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Daryoush Shahbazi-Gahrouei
Department of Medical Physics and Medical Engineering,
Isfahan University of Medical Sciences, Isfahan, Iran

Tel: +98-311-7922495

A Novel Method for Quantitative Analysis of Anti-MUC1 Expressing Ovarian Cancer Cell Surface Based on Magnetic Cell Separation

[1]Daryoush Shahbazi-Gahrouei and [1,2]Mohammad Abdolahi

Scope of this study is to describe a simple, rapid, accurate, inexpensive and easily available *in vitro* method based on magnetic nanoparticles and magnetic cell separation principle to quantitative analysis the cell surface antigen expression of ovarian cancer cells surface (MUC1). In this work, superparamagnetic iron oxide nanoparticles (SPIONs) attached to monoclonal antibody (mAb) C595 that binds to ovarian cancer cells. Conjugation of mAb C595 to commercial SPIONs was achieved using a heterobifunctional linker sulfo-SMCC. The capability of the method was compared with flow cytometry as a gold standard and showed that both methods provide approximately the same results. This demonstrates the high potential of antigen-specific (anti-MUC1 expressing ovarian cancer) magnetic cell separation of C595 mAb coated with SPIONs-C595 for quantitative cell surface antigen detection and analysis. Overall, SPIONs-C595 nano-probe is potentially both, a selective ovarian molecular imaging tool as well as a therapeutic agent.

Key words: Ovarian cancer, SPIONs, flow cytometry, C595 mAb, cell separation

[1]Department of Medical Physics and Medical Engineering, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
[2]Department of Medical Physics, School of Medicine, Bushehr University of Medical Sciences, Bushehr, Iran

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INTRODUCTION

Biomarkers reflect a disease state very specifically and sensitively, therefore, they could be used for the early diagnosis, differentiation between disease types with higher accuracy, disease monitoring during and after therapy and as possible therapeutic agents (Lee *et al*., 2008; Simon, 2011; Saito *et al*., 2012). Cell surface antigens play an important role in cellular functions of diseases in a variety of cancers and act as an ideal biomarker to produce against a specific cancer cell (Belov *et al*., 2010). Recently, superparamagnetic nanoparticles have attracted growing interest as high performance biomaterial which is used for the diagnosis and treatment of cancers, inflammatory and many other human diseases (Aries de Silva *et al*., 2008; Mody *et al*., 2009). It is clear that cancer patients would benefit when new diagnostic and therapeutic methods of molecular indicators is used (Schrevel *et al*., 2012). They have been used for various applications such as, immunoassay, cell separation and molecular biology.

Applications of cell surface antigens in cancer diseases have enough potential for cancer detection at early stages and enable improve the technology for cancer cells antigen discovery. Therefore, rapid, accurate and inexpensive detection methods of the relevant marker are interesting and vital. Now-a-days, a wide range of technologies is utilized for detection and characterization of surface antigens, but the most widely used approach analyzes cell surface antigens by flow cytometry (Ranzoni *et al*., 2012). Flow cytometry is the gold standard method for accurate measurements of cell surface antigens. However, this technique is too expensive, is not available in all laboratory centers and also needs to expert and highly trained personnel. In addition, in developing countries it is difficult to access to technical support and quality control programs of relevant devices (Karl *et al*., 2009; Gupta *et al*., 2012).

Recently, a new technique has been developed using magnetic nanoparticles coupled to Monoclonal Antibodies (mAb), as a non-flow cytometric method, that identifies cell surface antigen expression by specific antibody-antigen reaction as an easy, fast and low cost modality (Long *et al*., 2009). In addition, the use of magnetic nanoparticles as molecular imaging probes in particular, in Magnetic Resonance Imaging (MRI) enables non-invasive *in vivo* studies of antigen expression of diseases in various internal organs (Shahbazi-Gahrouei *et al*., 2006; Shahbazi-Gahrouei, 2009). Herein, a rapid and accurate *in vitro* assay based on magnetic nanoparticles and magnetic cell separation principle is described and developed to quantitatively analysis of the mucine-1 (MUC1) as a cell surface antigen specific for different ovarian cancer cells like OVCAR3, SKOV3 and A2780. This method relies on the fact that many types of ovarian cancer cells express high levels of MUC1 on their cell surface (Wang *et al*., 2007; Mirzaei *et al*., 2012). This study is the first to describe synthesis and *in vitro* investigation of the detection and analysis of a novel nanoconjugate contains using of Superparamagnetic Iron Oxide Nanoparticles (SPIONs), attached to C595 monoclonal antibody that binds to the MUC1, expressing ovarian cancer cell surface based on magnetic cell separation.

MATERIALS AND METHODS

Sulfo-SMCC cross-linker (sulfosuccinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate), Traut's Reagent (2-iminothiolane) and cysteine were purchased from Sigma (Chemical Co., USA). Nanomag®-D-spio nanoparticles in suspension (diameter: 20 nm, surface: CLD-NH_2, 5 mg mL^{-1}; 2.4 mg Fe mL^{-1}) were obtained from micromod Partikeltechnologie GmbH (Rostock, Germany). Midi MACS separator (MACS® Technology is the gold standard for magnetic cell separation) and High Gradient Magnetic Field (HGMF) columns were purchased from Miltenyi Biotec GmbH (Gladbach, Germany). PD-10 columns were prepared from GE Healthcare (Piscataway, NJ). The Bradford reagent was purchased from BioRad (Hercules, CA). Amicon centrifugal filters (0.5 mL capacity, 10 kDa MWCO) were purchased from Millipore (Billerica, MA).

