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Multiplexed magnetic nanoparticle-antibody conjugates (MNPs-ABS) based prognostic detection of ovarian cancer biomarkers, CA-125, β-2M and ApoA1 using fluorescence spectroscopy with comparison of surface plasmon resonance (SPR) analysis

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ABSTRACT

A multiplexed MNPs-Abs based fluorescence spectroscopic system in analysis of serum biomarkers; CA-125, β2-M and ApoA1 for the early detection of ovarian cancer was first time proposed. The lowest detection limits measured in multiplexed setup were 0.26 U/mL, 0.55 ng/mL and 7.7 ng/mL respectively for CA-125, β2-M and ApoA1. A comparative real sample analysis of healthy normal (Control), benign and ovarian cancer patients with SPR has also been done to validate the process. Moreover CA-125 detection only confirms 50–60% of early stage disease. This multiplexed system achieved sensitivity and specificity up to 94% and 98% respectively to distinguish early stage ovarian cancer patients from healthy individuals.

1. Introduction

Ovarian cancers are one of the most lethal gynecological malignancies worldwide. Due to the poor prognosis, identification of potential factors for prevention of ovarian cancer may have important clinical and public health implications. Ovarian cancers at early stage typically show only few specific symptoms therefore the 5-year survival rate among more than 70% patients at advance stage of diagnosis is less than 30% (Boente et al., 1999). In contrast, the 25% of patients who are diagnosed with stage I disease have a 5-year survival rate of up to 90%, and patients with stage II disease have a 5-year survival rate of up to 70% (Baker and Piver, 1994; Holschneider and Berek, 2000). Therefore, early detection of ovarian cancer has great promise to improve clinical outcome using different serum biomarkers.

A number of medical techniques to detect ovarian cancer at an early stage have been already in use for a long time like; laparoscopic test (Sarah et al., 2011; Rebecca et al., 2010) histological and biopsy (Robert et al., 2011; Huilin et al., 2014) ultrasound or ultrasonography (Paul and Christopher, 2003; Animesh et al., 2010; John and John, 2014). But all these tests remained presumptive even after quiet improvement in medical examination methods until the detection methodology reached to screening of specific biomarkers either alone or together with them. These biomarkers can either be tissue specific or serum based (Nolen and Lokshin, 2012; Jie et al., 2010; Brandon et al., 2011; Cecile et al., 2010; Kathleen and Ie-Ming, 2009; Weidong et al., 2013; Dan et al., 2011; Christine et al., 2008; Shapira et al., 2014; Yi-Wen et al., 2009). Screening and analysis of serum biomarkers is convenient in general compared to tissue specific as there is no need of biopsy or any other surgical methods. To analyze and quantify the ovarian cancer serum biomarkers, many methods have been used to detect the ovarian cancer at early stage such as; Quartz crystal microbalance (Sania et al., 2015; Jing et al., 2005), Mass spectroscopy (Charlotte et al., 2011; Wu et al., 2012; Soper et al., 2006; Ying et al., 2010), Immune assay (Archana et al., 2012; Shoumei et al., 2012; Siriwan et al., 2009; Zhifeng et al., 2008; Jesse et al., 2009), Electrochemical (Israa et al., 2013; He et al., 2013; Subramanian et al., 2012), surface Plasmon resonance (Brandon et al., 2011). Screening and quantification of serum biomarkers with routine medical examination methods enhanced the detection

