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# Sensitive Detection of Epithelial Ovarian Cancer Biomarkers Using Tag-Laser Induced Breakdown Spectroscopy

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## 1. Introduction

Epithelial ovarian cancer (EOC) is the 5th leading cause of death from cancer in women and the main cause of death from gynecological cancer (Barber, 1986). Women have a lifetime risk of ovarian cancer of around 1.5%, which makes it the second most common gynecologic malignancy after breast cancer. Ovarian cancer is often referred to as 'the silent killer' because it frequently causes non-specific symptoms, which contribute to diagnostic delay, diagnosis in a late stage and a poor prognosis. This is one of the reasons for the relatively low, approximately 40%, 5-year survival rate for women diagnosed with advanced EOC. However, when EOC is diagnosed at an early stage this rate increases up to 95% (McGuire *et al.*, 2000). This enhancement demonstrates that early detection of EOC is crucial and it is vital to develop novel diagnostic methods for higher throughput screening of human samples and new biomarkers discovery.

One of the important and promising strategies for early cancer diagnosis relies on the development of approaches that can provide accurate detection and identification of specific protein-biomarkers in the serum. These protein-biomarkers would be measured and monitored to yield specific signatures that can be used for the early detection of the disease. Recently, numerous reports demonstrated that a *single* biomarker (example: CA 125, biomarker of ovarian cancer) approach is highly unlikely to yield results that can accurately distinguish cancer samples from healthy ones. This led researchers to explore the idea of using a *basket* of biomarkers (Petricoin *et al.*, 2002; Mor *et al.,* 2005) with the expectation that this approach may yield increased specificity and sensitivity for cancer detection. Using this approach, G. Mor *et al.* reported 95% efficiency discrimination between disease-free and EOC patients, including patients diagnosed with stage I and II disease (Mor *et al.*, 2005). These authors used a blood test, based on the simultaneous identification of four biomarkers: leptin, prolactin, osteopontin, and insulin-like growth factor-II. Petricoin *et al.* reported the use of mass spectroscopy to develop a classifier that could identify serum from patients with ovarian cancer with 100% sensitivity and 95% specificity (Petricoin *et al.*, 2002). In a follow up study, Zhu *et al.* reported similar results (Zhu, 2003). However, questions were raised about tests reproducibility and reliability (Wagner, 2003; Garber,

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2004). Despite this roller coaster, the need for the development of high-throughput methods and multiplexing assays for the simultaneous detection of multiple analytes is real (Rubenstein, 2010).

Several approaches addressing the development of multiplexing assays have been reported in recent years. C.A. Mirkin *et al.* proposed using oligonucleotides as barcodes (DNA barcode) and nano-particles aggregates that can alter their physical properties (e.g. optical, electrical, mechanical) upon aggregation (Mirkin *et al.*, 2005). The simultaneous measurement of serum proteins and sequence-specific oligonucleotide probes by employing 16 sets of fluorescent microspheres and a flow cytometer has been demonstrated (Fulton *et al.*, 1997). Recently, flow injection inductively coupled plasma mass-spectrometry and macrocyclic metal chelate complexes loaded with different lanthanides have also been used to detect several model peptides and protein (Ahrends *et al.*, 2007). Further, application of multicolor quantum dots for molecular diagnostics of cancer was reported (Smith *et al.*, 2006) and barcodes striped metal nanoparticles were used to provide multiplexed data in various bioassays (Freeman *et al.,* 2005).

Many of the multiplex assays developed recently are based on antigen induced particle aggregation (Gosling *et al.*, 1990). Therefore, it is important to strive to better understand the aggregation mechanism. Various systems have been employed to study the aggregation process. Examples include lipid vesicles (Farbman-Yogev *et al.*, 1998) and metal/metalloid particles (Freeman *et al.*, 2005; Smith *et al.*, 2006). A simplified vesicle aggregation theory was developed (Farbman-Yogev *et al.*, 1998). This theory deals primarily with vesicles made of biotinylated lipid molecules. The presence of streptavidin molecules induces aggregation (flocculation). The vesicles are made of amphiphilic molecules combined to form shell-like bilayer structures. Therefore, the bonds between lipids and vesicles can be weaker than bonds between biotins on lipids and streptavidin molecules in a solution. This theory predicts the dissociation of initial cross-linked molecules from the vesicles and their re-aggregation into preferably smaller aggregates (Farbman-Yogev *et al.*, 1998).

