocity of Captured KG1a Cells. Microtubes were coated with 5 μg/ml P-selectin. ** Student t test, 2 tailed. $p < 0.05$.*

Finally, when comparing the number of cells captured at various imposed wall shear stresses, we found that the 100% NP surface consistently outperformed the 0% NP surface by capturing significantly more cells (Figures 6, 7). Since cell adhesion to P-selectin is calcium-dependent, the lack of cells captured in no-calcium negative control trials shows that all captured cells can be attributed to interactions with the P-selectin tube coating (data not shown).

Next, we tested the capture of HSPCs from primary human samples. Rolling velocities of cells captured from bone marrow aspirate samples resuspended in PBS+ were approximately 2 times greater on 0% NP tubes than on 100% NP tubes (Figure 8). These rolling velocities were also very similar to those seen with KG1a rolling cells (Figure 5).

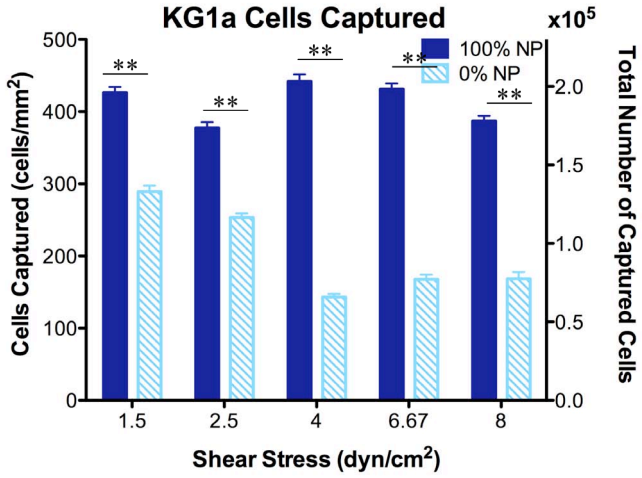

Figure 6. *KG1a Cells Captured. Microtubes were coated with 5 μg/ml P-selectin. ** Student t test, 2 tailed. $p < 0.05$.*

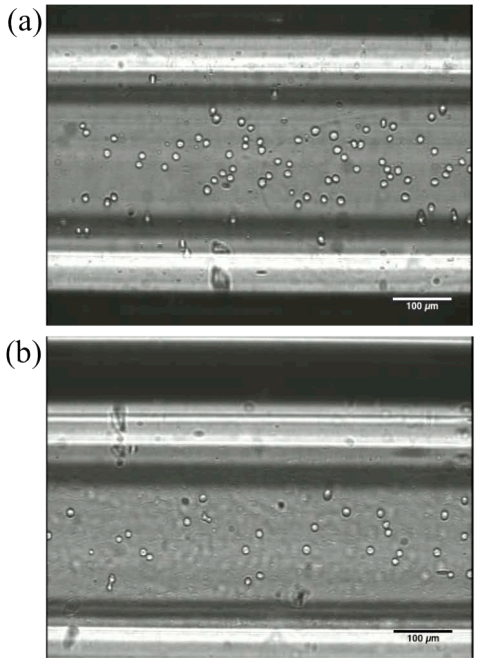

Figure 7. *Representative Images of Captured KG1a Cells. (a) 100% Nanoparticle-coated surface. (b) 0% Nanoparticle-coated surface. Microtubes were coated with 5 μg/ml P-selectin.*

We also found an increased total number of cells captured on nanoparticle-coated tubes than on P-selectin-only tubes (Figure 9). This is consistent with results obtained with KG1a cells (Figure 6).

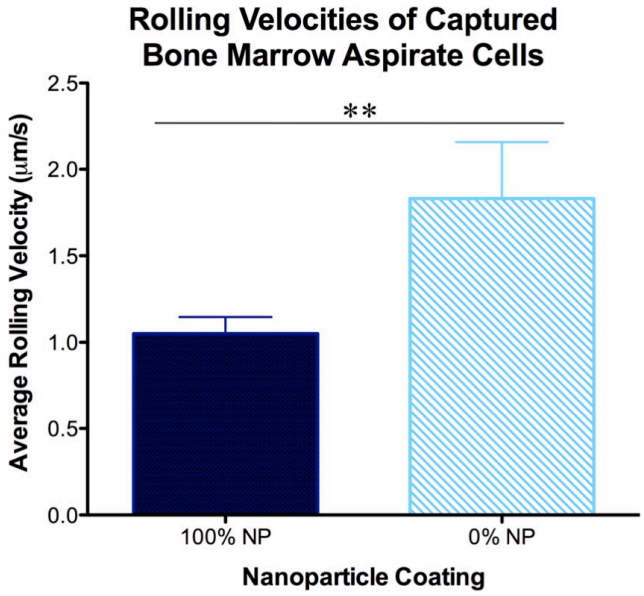

Figure 8. *Rolling Velocities of Captured Bone Marrow Aspirate Cells. Microtubes were coated with 20 μg/ml P-selectin. ** Student t test, 2 tailed. p < 0.05.*