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methodology of ovarian cancer at early stage. Of the serum biomarkers, CA-125 has been studied most extensively (Bast et al., 1983; Moore et al., 2010). CA-125 is shed from ovarian cancer cells and level can rise exponentially 10–21 months before diagnosis, but detects only 50–60% of early stage disease. Although CA-125 has a specificity of 99% in the postmenopausal population, in a premenopausal population, the specificity is further compromised by the presence of endometriosis, adenomyosis and retrograde menstruation that can elevate antigen levels. Elevated CA-125 levels do not always indicate ovarian cancer and levels can misrepresent a biological state. Therefore the detection of a single serum biomarker (like CA-125) is not always adequate to define status of cancer. Considering this fact, we build a multiplexed fluorescence spectroscopic based assay to detect biomarkers; β2-microglobulin (β2-M), Apo-lipoprotein A1(ApoA-I) and cancer antigen125(CA-125) at an early stage of ovarian cancer and comparatively analyzed with Surface Plasmon resonance spectroscopy (SPR). β2-M is synthesized by all nucleated cells and forms complexes with the heavy chain of MHC class I antigen through non-covalent linkage on cell surfaces (Pedersen et al., 1994). Down-regulation of MHC I occurs frequently in cancer cells, and this contributes to the immune evasiveness of cancer cells that allows them to escape from elimination by the attacking cytotoxic T cells (Seliger, 2005). However the raised serum levels of β-2M also has been described in some malignancies such as gastrointestinal (Hans et al., 1980) and breast cancer patient and correlate negatively with patient survival (Rasmuson et al., 1996). Similarly ApoA-I (28 kDa) is the major protein constituent of high density lipoprotein and plays an important role in reverse cholesterol transport by extracting cholesterol and phospholipids from peripheral cells and transferring it to the liver for excretion (Breslow et al., 1982). Decreased apoA-I levels were previously reported in the serum of patients with both ovarian cancer (Mahlck and Grankvist, 1994; Kuesel et al., 1992; Gadomska et al., 2005) as well as atherosclerosis (Dionyssiou et al., 2002). Serum lipid and lipoprotein association with cancer has been reported in numerous studies (Feinleib, 1981; Sorlie and Feinleib, 1982; Williams et al., 1981). Hence, the detection of β2-M and apoA-I may be useful as prognostic and therapeutic response indicators with CA-125 for the cancer patients. The purpose of proposed fluorescence spectroscopic based multiplexed assay is to make detection of early stage prognostic ovarian cancer biomarkers simple and cost effective compared to already available OVA1 test (U.S. Food and Drug Administration, FDA news release, 2011) that is quiet expensive, valued at roughly $650 per patient (Muller, 2010). Even though the proteomic based analysis is also expensive and needed pre sample treatment.

2. Material and methods

2.1. Patients and samples

All patients were enrolled at Vardhman Mahavir Medical College and Safdarjung Hospital had signed a declaration of consent stating that the patient's specimens may be used for scientific intentions. The blood plasma was collected from ovarian cancer patients (before surgery), benign and healthy women. The blood was collected in to red topped Vacutainer.

After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 15–30 min. Remove the clot by centrifuging at 1000–2000x*g* for 10 min at 4 °C.

The resulting supernatant is designated as a serum, and immediately transferred into a clean polypropylene tube using a Pasteur pipette. To avoid freeze-thaw, the serum was apportioned into 0.5 mL aliquots and stored at –20 °C or lower. The stages and grades of tumors from the ovarian cancer patients were assigned according to the guidelines provided by the International Federation of Gynecology and Obstetrics (FIGO), and the enrolled groups were then divided according to age.

2.2. Reagents

Ethanolamine hydrochloride (EA), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 2-(N-Morpholino)ethanesulfonic acid sodium salt (MES), Phosphate buffer saline buffer(BSA Buffer) 10X were purchased from Sigma-Aldrich. Bovine Serum Albumin (BSA, $\geq$95% protein, $\leq$5% H_2O) from thermoscientific. Carboxylated MNPs (0.1–0.2 μm, model EPR-FC-0.2) purchased from EPRUI (www.nanoparticles-microspheres.com). NaCl and NaOH from Merck Millipore, India. Alexa Dyes (405/532/680 nm) from Thermoscientific. Mouse monoclonal [1409] to Apolipoprotein A 1 from Abcam. polyclonal antibody APOA1[PAB9966], APOA1 (Human) Native Protein(P5326), AntiMUC16 monoclonal antibody [X325] (ab10033) from Abnova. AntiMUC16 polyclonal antibody (139531), MUC16 protein (Human, 138163), b2Microglobulin monoclonal antibody (M389002X), b2Microglobulin polyclonal antibody (139400AP) and Human b2Microglobulin (M389002J) from US Biological Lifesciences.

3. Experimental

3.1. Instrument and apparatus

All Fluorescence measurements were done on LS 45 Fluorescence Spectrometer, Perkin–Elmer. The SPR measurements were conducted on BiacoreX100 (GE Healthcare, Piscataway, NJ) at room temperature (25 °C) using 10 mM phosphate buffer pH 7.20. The UV–visible spectrum was recorded on UV-2450 ultraviolet–visible spectrophotometer (Shimadu, Japan).The FT-IR spectrum was recorded on spectrum 65 spectrometer (Perkin–Elmer,USA). Light scattering and zeta-potential measurements were performed on a Brookhaven Zeta PALS instrument at 25 °C.