Particle based immuno-assays have been reviewed in a number of publications (Gosling *et al.*, 1990; Smith *et al.*, 2006). Metal and metalloid particles are typically modified with the IgG molecules, which are strongly (irreversibly) attached to the surface. The presence of antigen molecules induces aggregation. In contrast to lipid vesicles, solid-state particles are unlikely to lose the cross-linked molecules and the particle aggregates are more stable (Farbman-Yogev *et al.*, 1998). From this perspective, the metal and metalloid nano- and micro-particles seem more appropriate for developing effective immuno-assays.

## 2. Tagging specific proteins: Multi-element coded nano- and micro-particle assay

As discussed above, multiplexing combined with particle-based assay provides an effective and promising approach for developing diagnostics. After thorough consideration of advantages and disadvantages of the existing technologies briefly described above, we present a novel type of assay on a base of multi-element coded nano- and micro-particle tags and Laser-Induced Breakdown Spectroscopy (LIBS) as a detection method (Meelikechi and Markushin, patent pending). This approach, developed in our laboratory, relies on the use of nano- and micro-particles composed of different chemical elements to yield single and multi-element code for labeling of the molecules of interest.

To illustrate this approach, we show in Figure 1 a schematic of the multi-element coded nano- and micro-particle based assay composed of *2 elements: Si and Fe*. To perform immunoassay we used ovarian cancer biomarkers *Leptin and CA 125* with pairs of relevant monoclonal antibodies. Monoclonal antibodies were biotinylated prior to performing the assay. To mimic blood conditions, all buffers contained about 5% of bovine serum albumin (BSA) (Majoor, 1946). For separation of single and aggregated particles we used test tubes equipped with 5 μm pore size filters or magnetizing.