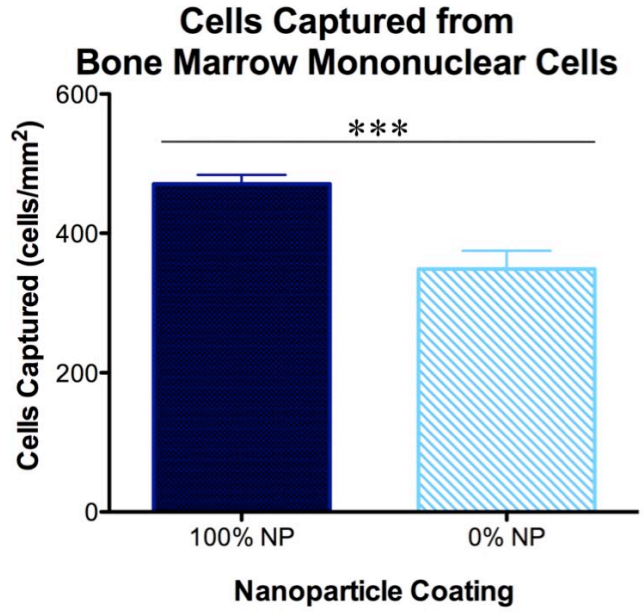

Figure 9. *Cells Captured from Bone Marrow Mononuclear Cells. Microtubes were coated with 20 μg/ml P-selectin. *** Student t test, 2 tailed. p < 0.001.*

Labeling captured cells for CD34 showed 6-7 fold enrichment of HSPCs from primary samples. There was no significant difference in purity between the 100% and 0% NP tubes (Figures 10,11).

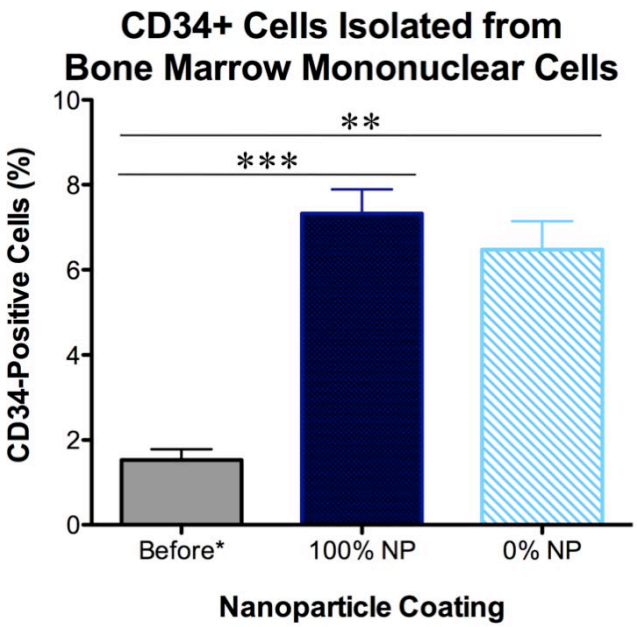

Figure 10. *CD34-positive Cells Isolated from Bone Marrow Mononuclear Cells. Microtubes were coated with 20 μg/ml P-selectin. * Percent CD34-positive cells in before samples determined in separate experiments, by flow cytometric analysis of identically prepared samples of bone marrow mononuclear cells. (n=7); ** Student t test, 2 tailed. p < 0.05; *** Student t test, 2 tailed. p = 0.001.*