All other chemicals were supplied by Aldrich and used as received. C595 monoclonal antibody was obtained from Professor Barry Allen (UNSW, Sydney, Australia). Cell culture media and fetal bovine sera (FBS) were obtained from GIBCO, Invitrogen Corporation (Carlsbad, CA, USA). Ovarian cancer cell lines, OVCAR3, SKOV3 and A2780, were purchased from national cell bank of Iran (Pasture Institute, Tehran, Iran).

Conjugation of antibody with nanoparticles: The monoclonal antibody C595 was thiolated and conjugated to maleimide functionalized nanomag®-D-spio nanoparticles. The scheme of SPIONs conjugation to C595 mAb is illustrated in Fig. 1. Therefore, the sulfo-SMCC cross-linker was first added to nanomag®-D-spio particles with CLD-NH2 surface to introduce maleimide groups. Specifically, 100 μL of 14.35 μM Sulfo-SMCC solution in DMSO was added to 5 mg of nanoparticles in PBS-EDTA buffer under gentle shaking for 1 h at room temperature. After incubation the suspension was washed with PBS-EDTA buffer with

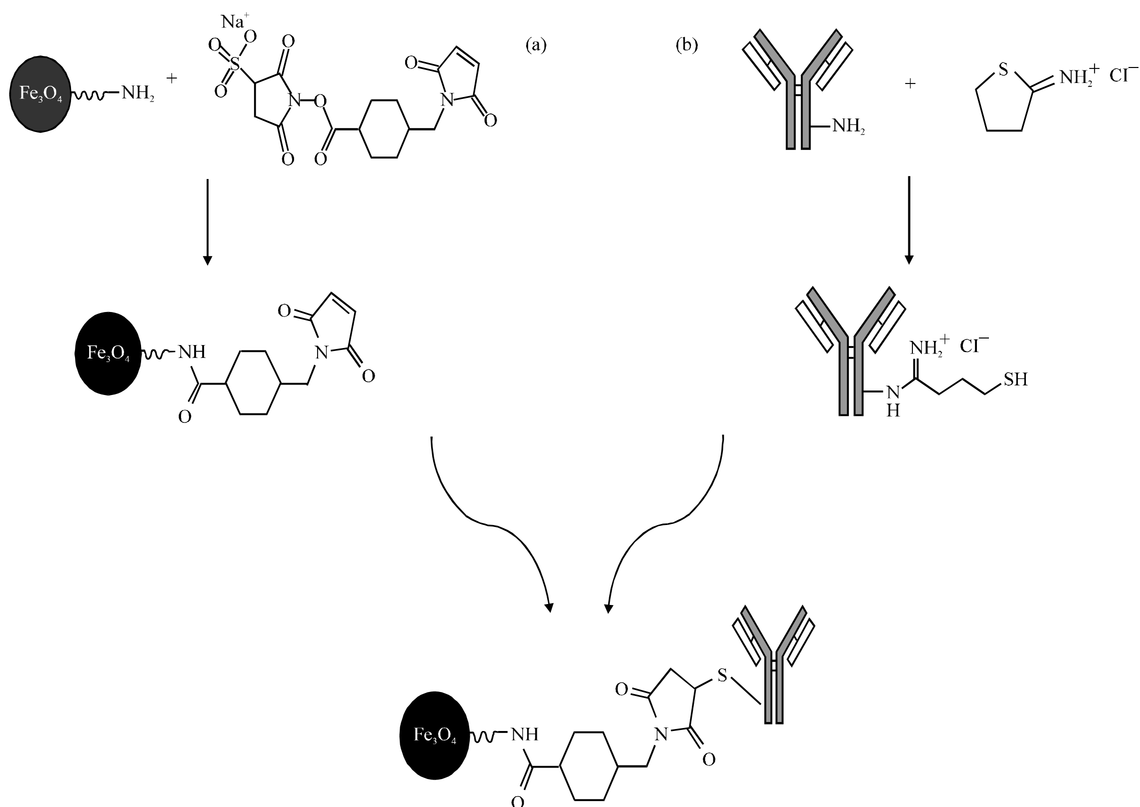

Fig. 1(a-b): Scheme of SPIONs conjugation to C595 antibody: (a) Functionalization of SPIO-CLD-NH_2 with Sulfo-SMCC and (b) Functionalization of antibody with SH groups using Traut's reagent. Conjugation of thiolated antibody to maleimide functionalized SPIONs

PD-10 size exclusion columns to remove unreacted sulfo-SMCC. In the next step primary amines of C595 monoclonal antibodies were modified with 2-iminothiolane to introduce sulfhydryl groups. Typically, 8 μL of 7 mM Traut's reagent solution was added to 400 μL of pure antibody solution in PBS-EDTA buffer (1 mg mL^{-1}) and shaken for 1 h at room temperature. To remove unconjugated 2-iminothiolane, the solution was washed three times by 10 kDa cut-off Amicon centrifugal filter units with PBS as an eluent.