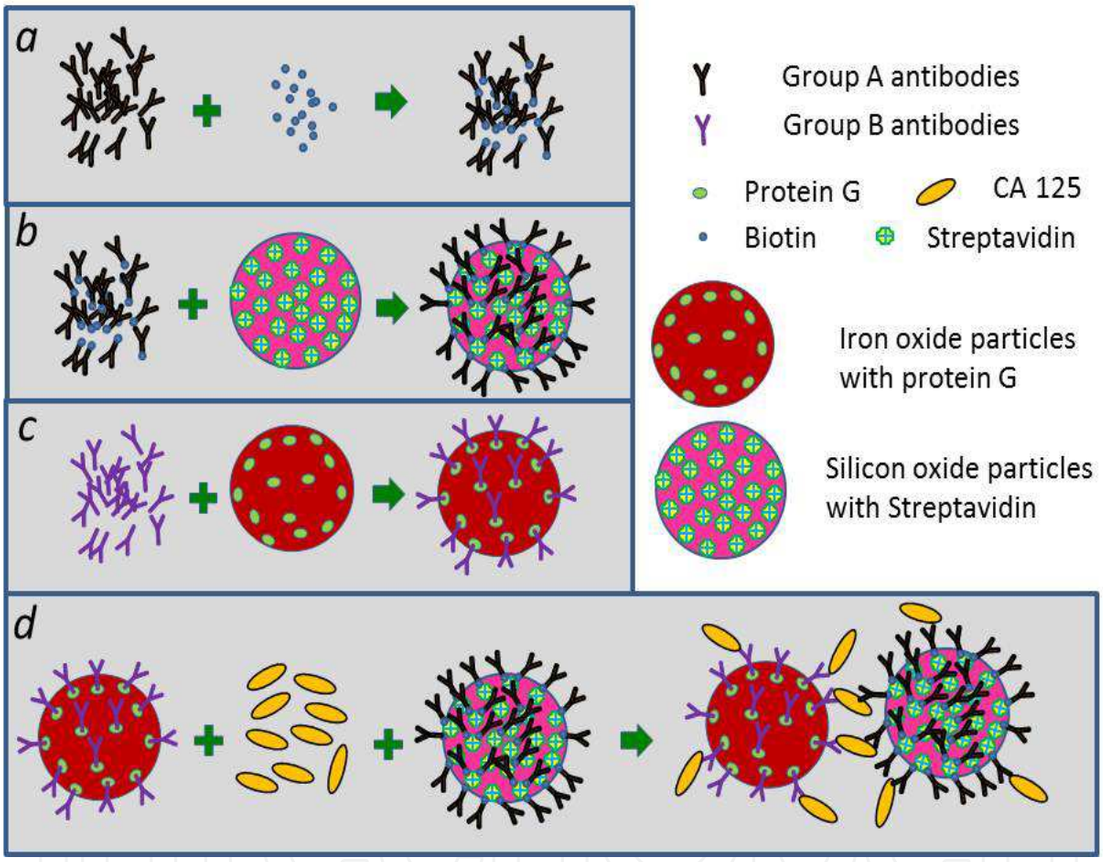


Fig. 1. Tag-LIBS experimental schematics. **Step a** – biotinylation of the group A antibodies (M86429M); **step b** – attaching biotinylated group A antibodies (M86429M) to silicon oxide particles modified with streptavidin (suspension B); **step c** – attaching of the group B antibodies (M86306M) to iron oxide particles modified with protein G (suspension A); **step d** - incubation (following by magnetizing and washing) of the mixture of suspensions A and B with various concentrations of CA 125.

Many optical detection techniques developed to perform assays are based on the use of fluorescent tags (Zal and Gascoignea, 2004). Although these techniques have many advantages, they often suffer from photo bleaching and it is therefore advantageous and

useful to explore the development of approaches that do not suffer from such a limitation. To do this, it is useful to note that there is a broad class of materials (metals and non-metals), which are neither fluorescent nor effectively chemically active. These materials can be combined to yield composites that can also be used as tags. This provides a large number of possibilities: combining only 11 of such materials (i.e. iron, gold, silver, platinum, aluminum, copper, titanium, nickel, zinc, tin and copper) into micro- and nano-particles can yield more than 1000 types of different composite particles, some of which are well known alloys such as brass (copper and zinc), bronze (copper and tin), and duralumin (aluminum and copper). Each of these alloys and composites is unique by chemical content and potentially can be used as micro-tags for labeling and detection of ovarian cancer biomarkers.

Below we provide examples of the use of the multi-element coded particle assay for the detection of analytes.

## 3. Reading the tags: Laser induced-breakdown spectroscopy for early cancer diagnosis

Following the sample preparation described in the previous section, we focus our discussion on the detection of the micro- and/or nano-particles attached to the specific biomarkers of interest. To perform this step, we use an all-optical technique widely used in many applications ranging from space exploration to quality control but only rarely for medical applications. This technique, known as Laser Induced Breakdown Spectroscopy (LIBS) is well described in the literature and only a brief description is provided in this chapter. For more information, we refer the reader to the excellent body of literature available (Cremers and Radziemski, 2006, Miziolek *et al.* 2006, and Markushin *et al.*, 2009).