3.2. Functionalization of magnetic nanoparticles (MNPs)

We did the functionalization of MNPs using classical EDC/NHS blocking chemistry (Sam et al., 2010) (Fig. S1) in improved way (Puertas et al., 2011) completed in two steps, initial rapid ionic adsorption of the Antibodies (Abs) on partially activated MNPs followed by a much slower Abs covalent attachment (subsection 1. S1 and 1.S2 of supplementary information). Thus, the irreversible binding (covalent) only occurs through the region of the Abs surface where ionic adsorption took place.

3.3. Characterization of covalently immobilized antibodies on the surface of MNPs

The UV–vis spectra of the Ab, MNPs (Fe_3O_4), and Abs-MNPs conjugates is shown in Fig. S5. There is an obvious absorption band at 255–290 nm in the spectra of Ab. However, a broad absorption band at 245–265 nm is clearly observed in the spectrum of MNP-Ab. This may be attributed to the formation of complex between Ab and MNPs. The shift of the absorption peak originates primarily from the light absorption and scattering of MNPs, and is the characteristic of semiconductors within direct band gap (Kaushik et al., 2008; Zhao et al., 2006). This suggests the successful immobilization of Ab on the MNPs surface.

The FT-IR spectra of MNPs and MNP-Ab conjugates as shown in

Fig. S6 exhibits a typical Fe–O–Fe absorption bands of HOOC–MNP at 548 cm^{-1}, and the C=O and O–H stretches of the COOH at ~1710 cm^{-1} and ~3500 cm^{-1}(Wang et al., 2010), which indicates the Carboxylic acid group was chemical bound on the surface of the Fe_3O_4 MNP. Comparing with the FT-IR spectra of carboxyl-Fe_3O_4 MNP, the characteristic peaks of N–H of the CONH at 3100–3600 cm^{-1} were observed in the FT-IR spectrum of MNP–Ab. Therefore, Ab is believed to coat on the surface of MNP by CONH formed through reaction between $-NH_2$ of the Ab and –COOH on the surface of MNP.

3.4. Antibodies labeling with dyes

Polyclonal antibodies (Anti-CA-125/Anti-β-2 M/Anti-ApoA1) labeling with Alexa succinimidyl esters 405 nm/532 nm/680 nm respectively:

Polyclonal antibodies (in PBS buffer pH 7.2 supplemented with .05% sodium azide) were diluted with 1/10th volume of 1 M solution of sodium bicarbonate pH ~8.3 to final concentration 1 mg/ml. Transfer 100 µL of Polyclonal Antibody solution to the vial of reactive dye (dissolved in DMSO). Cap the vial and incubate the solution for 1 h at room temperature by gently inverting the vial several times after every 10–15 min. In the meanwhile G-25 sephadex column was prepared by adding the slurry of G-25 beads (made by dissolving appropriate amount of bead in PBS buffer pH 7.2) in the empty column to make ~1.5 mL bed volume. Now wash the column with same buffer twice at 1100 g for 3 min and loaded with the whole reaction mixture drop-wise. After the spin down at 1100 g for 5 min, collect the labeled antibodies (polyclonal Anti-CA-125-Alexa405/polyclonal Anti-β2M-Alexa532/polyclonal Anti-ApoA1-Alexa680). The degree of labeling measured according to manufacturer supplemented protocol were found; 95%, 98% and 86% respectively for Alexa 405, Alexa 532 and Alexa 680 to the used antibodies. QY measurements of Anti-CA-125-Alexa405 (0.49), Anti-β2M-Alexa532(0.59) and Anti-ApoA1-Alexa680(0.34) were made in PBS (50 mM potassium phosphate, 150 mM NaCl, pH 7.2) at 22 °C relative to fluorescein in 0.01 M NaOH (QY=0.89).

4. Result and discussion

4.1. Calibration against standard CA-125, β2M and ApoA1 proteins

We used a simple fluorescence spectroscopic based methodology to detect level of blood antigenic markers (CA-125, β2M and ApoA1) for ovarian cancer. The experimental theme was presented in Fig. 1(a) and (b). In order to quantitatively measure the level of blood antigenic markers and to check the activity of labeled Abs and functionalized MNPs, we calibrated our proposed system against different concentrations of standard CA-125, β-2M and ApoA1 proteins as a control. The polyclonal antibodies; Anti-CA-125-Alexa405, Anti-β2M-Alexa532 and Anti-ApoA1-Alexa680 were diluted to 1:600, 1:500 and 1:600 respectively in PBS, pH 7.2 with 0.05% between 20, 150 mM NaCl and 0.06% sodium azide. Similarly MNPs labeled with monoclonal antibodies (Anti-CA-125, Anti-β2M and Anti-ApoA1) and the protein standards diluted up to different concentration ranges in the same buffer (PBS pH 7.2 with 0.05% between 20, 150 mM NaCl and 0.06% sodium azide).