The antibody conjugation to SPIONs was achieved by addition of the maleimide functionalized particles to the SH-labeled antibody and incubation under gentle shaking for 3 h at room temperature. Remaining functional groups were blocked by addition of 100 μL of 20 mM freshly prepared cystein solution. Finally the antibody-labeled SPIONs were purified on magnetic columns (MACS separator).

Characterization: The hydrodynamic particle size and the width of the particle size distribution (polydispersity index) of nanoparticles were obtained via Photon Correlation Spectroscopy (PCS) using a Malvern Nano Series ZS particle size analyzer (Malvern Instruments, Worcestershire, UK). Samples morphology was observed by Transmission Electron Microscopy (TEM) on a Tecnai 10 TEM (FEI Company, USA) operating at 80 kV. To conform the feasibility and sensitivity as magnetic cell separation nanoprobe, magnetic properties of synthesized nanoprobe was studied by use of Nuclear Magnetic Resonance Dispersion (NMRD) profiles (Spinmaster FFC2000, STELAR, Italy), in a field range extending from 200 μT to 1.2 T. Additional measurements of relaxation rate ($R_{1,2}$) were performed at 20 and 60 MHZ on Bruker Minispec system (Bruker, Karlsruhe, Germany).

The binding of mAb to SPIONs and the amount of immobilized antibody were determined by the Bradford assay. In brief, 40 μL of Coomassie Plus reagent concentrate were added to 160 μL of dispersion of nanoparticles, either mAb-coated or non-coated. After 10 min of incubation, the absorbance was measured at 595 nm using a microplate reader (Stat Fax, Awareness Technologies, USA). The results were compared to a standard curve of BSA solution in the concentration range from 10-150 μg mL^{-1} as illustrated in Fig. 2.

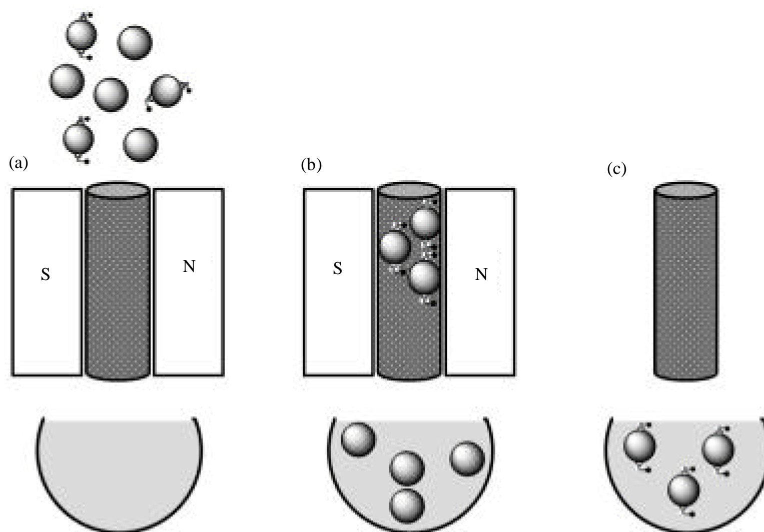

Fig. 2(a-c): Scheme of the magnetic bead-based cell separation: (a) A mixture of magnetically labeled and non-labeled cells is applied on a separation column, (b) Specific cell selection using MACS columns. Magnetically labeled cells are retained in the magnetic field of the separation column; unlabeled cells pass through the column as negative fraction, (c) After removal of the column from the magnetic field, the desired cells are eluted as the enriched positive fraction

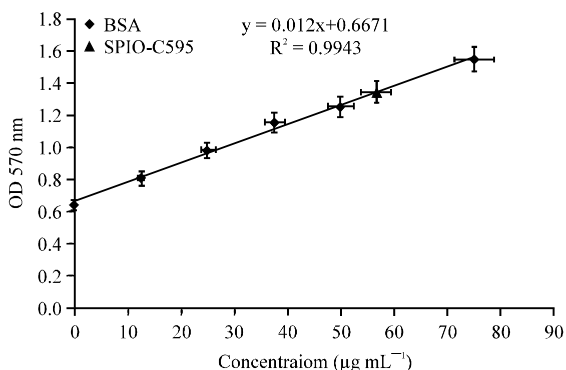

Fig. 3: Antibody concentration measurement by Bradford protein assay

The iron concentrations of the samples were measured by relaxometry measurements at 20 MHZ after digestion of samples by microwave oven. This was achieved by mineralization of sample in acidic conditions (0.2 mL sample, 0.6 mL HNO_3 and 0.3 mL H_2O_2) by microwave oven (Milestone MLS-1200, Sorisole, Italy). The millimolar iron concentration was determined from the longitudinal relaxivity (R_1) of samples which described previously (Abdolahi *et al*., 2013) and iron concentration was obtained as shown in Fig. 3.

Cell culture: OVCAR3, SKOV3 and A2780 adherent human ovarian cancer cell lines were grown in Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA). For OVCAR3, 10 µg mL^{-1} of insulin was used. The cells were cultured in 250 mL flasks, at 37°C in a humidified atmosphere with 5% CO_2. For subculture and harvesting the cells, they were washed with PBS followed by treatment with 3 mL Trypsin (Gibco, Grand Island, NY, USA) for 3 min to detach the cells. About 10 mL of culture medium was added to neutralize the Trypsin. The cells were then centrifuged at 2000 RPM for 10 min, followed by removing the medium and resuspended in complete media and reseeded into new culture flasks.