LIBS is an analytical technique based on the use of laser pulses intense enough to breakdown the chemical bonds of the constituents of a sample to be interrogated. The experimental arrangement used in our laboratory is shown in Figure 2. By focusing nanosecond long laser pulses onto a sample, a short-lived plasma of the sample is generated. These laser pulses ablate a small quantity of the sample - a few hundreds of nanograms - located on an automated 3-D translational stage. Light emitted by the plasma during cooling is collected by a bundle of optical fibers, which transmits it to a 7-channel Ocean Optics LIBS HR2000+ spectrometer (190 - 970 nm) for analysis. The 10 nsec Q-switched Nd-YAG laser (BM Industries Serie 5000) operating at 1064 nm was used for the sample ablation. About 100 laser shots on 100 different spots on a filter were used to collect the LIBS spectra. For the purpose of this investigation, we used average laser pulse energies of about 70 mJ/pulse, a double convex BK7 focusing lens with a focal length of 70 mm and antireflection coating for 1064 nm was positioned at about 61.7 mm distance from the surface of the filter. LIBS spectra were obtained at ambient atmospheric conditions. To identify the measured atomic and ionic lines, we use the LIBS spectral database developed by our group (OSCAR website, n.d., Rock *et al.*, 2008). Potentially, the multi-element coded assay is able to detect and identify numerous analytes in parallel with minimum interference. This method has the added advantage of requiring as little as a few micro liters of serum specimen. More details of the experiment can be found elsewhere (Markushin *et al.*, 2009, Rock *et al.*, 2008).

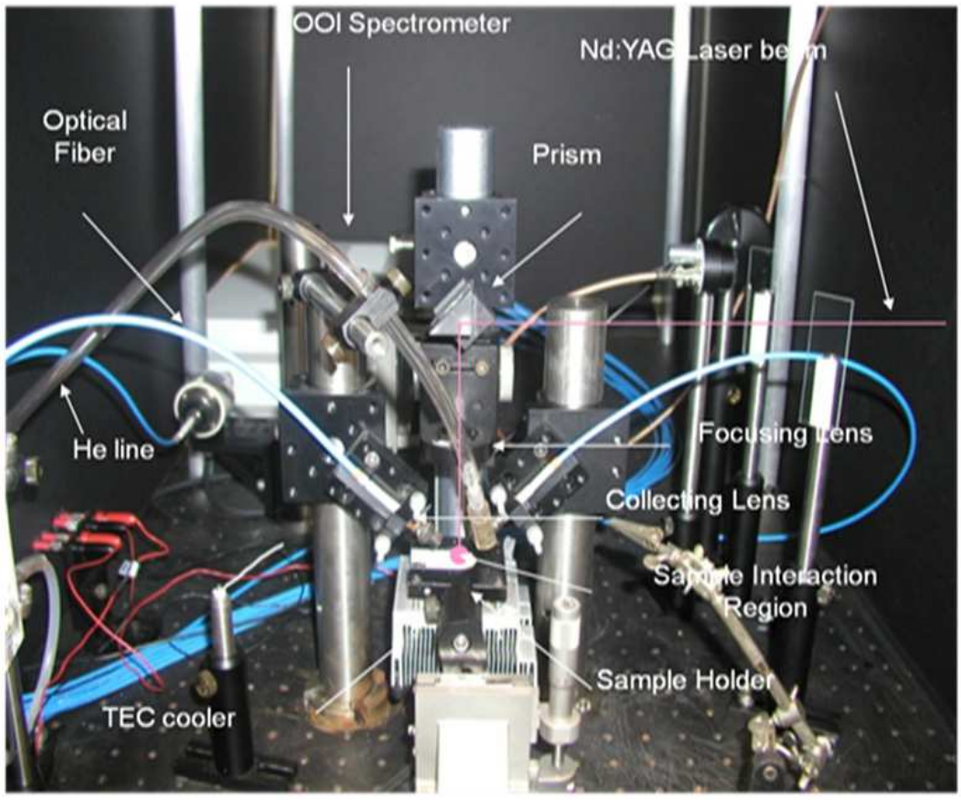


Fig. 2. LIBS experimental set-up consisting of a Nd-YAG Laser pulse laser, prism, focusing lens, bundle of light collecting optical fibers, thermoelectric cooler (TEC), a Helium line and the Ocean Optics 7-channel spectrometer (OOI spectrometer).