4.2. Experimental conditions

During the all measurements, the same experimental conditions were set up like; used buffer, excitation and emission slit numbers and cuvette type and temperature. We used 1 mL right angle cuvette. In experimental measurement, 200 µL diluted monoclonal antibody labeled MNPs (~7000) were added in the cleaned and dry cuvette followed by addition of 50 µL of 0.1%BSA and incubated for 25 min. After that 100 µL of the standard protein samples (CA-125, β2M and ApoA1) of particular concentration were added and the system again was incubated for one hour to allow the proper binding of antigen and antibody. After that 100 µL polyclonal antibodies; (Anti-CA-125-Alexa405, Anti-β2M-Alexa532 and Anti-ApoA1-Alexa680) were added and system again incubated for 45 min. After this the cuvette was inserted in the LS 45 system and fluorescence was measured. A magnetic force is applied to settle down the sandwich (MNPs–monoAbs–Antigen–polyAbs) by putting the magnet at the base of cuvette holder and the fluorescence change was live monitored against the

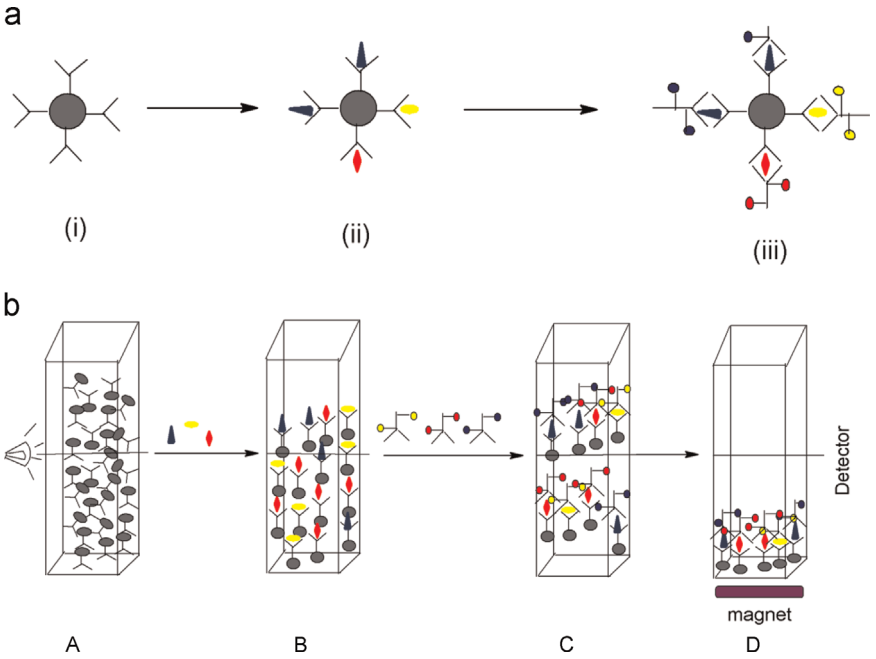

Fig. 1. (a) and 1(b). Experimental theme of Abs-MNPs conjugate based Fluorescence spectroscopic detection system, (i) and (A) MNPs-monoAbs, (ii) and (B) MNP-monoAbs–Antigenic biomarkers, (iii) and (C) sandwich of MNPs-monoAbs–Antigenic biomarkers-Alexa labeled poly Abs, (D) magnetic separation.