***In vitro* cytotoxicity:** To evaluate the effect of nanomag®-D-spio particles and synthesized nanoprobe on cell proliferation, the 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) assay (Sigma Aldrich, USA) was used (20). Three ovarian cell lines (OVCAR3, SKOV3 and A2780) were seeded at a density of 2×10^4 cells/well in 96-well plates and allowed to proliferate. Once adhered, the cells were incubated with either 0.1 mL of medium containing nanomag®-D-spio or

SPIONs-C595 at iron concentrations ranging from 0.2 to 2 mM for 2, 8 and 24 h. The culture medium without any particle was used as the control.

After incubation time 10 μL well^{-1} (5 mg mL^{-1}) MTT was added and incubation was continued for further 3 h. The medium was carefully removed and the formazan crystals (indicating cell viability) were solubilized by adding 100 μL DMSO (Sigma-Aldrich) per well. The absorbance was determined at 570 nm by the Statfax-microplate reader (Awareness Technology, USA). Experiments were performed in triplicate and cell survival was determined as a percentage of viable cells in comparison with control wells. One-way analysis of variance followed by Duncan's multiple range tests was used to determine whether the SPIONs caused any significant cytotoxicity.

Prussian blue staining: The principle of this method is that the ferric iron (Fe^{3+}) in the presence of ferrocyanide ion is precipitated as the highly colored and highly water-insoluble complex, potassium ferric ferrocyanide, Prussian blue. The ovarian cancer cells were detached and washed three times with PBS and about 10^6 cells/tube of each cell were suspended in 15 mL tube and incubated with culture medium containing nanomag®-D-spio or SPIONs-C595 at Fe concentrations of 2 mM (1 tube control) for 1 h at room temperature.

After incubation, the cells were washed three times with PBS to remove excess nanoparticles. Then, cells were fixed on 22×22 mm square glass coverslips with 4% glutaraldehyde, washed and stained using Prussian blue solution comprising equal volume of 2% hydrochloric acid aqueous solution and 2% potassium ferrocyanide (II) trihydrate. Slides were then washed and cells counter stained with nuclear fast red. The iron particles in the cells were observed as blue dots under a Nikon Eclipse TS100 microscope (Nikon Corp., Tokyo, Japan).

Surface antigen expression: To confirm the MUC1 expression on ovarian cancer cell lines as well as the capability of functionalized SPIONs to targeting a positive MUC1 ovarian cancer cells, Prussian blue staining were used. Also, quantitative expression of MUC1 on the cell lines was investigated by flow cytometry and new proposed method based on magnetic cell separation.

All cells at passage 4 were used for flow cytometric analysis. Briefly, the cells trypsinized, washed with PBS containing 10% BSA (washing buffer) and approximately 10^6 cells/tube of each cell type were plated into each tubes. The cells were resuspended in 90 μL of washing buffer and were preblocked with FcR Block (human) reagent (Miltenyi) for 10 min at room temperature in the dark. After blocking, appropriate amount of primary C595 mAb was added to each cell tube (one tube of each cell line as a control), incubated for 30 min in the dark at room temperature and then washed 3 times using a washing buffer.

After washing, the cells were resuspended and incubated in goat anti-mouse FITC (Fluorescein Isothiocyanate) mAb for an additional 30 min at room temperature in the dark. Cells were then washed, resuspended in 0.5 mL of PBS and examined immediately using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Data files were acquired and analyzed using BD CellQuest™ software.

Magnetic cell separation: Human ovarian cancer OVCAR3, SKOV3 and A2780 cells were detached and washed three times with PBS. A approximately 1-2×10^6 cell/tube of each cell type were plated in 15 mL tube and incubated with culture medium containing the synthesized nanoprobe (SPION-C595) at Fe concentrations of 2 mM. After 2 h incubation at room temperature, cells were washed with PBS three times and resuspended in 1 mL PBS containing 0.1% FBS. The magnetic cell separation was carried out on a midi MACS system. The LS separation column was set in the Midi MACS sorting device, washed twice with 1.0 mL of PBS solution. Then, the cell suspension was added to the separation column, washed three times to obtain the non-magnetic cells flowing through the sorting column. Finally, the LS separation column from the magnetic field was removed and eluted the double positive cells, then the $MUC1^+$ cells from the MS separation column was also separated. The number of $MUC1^+$ and $MUC1^-$ cells were detected and counted using conventional trypan blue staining, under an optical microscope. The percentage of MUC1 expression on the cell surface was determined as equation which described previously (Shahbazi-Gahrouei *et al*., 2013).