To implement the multi-element coded approach and demonstrate its potential, we prepared and analyzed two-element composite particles (Markushin *et al.*, 2009). We used 1.5 μm iron oxide biotinylated particles and 3 μm silicon particles with attached avidin. First, two types of control experiments (experiments #1 and # 3) were performed. In control experiments we checked for the possibility of nonspecific binding of iron oxide biotinylated particles to the plastic lab ware components (LIBS spectrum of experiment # 1 on Fig. 3a, 3b). Nonspecific binding was found to be insignificant. In another control experiment, # 3, (Fig. 3a, 3b) iron oxide biotinylated particles were pre-incubated with an excess of avidin molecules that allowed neutralizing the biotin groups of iron oxide particles with avidin. The following incubation of the neutralized iron oxide particles with Si-avidin particles and LIBS analysis demonstrates that nonspecific interactions between both types of micro-particles are limited. Some portion of silicon particles with probably bigger than the 3 μm particle size can be trapped by 5 μm pore size filter (short-dashed line on Fig. 3a, 3b). This was confirmed by additional control experiment with filtering of the 3μm size silicon particles over 5 μm pore size filters (data not shown).

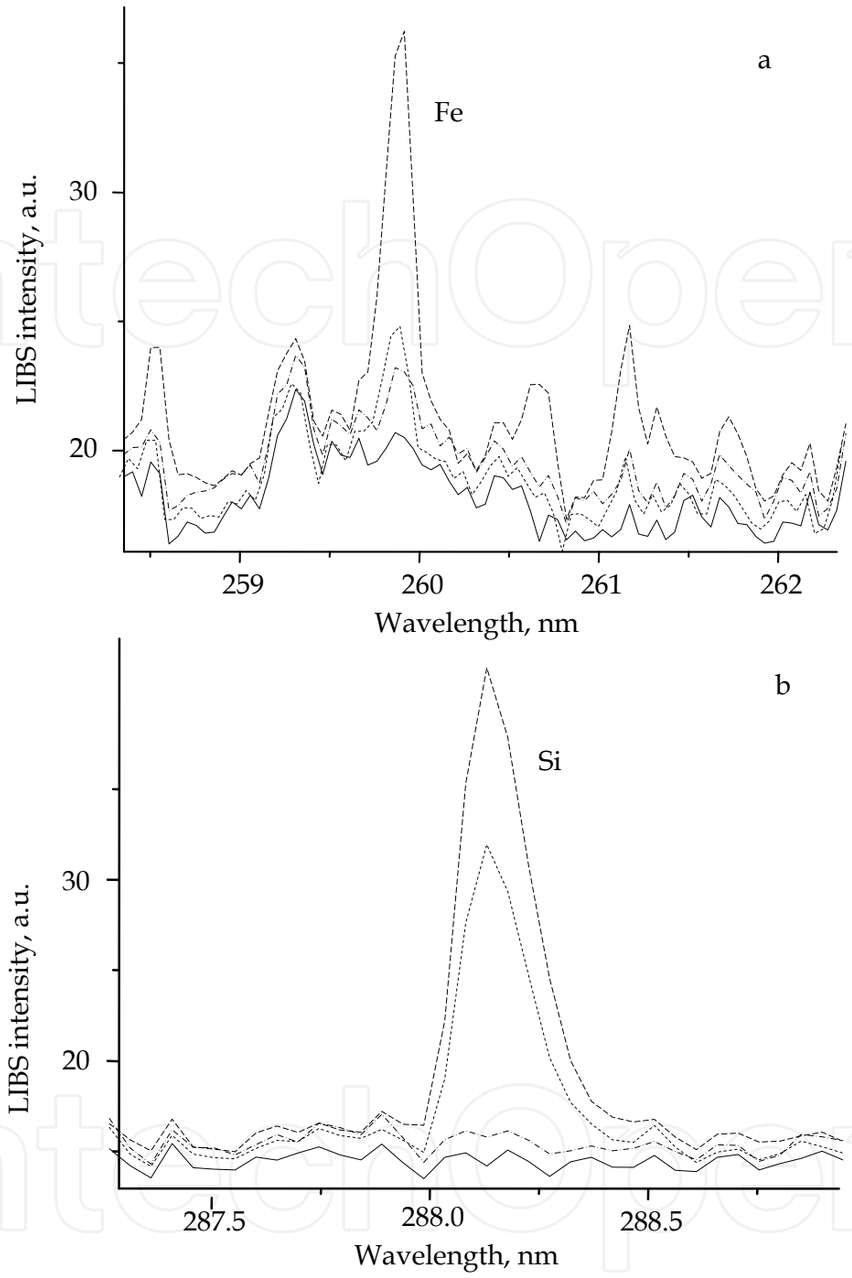


Fig. 3. LIBS based identification of two-element Fe-Si composite micro-particles. The fragment of the LIBS spectrum around 288.1 nm Si line (a). The fragment of the LIBS spectrum around 259.9 mn Fe line (b). Solid line – empty filter, dot-dashed line - experiment # 1, dashed line - experiment # 2, short-dashed lines - experiment # 3 (adapted from (Markushin *et al.*, 2009))

The two-element coded composite micro-particles were prepared by allowing the iron oxide biotinylated particles to interact with the silicon particles modified by avidin (experiment # 2). We monitored the amount of aggregates by taking 140 laser shots at the surface of 5 μm