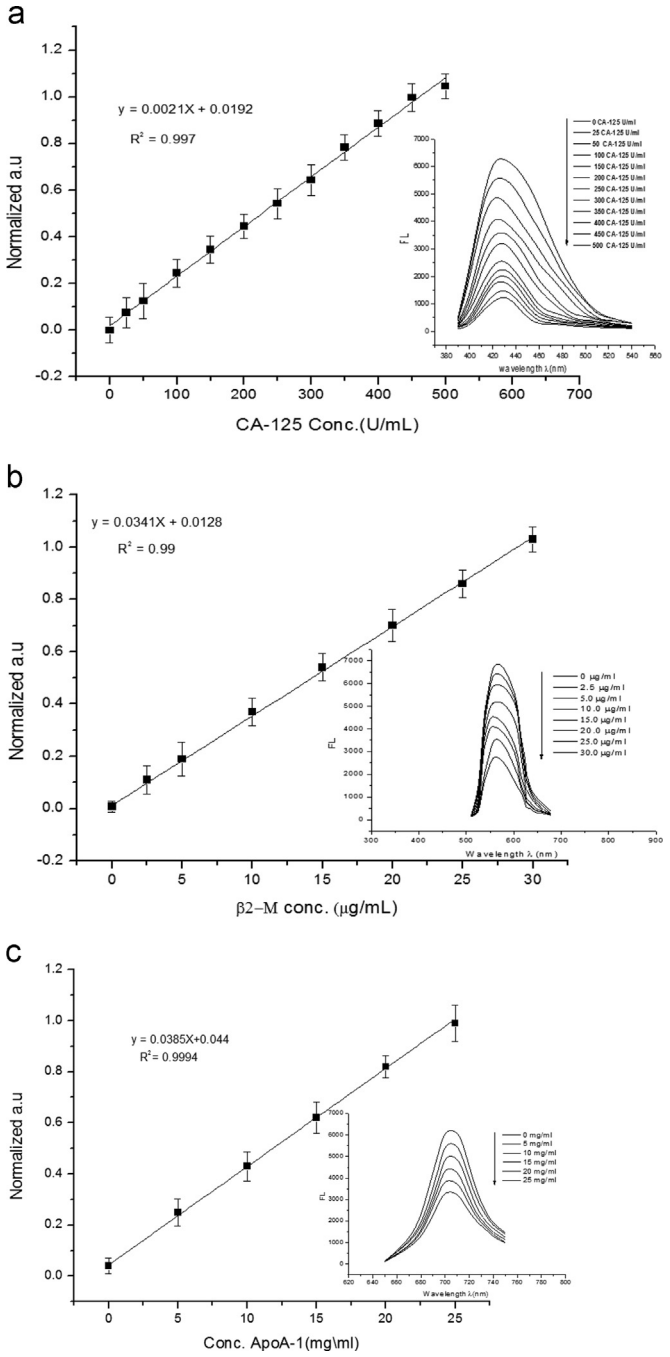

Fig. 2. [*,£]Normalized calibration of (a) CA-125, (b) β-2M and (c)ApoA1 biomarkers with increasing concentration (0.0–500 U/ml), (0.0–50.0 μg/ml) and (0.0–25.0 mg/ml) respectively, [*]Triplicate measurements, [£]The plotted intensities were normalized by dividing, all the changed intensities (after addition of biomarkers) from maximum changed in intensity (at higher concentration of biomarker). At "0" concentration of biomarker no change in intensity was observed so at this point normalized intensity should be considered as "0".

used concentration of standard. The system was calibrated considering the fact that labeled MNPs and polyclonal Abs should be two to three fold higher concentrated than standard and test protein sample. Their corrected responses for CA-125, β-2M and ApoA1 were mentioned in the Fig. 2(a)–(c) respectively.

4.3. Multiplexing of functionalized-MNPs and sample analysis

In order to make analysis of sample fast and validated, we proposed multiplexing of functionalized MNPs. 100 μL of each diluted, monoclonal antibody labeled MNPs (~3500) specific for CA-125, β-2 M and ApoA1 were added to the cuvette followed by addition of 100 μL of 0.1% BSA and incubated for 25 min at room temperature at room tepperature. After that 100 μL plasma sample drawn from healthy, benign and ovarian cancer patients was added and incubated for one hour. After this 100 μL of each polyclonal antibodies; (Anti-CA-125-Alexa405, Anti-β2M-Alexa532 and Anti-ApoA1-Alexa680) were added and system again incubated for 45 min at room temperature. The expected decrease in fluorescence intensities were recorded at room temperature for each targeted protein by changing the excitation and emission wavelengths; however the slit number kept constant throughout the experimental measurements. The obtained results were summarized in Table 1. The fluorescent scattering effect of sandwich system was also studied in subsection 1.S3 of supplementary information, In our multiplexing system we observed 10–13% scattered contribution in each case of standard protein analysis (Fig. S7 (a)–(c)). All the calibration was done after subtracting this artifact.

4.4. Detection limit and detection range

To measure the sensitivity of multiplex, the system was calibrated to measure the lowest limit of detection (LOD) as did in subsection 4.1. 250 μL of the alexa labeled polyclonal antibodies (Anti-CA-125-Alexa405, Anti-β2M-Alexa532 and Anti-ApoA1-Alexa680) diluted to 1:10,000, 1:8000 and 1:10,000 respectively in PBS, pH 7.2 with 0.05% tween 20, 150 mM NaCl and 0.06% sodium azide and 200 μL diluted monoclonal antibody labeled MNPs (~7000) blocked with 50 μL of 0.1%BSA. We measured the lower detection limit for proposed biomarkers (CA-125, β-2M and ApoA1) 0.26 U/mL, 0.55 ng/mL and 7.7 ng/mL respectively. All the measurements of standard proteins (Fig. S8(a)–(c) calibrated, covering lowest to highest ranges are suitable for the analysis of real blood samples.