RESULTS

Conjugation of antibody with nanoparticles: The C595 monoclonal antibody was thiolated with Traut's reagent and conjugated to maleimide functionalized SPIONs (Fig. 1). The feasibility of successfully grafting of antibody molecules to SPIONs was confirmed by the Bradford assay as well as the measurements of the hydrodynamic size and shape of SPIONs by using PCS and TEM. Analyses by Bradford protein assay and spectrophotometric readings show an amount of immobilized antibody of 56-58 μg Ab/mL of synthesized nanoprobe (Fig. 3). By the use of standard curve which

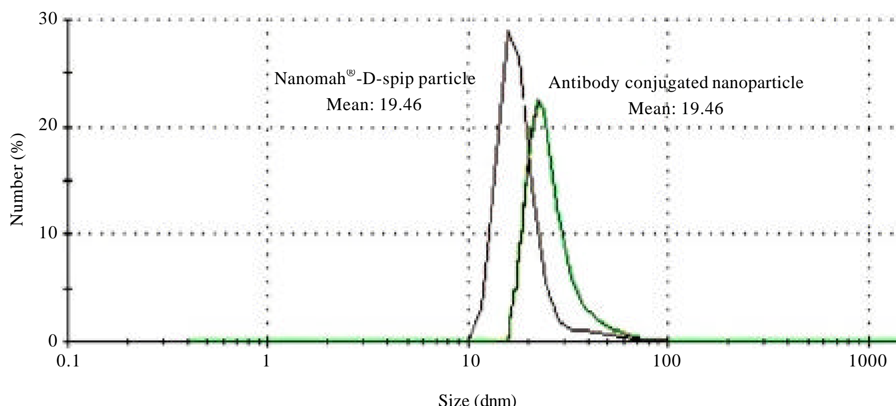

Fig. 4: Hydrodynamic diameter of nanomag-D-spio (19.46 nm) and C595-SPIONs (27.22 nm)

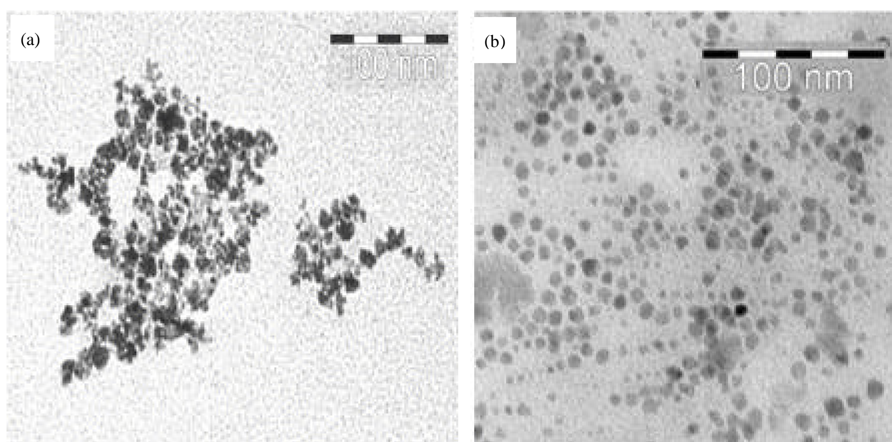

Fig. 5(a-b): TEM images for plain and antibody conjugated SPION: (a) nanomag-D-spio and (b) SPION-C595, antibody binding causes a significant reduction of particle agglomeration. The average particle size of particles estimated from TEM images was about 10-20 nm

prepared according to commercially available iron standard solution (ICP standard, Sigma Aldrich), the iron concentration of particles was measured the same as previous work (Shahbazi-Gahrouei *et al.*, 2013).

Characterization: The particle size distribution of SPIONs before and after antibody conjugation was determined by PCS (Fig. 4). The hydrodynamic particle diameters are determined to be 19.46±0.80 and 27.22±1.22 nm for nanomag®-D-spio and C595-SPIONs, respectively. Figure 5a and b show TEM images for the spherical shaped plain and antibody conjugated SPIONs, respectively. The average particle size calculated from TEM was 10-20 nm for both nanomag®-D-spio and C595-SPIONs. The morphology study of particles from TEM image suggests that antibody molecules conjugated to SPIONs reduce the agglomeration of nanomag®-D-spio particles.

Table 1: Longitudinal and transversal relaxivities (r_1 and r_2) of nanomag-D-SPIO and synthesized nanoprobe at 20 and 60 MHZ at 37 °C (Minispec) and the saturation magnetization and size of particles estimated by NMRD profiles data

	r_1 (s^{-1} mM^{-1})		r_2 (s^{-1} mM^{-1})		r_2/r_1	
Particle	60 MHz	20 MHz	60 MHz	20 MHz	60 MHz	20 MHz
Nanomag-D-SPIO	7.6	21.6	121.8	112.0	16	5.2
SPION-C595	7.2	14.9	106.5	83.7	14.8	5.6

R_1 and R_2 relaxation rate measurements of particles were performed on a Bruker Minispec operating at 20 MHZ and 60 MHZ. A summary of the longitudinal and transversal relaxivity is provided in Table 1.