pore size centrifuge filters. After overnight incubation the filtrate with unbound micro-particles was removed by centrifuging over 5 μm pore size filters, then the top part of the test tube was cut off and the bottom part with the particles being left on a filter (residue particles) was checked by LIBS for the presence of Fe and Si elements (dashed line on Fig. 3a, 3b). The presence of both Fe (259.9 nm) and Si (288.1 nm) emission lines in the same sample proves that we generated the two-element coded composite micro-particles. Thus, the ability of LIBS to detect the presence and composition of the micro-particles is demonstrated. Further, we suggest that this technique can be employed as a detection method for the future element coded assay development.

Sensitivity is a key factor of any analytical method. To determine the sensitivity of the element-coded approach, we used the model protein avidin. We performed detection and quantification of avidin molecules using LIBS based iron oxide micro-particle assay (Fig. 4). The details of the experiment can be found elsewhere (Markushin *et al.*, 2009, Rock *et al.*, 2008). 1.5 μm iron oxide micro-particles coated with biotin were purchased from Bangs Laboratories and their aggregation was induced upon the addition of avidin. We monitored the amount of aggregates by using 140 laser shots at the surface of 5 μm pore size centrifuge filters after removing the filtrate with non-bound micro-particles. Figure 4 shows the avidin concentration dependence of the LIBS based intensity of Fe emission line at 259.9 nm integrated over the filter surface. The iron oxide micro-particle assay demonstrated limit-of-detection about 30 ppb of avidin. This Figure has a significant maximum at about 155 ppb and is discussed below.