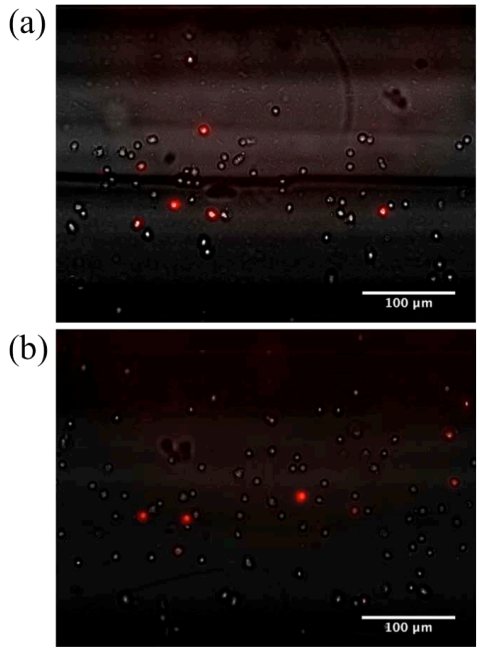

Figure 11. *Representative Micrographs of Fluorescently-labeled Bone Marrow Mononuclear cells in tubes. (a) 100% Nanoparticle-coated surface. (b) 0% Nanoparticle-coated surface. Both surfaces were incubated with 20 μg/ml P-selectin.*

Finally, flow cytometry of apheresis samples before and after perfusion through tubes coated only with P-selectin showed a 20-30 fold enrichment of CD34-positive cells (Figure 12). Due to limited availability of sample, nanoparticle-coated tubes were not tested. However, the above result suggest that enrichment from nanoparticle-coated tubes could yield similar levels of purity.

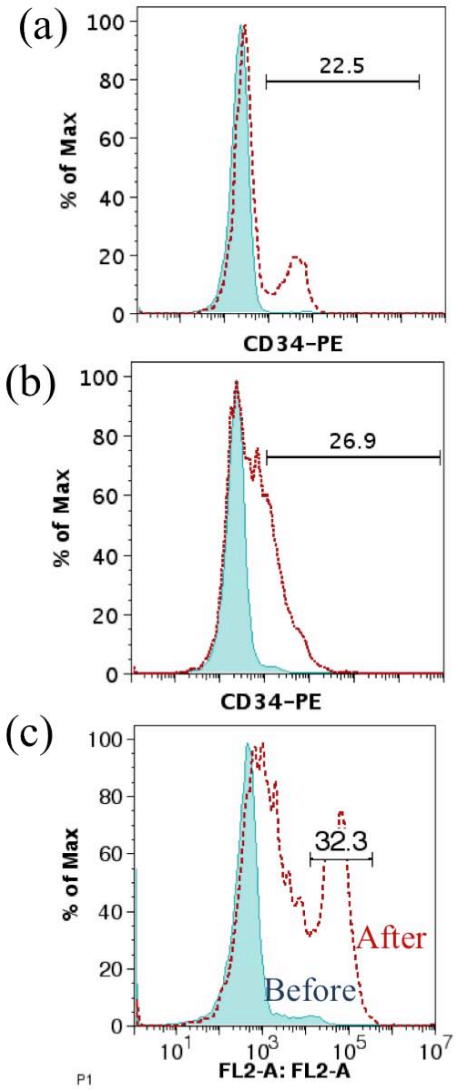

Figure 12. *Enrichment from Apheresis Samples on P-selectin-only Surfaces. We saw 20-30 fold enrichment of CD34-positive cells from frozen apheresis samples in microtubes coated with 40 µg/ml P-selectin only. Due to limited availability of sample, nanoparticle-coated tubes were not tested. (a) Flow rate of 25 µl/min (1.5 dyn/cm^2); (b,c) Flow rate of 40 µl/min (2.5 dyn/cm^2).*

DISCUSSION

We have demonstrated that 50 nm colloidal silica nanoparticle coatings increase cell capture of the P-selectin based stem cell enrichment process without decreasing purity, and that these protocols are effective for enrichment of human adult CD34-positive HSPCs from primary apheresis and bone marrow aspirate samples. Microtubes with nanoparticle coatings increased both number of cells captured and strength of capture.

P-selectin-coated microtubes can enrich stem cells from apheresis sample by up to 20 times (Figure 12), and microtubes also coated with nanoparticles captured more cells (Figure 9) with lower rolling velocities (Figure 8), while maintaining purity of CD34-positive cells (Figures 10,11). Since P-selectin binding is calcium-dependent, captured cells can be easily and gently removed from the microtubes without affecting surface markers, and can be further studied or used for HSPC transplantations. Previous studies have confirmed stem cell activity of cells isolated from P-selectin coated microtubes[1], which should be confirmed for cells from nanoparticle-coated tubes. While unlikely, nanoparticle-coated tubes should be tested for immunogenicity, as well as cyto- and genotoxicity to captured cells. As only a limited selection of nanoparticle sizes and geometries have been tested thus far[3][16], further testing should seek to identify optimal nanoparticle parameters. Computational fluid dynamics could also be employed to determine the effect small protrusions on the tube surface, like those created by nanoparticle coatings, on cell capture.