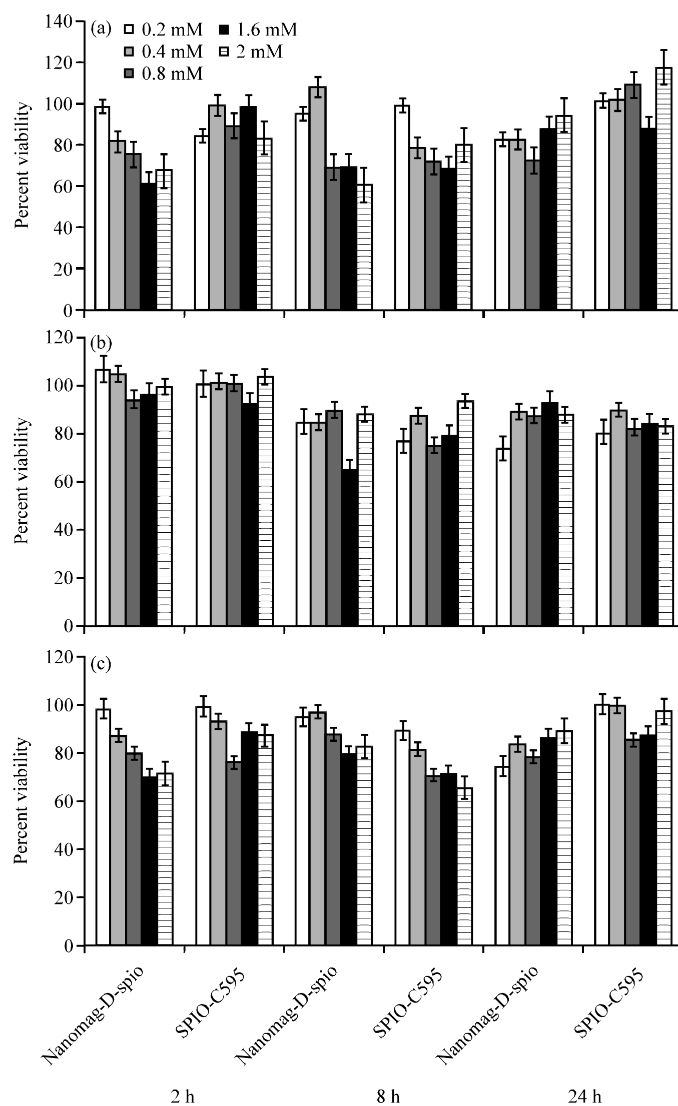

Fig. 6(a-c): *In vitro* assessment of cytotoxicity of Nanomag-D-SPIO and SPION-C595 in ovarian cancer OVCAR3, SKOV3 and A2780 cell lines by the MTT assay. The cells were incubated with Nanomag-D-SPIO or SPION-C595 at equivalent iron concentrations ranging from 0.2 to 2 mM for 2, 8 and 24 h

***In vitro* cytotoxicity:** The *in vitro* cytotoxic effect of nanomag®-D-spio and the synthesized nanoprobe was assessed using the standard Methyl Thiazol Tetrazolium bromide (MTT) assay, using ovarian cancer OVCAR3, SKOV3 and A2780 cell lines. As indicated in Fig. 6, the results after different incubation times with different iron concentrations for all cell lines show higher than 60% cell viability in relation to the control. The statistical analysis showed statistically significant evidence of non-functionalized or functionalized SPIONs toxicity to cells ($p = 0.02$). The P-value between 2-8, 2-24 and 8-24 h incubation of OVCAR3 cell line with SPIONs-C595 and nanomag®-D-spio was 0.01-0.15, 0.02-0.08 and 0.01-0.1, respectively. In comparison to the SKOV3 cell line these values were 0.02-0.21, 0.02-0.04 and 0.01-0.09. These values to the A2780 cell lines was 0.01-0.2, 0.01-0.52 and 0.001-0.07. The results of the MTT assay show a moderate negative correlation ($r = (-0.1)-(-0.8)$) between concentration and viability for the most of assays after 2 and 8 h incubation, but after 24 h incubation a positive correlation ($r = 0.08-0.6$) between the concentration and viability was found.

Cell Surface antigen expression: The qualitative and quantitative expression of MUC1 on ovarian cancer OVCAR3, SKOV3 and A2780 cell lines was investigated by Prussian blue staining, flow cytometry and proposed method based on magnetic cell separation.