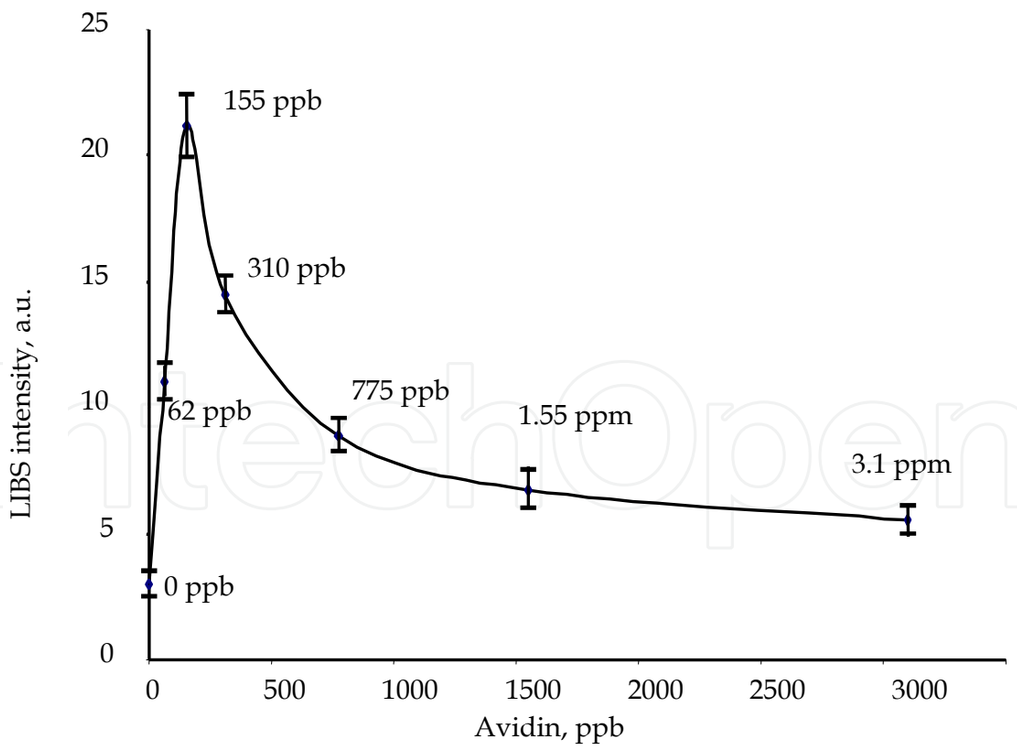


Fig. 4. Avidin concentration dependence of the micro- particles aggregation (adapted from (Markushin *et al.*, 2009))

We employed the element coded LIBS based approach for the detection of biomarker CA 125. For this particular measurement, we express the concentration of CA 125 in International Units (IU or U) per milliliter. Typically these units are used for quantification of biologically active substances (e.g. CA 125) instead of grams or moles (World Health Organization (WHO) Expert Committee on Biological Standardization, n.d.) According to the WHO Expert Committee on Biological Standardization, an International Unit is the specific biological activity of a substance, i.e. the quantity of a biologically active molecules required to produce a defined response. The use of such internationally accepted units ensures that biologically active substances with the same measured response will contain the same quantity expressed in International Units.

We also used the two-element micro-particle coding method to detect ovarian cancer biomarker CA 125. To do this, we prepared two bio-suspensions for groups A and B IgG antibody tagging (Fig. 1). In suspension B the biotinylated group A antibodies were immobilized to the silicon oxide micro particles with streptavidin. In suspension A the group B antibodies were immobilized through their Fc (fragment crystallizable region) fragments to iron oxide micro particles modified with immunoglobulin-binding protein G. Protein G mediates attachment of antibodies to the micro-particles and ensures the proper orientation of IgG molecules for the better immuno-assay efficiency (Bjorck & Kronvall, 1984). The buffer used contained 5% of BSA to mimic blood conditions (Majoor, 1946).

The following is a step-by-step description of the two-element micro-particle coding assay. To perform the analysis on the presence of CA 125 in a solution we follow the following procedure:

Step 1. the monoclonal antibodies M86429M were biotinylated prior to doing assay. EZ-Link Sulfo-NHS-Biotinylation Kit (Pierce Biotechnology, Inc.) was used for this purpose. PBS buffer used for dilutions contained 5% of BSA to mimic blood conditions (Fig 1 a).

Step 2. we prepared suspension B: the 1 μm silicon oxide particles (Bangs Laboratories, Inc.) modified with streptavidin were added to the biotinylated monoclonal antibodies M86429M solution for overnight incubation at 4° C (Fig 1 b). Following incubation, unbound IgG molecules were washed away by three wash-centrifugation cycles using spin-filters with a pore size about 100 nm (Millipore).

Step 3. we prepared suspension A by adding the 1.5 μm iron oxide particles (Polysciences, Inc.) modified with protein G to the monoclonal antibodies M86306M solution for overnight incubation at 4° C (Fig 1 c).

Step 4. CA 125 solutions of defined concentrations were added to mixtures of suspension A and suspension B taken in equal volumes and were incubated and shaken 4 hours at room temperature and overnight at 4° C, then were stored at 4° C. For the control experiment the PBS buffer with 5% of BSA (no CA 125) was added to a mixture of suspension A and suspension B and were incubated and shaken 4 hours at room temperature and overnight at 4°C then were stored at 4° C.