Since there is evidence of more primitive stem cells rolling on P-selectin, and the technique employed here is not based on isolating only CD34-positive cells, it would be interesting to investigate whether more primitive CD34-negative and CD34-dim cells can be isolated in a similar manner. Preliminary observations from our experiments with bone marrow mononuclear cells indicate that a number of captured cells are CD34-dim, and may be of the rarer, more pluripotent population of HSPCs. The increased number of cells captured on nanoparticle-coated surfaces may allow future studies to quantify this effect.

ACKNOWLEDGMENTS

We would like to thank Karen Rosell at the University of Rochester Medical Center for providing primary samples, and Kuldeepsinh Rana, Srinivas Narasipura, and Woojin Han for their contributions to this work.

REFERENCES

[1] Narasipura S. D., Wojciechowski J. C., Charles N., Liesveld J. L., and King M. R. , 2008, "P-Selectin coated microtube for enrichment of CD34+ hematopoietic stem and progenitor cells from human bone marrow.," Clinical chemistry, **54**(1), pp. 77-85.

[2] Wojciechowski J. C., Narasipura S. D., Charles N., Mickelsen D., Rana K., Blair M. L., and King M. R. , 2008, "Capture and enrichment of CD34-positive

haematopoietic stem and progenitor cells from blood circulation using P-selectin in an implantable device.," British journal of haematology, **140**(6), pp. 673-81.

[3] Han W., Allio B. A., Foster D. G., and King M. R. , 2010, "Nanoparticle coatings for enhanced capture of flowing cells in microtubes.," ACS nano, **4**(1), pp. 174-80.

[4] Krause D. S., Fackler M. J., Civin C. I., and May W. S. , 1996, "CD34: structure, biology, and clinical utility.," Blood, **87**(1), pp. 1-13.

[5] Weissman I. L., and Shizuru J. A. , 2008, "The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases.," Blood, **112**(9), pp. 3543-53.

[6] Little M., and Storb R. , 2002, "History of haematopoietic stem-cell transplantation.," Nature reviews. Cancer, **2**(3), pp. 231-8.

[7] Copelan E. A. , 2006, "Hematopoietic stem-cell transplantation.," The New England journal of medicine, **354**(17), pp. 1813-26.

[8] de Wynter E. A., Ryder D., Lanza F., Nadali G., Johnsen H., Denning-Kendall P., Thing-Mortensen B., Silvestri F., and Testa N. G. , 1999, "Multicentre European study comparing selection techniques for the isolation of CD34+ cells.," Bone marrow transplantation, **23**(11), pp. 1191-6.

[9] Nakauchi H. , 1998, "Hematopoietic stem cells: are they CD34-positive or CD34-negative?," Nature medicine, **4**(9), pp. 1009-10.

[10] Goodell M. A., Rosenzweig M., Kim H., Marks D. F., DeMaria M., Paradis G., Grupp S. A., Sieff C. A., Mulligan R. C., and Johnson R. P. , 1997, "Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species," Nature Medicine, **3**(12), pp. 1337-1345.

[11] Sonoda Y. , 2008, "Immunophenotype and functional characteristics of human primitive CD34-negative hematopoietic stem cells: the significance of the intra-bone marrow injection.," Journal of autoimmunity, **30**(3), pp. 136-44.

[12] Greenberg A. W., Kerr W. G., and Hammer D. A. , 2009, "Relationship between selectin-mediated rolling of hematopoietic stem and progenitor cells and progression in hematopoietic development," Blood, **95**(2), pp. 478-486.

[13] Koeffler H. P., Billing R., Lusis A. J., Sparkes R., and Golde D. W. , 1980, "An undifferentiated variant derived from the human acute myelogenous leukemia cell line (KG-1).," Blood, **56**(2), pp. 265-73.

[14] Civin C. I., Strauss L. C., Brovall C., Fackler M. J., Schwartz J. F., and Shaper J. H. , 1984, "Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells.," Journal of immunology (Baltimore, Md. : 1950), **133**(1), pp. 157-65.

[15] Alon R., Hammer D. A., and Springer T. A. , 1995, "Lifetime of P-selectin carbohydrate bond and reaction to tensile force in hydrodynamic flow," Nature, **374**, pp. 539-542.

[16] Hughes, A., and King, M.R. Unpublished data.