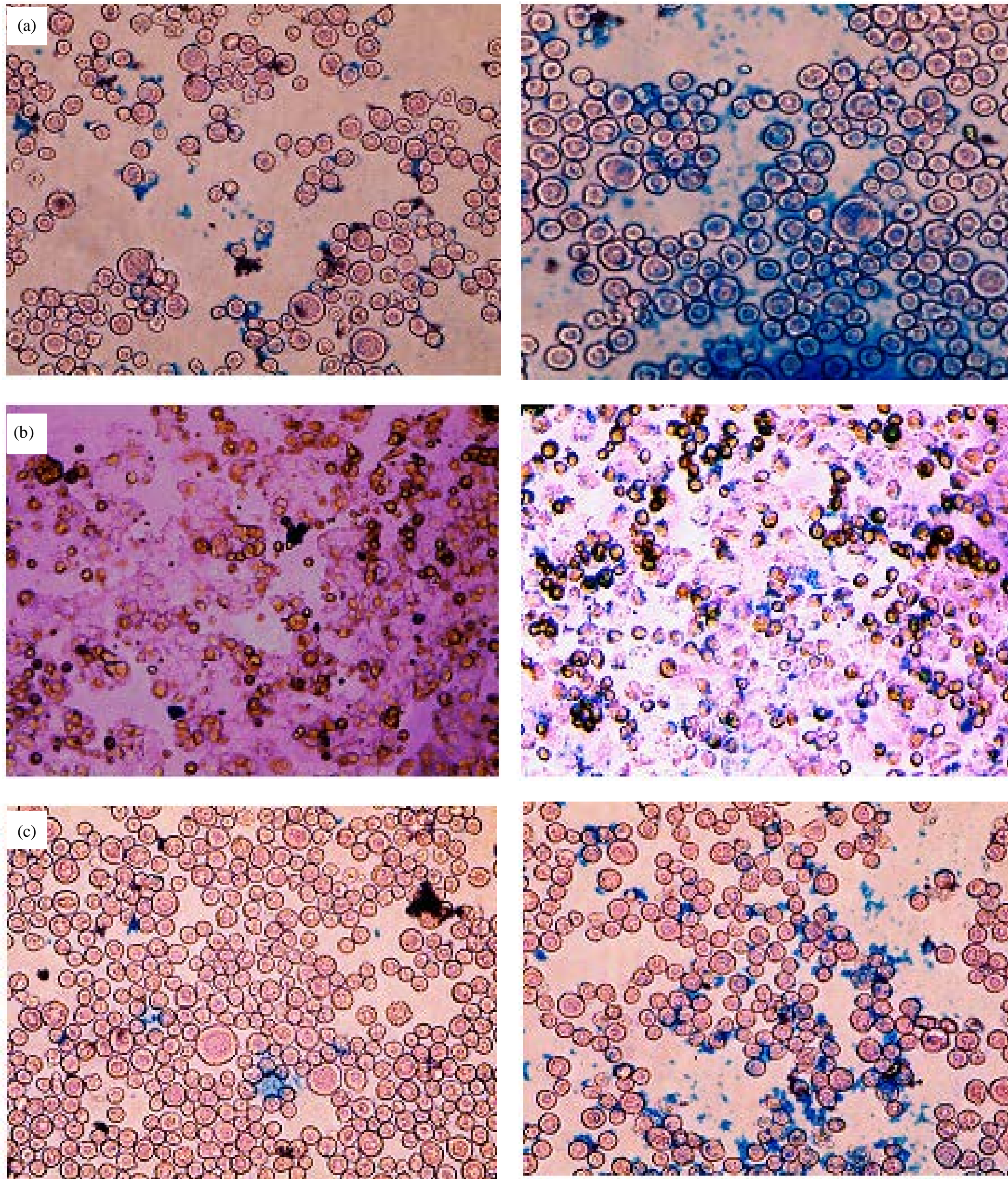

Fig. 7(a-c): Prussian blue staining images (objective magnification: ×40): (a) OVCAR3 cells after 1 h incubation with Nanomag®-D-spio (left) and SPIONs-C595 nanoprobe (right) (b) A2780 cells after 1 h incubation with Nanomag®-D-spio (left) and SPIONs-C595 nanoprobe (right), (c) SKOV3 cells after 1 h incubation with Nanomag®-D-spio (left) and SPIONs-C595 nanoprobe (right), SPIONs-labeled ovarian cancer cells show blue spots located inside cells, suggesting presence of iron oxide particles

The qualitative information on the cell surface antigen expression as well as specificity and cellular uptake of C595 functionalized and non-functionalized SPIONs to the cells were examined by Prussian blue staining. Blue areas or spots as shown in Fig. 7, represented the targeting effect and SPIONs uptake of functionalized particles on the cellular uptake behavior.