4.5. Life time and reproducibility study

Life time and reproducibility was measured in terms, the persistent of immobilized antibodies on the surface of MNPs, photostability or bleaching effect of dyes labeled antibodies and activity with course of time. The % immobilization defined as: [(initial concentration of protein–protein concentration with course of time)/initial concentration of protein] × 100 and auto-desorption was determined by Bradford assay using standard calibration graph. It is well known that some MNPs could interfere with the Bradford assay. To be sure that the obtained results are not due to traces of MNPs present on the supernatants, despite having used a magnet and also centrifuged. Immobilization of antibodies on MNPs surface is quiet stable, less than ~12% desorpted (checked up to 4 months). Photostability test was done in similar buffer conditions; PBS, pH 7.2 with 0.05% between 20, 150 mM NaCl and 0.06% sodium azide, same power and exposure of lamp for each labeled antibody. The % of decrease of initial fluorescence was measured for one hour, Fig. S(9). The % decrease of initial fluorescent measured for Anti-β2M-Alexa532, Anti-CA-125-Alexa405 and Anti-ApoA1-Alexa680 were respectively ~18%, ~23% and ~30%. However the multiplexed system just needs maximum ~5 min to take a reading and to get a conclusion from calibration graph. The alexa labeled and MNPs functionalized antibodies in working buffer analyzed for six months without any significant error. The alexa labeled Abs can be stored at −20 °C and MNPs-Abs at 4 °C in opaque color vials for long term use.

4.6. Comparative analysis with SPR

To validate the process of MNPs-Abs fluorescence spectroscopic

Table 1
Analysis of serum biomarkers (CA-125, β-2M and ApoA1) using fluorescence spectroscopic multiplexed system and comparison with SPR.

Patient status		*Mean age (years)	**CA-125 (U/mL)		**β-2M (μg/mL)		**ApoA1 (mg/mL)	
			MNPs-Abs multiplexed	SPR	MNPs-Abs multiplexed	SPR	MNPs-Abs multiplexed	SPR
Healthy normal(Control)		42.5	15.2 ± 0.67	16.1 ± 0.67	1.84 ± 0.78	1.87 ± 0.57	7.34 ± 0.57	7.37 ± 0.52
Benign patients		35.6	43.3 ± 0.72	44.5 ± 0.47	2.13 ± 0.82	2.23 ± 0.48	1.43 ± 0.62	1.45 ± 0.63
Ovarian cancer patients		53.4	292 ± 0.58	295 ± 0.52	2.68 ± 1.23	2.71 ± 0.56	0.88 ± 0.43	0.90 ± 0.72
FIGO stage	i	46.4	30.2 ± 0.65	32.4 ± 0.37	2.18 ± 0.86	2.24 ± 0.53	0.98 ± 0.73	0.96 ± 0.43
	ii	56.6	181.5 ± 0.72	183.5 ± 0.67	2.28 ± ± 0.67	2.31 ± 0.46	3.42 ± 1.23	3.45 ± 1.3
	iii	57.0	565 ± 0.68	567 ± 0.87	2.86 ± 1.31	2.85 ± 0.52	1.35 ± 0.95	1.37 ± 0.77
	iv	55.1	864.5 ± 0.73	867 ± 0.37	3.34 ± 1.67	3.36 ± 0.62	1.07 ± 0.63	1.02 ± 1.2

* Values presented as mean.
** Values presented as mean of triplicate measurement, ± = S.D.