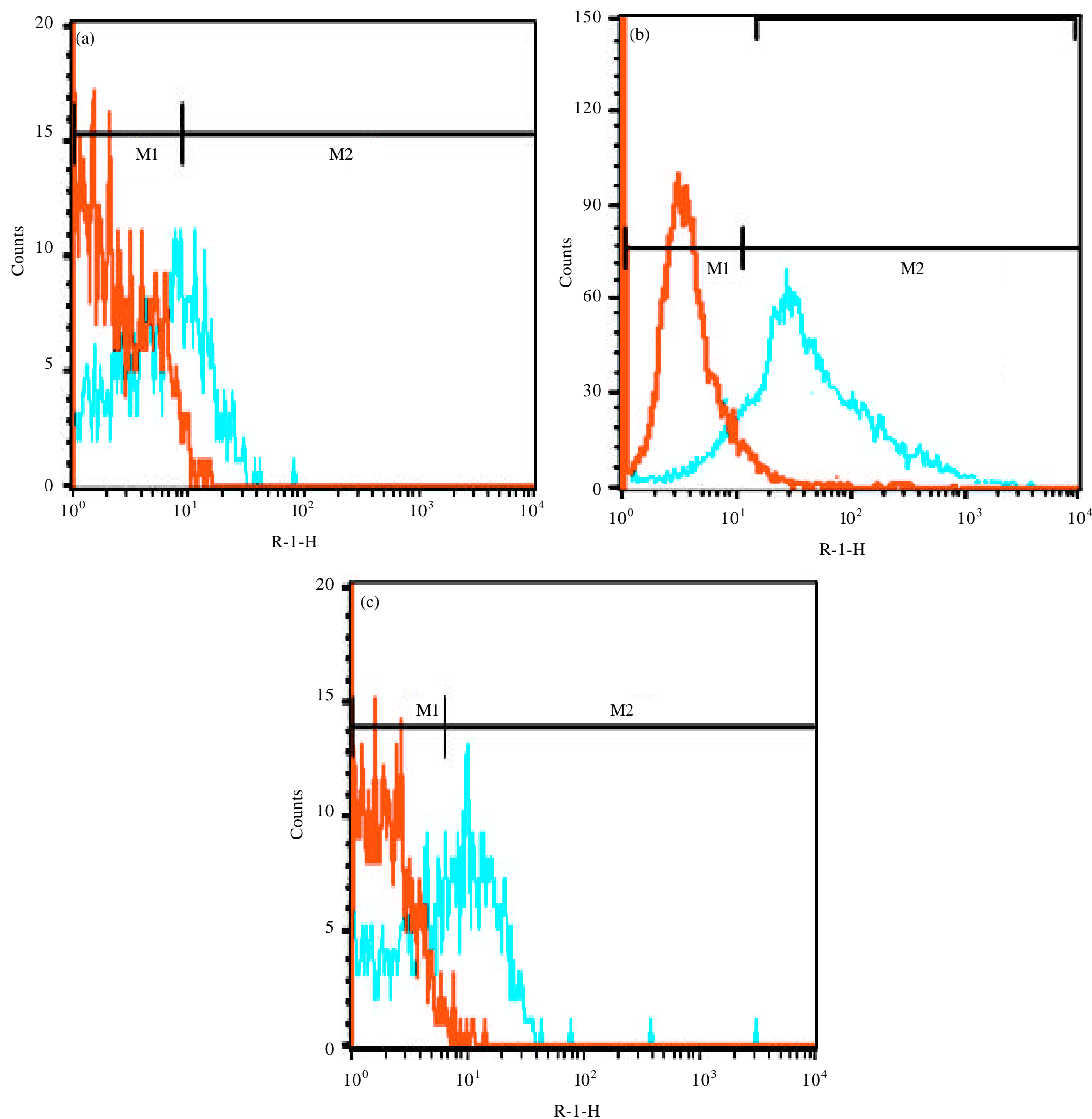

Fig. 8(a-c): Graphs of flow cytometry test for three studied cell lines: (a) A2780, (b) OVCAR3 and (c) SKOV3

Flow cytometric analysis was performed to confirm the availability and quantitative analysis of desired ovarian cancer cell surface antigen (MUC1). Immunofluorescence staining of OVCAR3, SKOV3 and A2780 cell lines showed that OVCAR3 cells express high levels of MUC1 on their cell surface (88.6±4.6)%, whereas SKOV3 and A2780 cell lines have a moderate MUC1 expression on their surface. These values were (48.18±2.03) and (28.3±1.23)% for two later cell lines, respectively (Fig. 8).

Immunomagnetic cell separation with use of synthesized SPIONs-C595 was used as a proposed method for detection and quantitative analysis of desired antigen expression on the ovarian cancer cells surface. Measurements based on magnetic cell separation method showed a (91.6±5.4)% expression of MUC1 on the surface of OVCAR3 cell line. For SKOV3 and A2780 cell lines these values were (42.1±3.4) and (27.7±2.4)%, respectively.

DISCUSSION

Detection and quantitative analysis of desired antigen expression of specific cell populations is a

prerequisite for many analytical and functional studies in basic research as well as for diagnostic and therapeutic applications. It has become obvious that these cell surface structures play an important role in early diagnosis, characterization, disease monitoring during and following therapy and as a therapeutic targets for various cancers (Sagara *et al*., 1999; Hoelzer, 2011). However, the difficulty and costs of detection, characterization and validation of new cell surface antigens has held back rapid development in this field. Hence, the development of a more efficient and inexpensive detection methods of the relevant marker is very basic and important.

The detection and quantitative analysis of MUC1 on the ovarian cancer cells as an example of a cell surface antigen as a highly specific, gentle and fast way was described. Functionalized iron oxide nanoparticles with anti-MUC1 monoclonal antibody (mAb C595) was done to serve as a MUC1-specific molecular probe for *in vitro* detection and separation of $MUC1^+$ ovarian cancer cell based on magnetic cell separation technique.

In addition, magnetic separation techniques have several advantages in comparison to traditional techniques for biomarker discovery. It is possible to coat nanoparticles with the ligand of interest, such as peptides (Jie *et al*., 2012), aptamers (Wu *et al*., 2011), dendrimers (Mirzaei *et al*., 2012) and folic acid (Saltan *et al*., 2011) and use this method to detect and analyze other biomarkers, that is not impossible by flow cytometry. Also molecular imaging enables non-invasive in vivo studies of biomarkers of diseases in various internal organs by use of magnetic nanoparticles coupled to targeted reagents (Ren *et al*., 2012; Abdolahi *et al*., 2013).

The results obtained in this study confirm and prove that the use of antibody-coated magnetic nanoparticles for isolation of antigen-specific cells is a convenient and simple method for quantitative cell surface antigen detection and analysis.

CONCLUSION

Results of this study confirm and prove that the use of C595 mAb targeted SPIONs conjugates for isolation of antigen-specific cells is a convenient and simple method for quantitative cell surface antigen detection and analysis for ovarian cancer cells as scheme are shown in Fig. 1.

In conclusions, findings of study showed that, through functionalization of SPIONs by C595 mAb specific binding to the MUC1-expressing ovarian cancer cells is achievable *in vitro* conditions. Prussian blue staining results and determination of iron uptake by cells also showed high affinity of SPIONs-C595 to MUC1-positive ovarian cancer cells. Overall, SPIONs-C595 nano-probe is potentially both, a selective ovarian molecular imaging tool as well as a therapeutic agent. Further subsequent in vivo studies and clinical trials appear warranted.

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