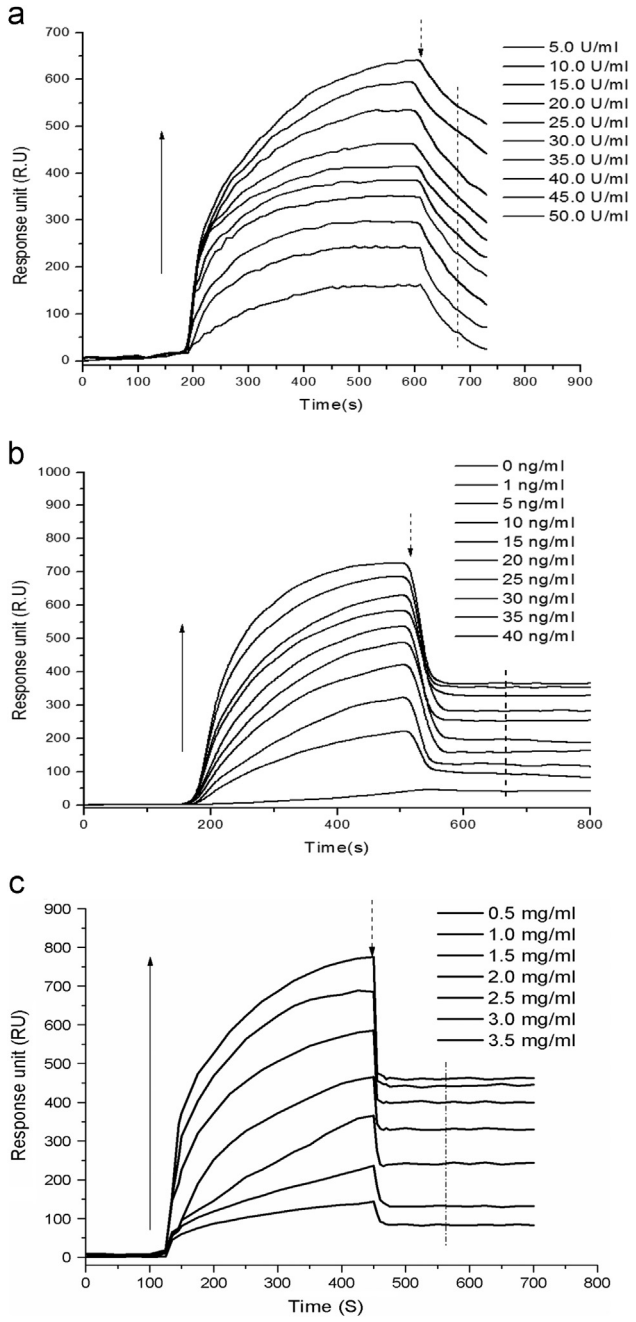

Fig. 3. (a), (b) and (c). surface plasmon resonance (SPR) response of standard CA-125 (5.0–45.0 U/ml), β-2M (0.0–40.0 ng/ml) and ApoA1 (0.5–3.5 mg/ml), upright solid arrow (↑), order of increasing concentration; down dashed arrow (⇣), off flow; dashed line (⋮) point of data measurement.

Table 2
Statistical analysis for ovarian cancer patients.

Biomarker tested	Sensitivity (%)	Specificity (%)	95% CI	NPV (%)	PPV (%)
CA-125	72	48	78–82	84	35
β-2M	42	44	80–92	69	31
ApoA1	35	38	85–92	48	29
CA125+ β-2M	86	59	92–93	94	41
CA-125+ ApoA1	88	68	89–94	96	53
β-2M+ ApoA1	65	58	86–90	76	43
CA125+β-2M+ ApoA1	94	98	92–94	96	81

based multiplexed system a comparative analysis was done using SPR.

4.6.1. Immobilization of antibodies on the SPR biosensor chips

All antibodies were immobilized on the CM-5 sensor chip (BiacoreX100, GE Healthcare, Piscataway, NJ)using the *N*-ethyl-*N*-(dimethylaminopropyl)carbodiimide(EDC)/*N*-hydroxysuccinimide (NHS) method according to the recommendation of the manufacturer. The carboxylated dextran matrix was activated by injection of 35 μl 200 mM EDC/50 mM NHS at a flow rate of 5 μl/min. A specified volume of unlabeled monoclonal antibodies (Anti-CA-125, Anti-β2M and Anti-ApoA1 was injected (100 μg/ml) in 10 mM sodium acetate buffer pH 4.5–5.5 depending on the isoelectric points of the antibodies. Residual activated groups were blocked by injection of 35 μl ethanolamine (1.0 M, pH 8.5), finally any pinholes on the surface were blocked with 1% BSA for 1 h. Approximately 7500 RU of antibodies were immobilized on the surface of the sensor chip. The sensor chips were stored at 4 °C until use.

4.6.2. Calibration of SPR response

Standard protein samples (CA-125, β-2M and ApoA1) were diluted in 10 mM Na-phosphate buffer pH 7.2 in different concentration ranges. Parameters affecting the performances of SPR were optimized. Initially 250 μl of 15 U/ml of CA-125, 300 μl of 20 ng/ml of β-2M and 250 μl of 1.5 mg/ml of APoA1with 10 mM phosphate buffer pH 7.20 at a flow rate of 5 μl/min, 10 μl/min and 10 μl/min respectively used. The response measured was the average of three injections. The optimization was performed by changing a single parameter while keeping other parameters constant. The optimum operating condition was considered by balancing between the signal and analysis time for one analysis. All the responses respective to different concentration of standards were measured; Fig. 3(a)–(c) and calibration plots drawn in Fig. S10(a)–(c).

4.6.3. Ovarian cancer patient's blood samples analysis by SPR

Before to measure the binding response of CA-125, β-2M and

Table 3
Comparison with already available multiplexed methods.

Method applied	Markers detected	Detection limit	Reference
Optical microresonators	Osteopontin, CA-125 and prolactin	Osteopontin(~270 pg/ml), CA-125(~1.8 U/ml) and prolactin (~290 pg/ml)	Huckabay et al., 2013
Electrochemical	IL-8 mRNA and IL-8 protein	IL-8 mRNA(3.9 fM), IL-8 protein(7.4 pg/ml)	Fang et al., 2009
Proteomic analysis	apolipoprotein A1(122 ± 42 mg/dl) and transthyretin,	apolipoprotein A1(122 ± 42 mg/dl), transthyretin(20 ± 7 mg/dl),	Zhang et al., 2004
Multiplexed immunoassay	Interleukin (IL)-6, IL-8, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1) and CA-125,	–	Elieser et al., 2005
Electrochemical Biosensor	alpha-fetoprotein, ferritin, carcinoembryonic antigen, human chorionic gonadotropin, CA 15-3, CA 125, and CA 19-9	< 2 ng/ml	Chandra, 2013
A microfluidic paper-based electrochemical biosensor	Glucose, lactate and uric acid	lucose (0.35 mM), lactate (1.76 mM) and uric acid (0.52 mM)	Chen et al., 2013
Fluorescence spectroscopy	CA-125, β2M and ApoA1	CA-125(0.26 U/ml), β2M (0.55 ng/ml) and ApoA1(7.7 ng/ml)	This paper

ApoA1 on respectively immobilized antibodies, we studied the nonspecific affinity of blood serum on the sensor surface, a 10% human blood serum diluted in 10 mM Na-phosphate buffer pH 7.20 buffer containing 1% BSA was pumped through the flow chamber for 10 min. The sensor response was measured in all sensing and reference channels and the level of nonspecific adsorption was estimated from the difference of the sensor response prior to the plasma injection and at the end. The average sensor response to nonspecific binding calculated from all channels was 0.042 mRIU. All plasma samples of ovarian cancer patients's were analyzed and responses were recorded after subtracting the background responses. The obtained responses of different plasma samples were used to measure the concentration of proposed biomarkers using their respectively drawn calibration graphs in different ranges; CA-125 (5.0–40 U/ml), β2M (1.0–30.0 ng/ml) and ApoA1(0.5–3.0 mg/ml) in Table 1.

4.7. Statistical analysis and comparison with reported multiplexed

The results obtained from patients with different stages (Table 1) of ovarian cancer were statistically analyzed. The sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) of the level of serum CA-125, β-2M and ApoA1 were compared among different stages. In Table 2; statistical data for ovarian cancer patients with the mean age of 53.4 years presented. Most of them were premenopausal (161 women or 88.4%). We measured sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) for all together (CA-125+β2M+ApoA1) 94%, 98%, 96% and 81% respectively using multiplex system. The individual calculated sensitivity and specificity of CA-125, β2M and ApoA1 was low compared to in groups. We also compared the reported multiplexed detection methods with our proposed method. The detail of comparison was reported in Table 3.

5. Conclusion

In the present study, first time multiplexed MNPs-Abs based fluorescence spectroscopic quantitative analysis of three serum biomarkers; CA-125, β2M and ApoA1 to detect ovarian cancer at an early stage has been performed. However CA-125 serum based biomarker has been extensively studied but its level can be raised in non-cancer patients and also vary in different stages of ovarian cancer patient. Moreover CA-125 detection only confirms 50–60% of early stage disease. In this study, a combination of; CA-125, β2M and ApoA1 achieved a sensitivity of up to 94% at a specificity of 98% to distinguish patients with early stage ovarian cancer from healthy individuals. Proposed multiplexed assay is also cost effective as compared to cost of single OVA1 test/patient.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at 10.1016/j.bios.2015.05.051.

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