Unbound silicon oxide particles and dissolved CA 125 molecules were separated from unbound iron oxide particles and aggregates of iron oxide and silicon oxide particles by using strong magnets (residual flux density about 14.5-14.8 KGs (K&J Magnetics, Inc. website, n.d.). The separation-washing cycles were repeated 3 times.

After completing magnetizing and pipetting, the top part of the test tubes was cut off and the bottom filters with particles deposited on it (residue particles) were checked by LIBS for the presence of Si elements.

To obtain better sensitivity, we employed the magnetizing type of assay. In this approach, following the incubation, the unbound molecules, the single silicon particles, the single iron oxide particles and particle aggregates were separated using strong magnets. After completing steps of magnetizing and pipetting, the residue particles left on the filters were analyzed using LIBS for the presence of silicon. Fig. 5 shows the fragment of LIBS spectra around 288.1 nm silicon emission line obtained by the two-element (Si and Fe) Tag-LIBS assay for detection of CA 125 biomarker. The control lowest black line on Fig. 5 was obtained from the empty filter. The red line curve is a LIBS spectrum of control sample where instead of CA 125 the buffer was added. Other lines represent various concentrations of CA 125 in a solution (see Fig. 5). The presence of some Si in the control sample is possibly caused by non-specific interaction of micro-particles.

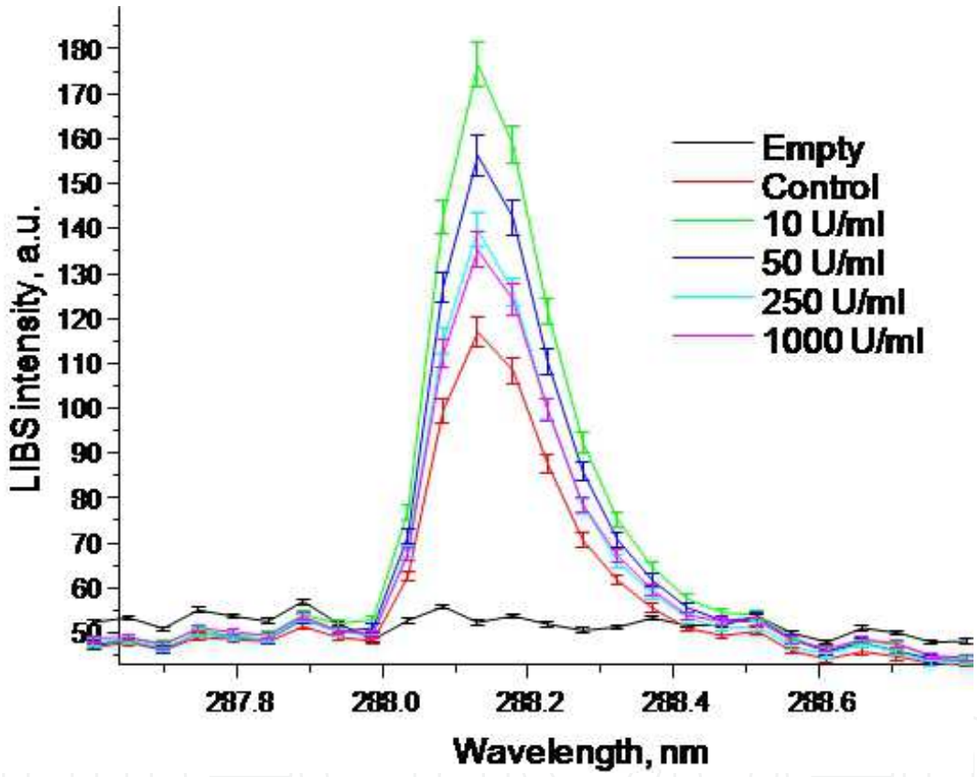


Fig. 5. The fragment of LIBS spectra around 288.1 nm silicon emission line in the two-element (Si and Fe) Tag-LIBS assay for detection of CA 125. The black line represents LIBS of the empty filter. Other lines represent various concentrations of CA 125 in a solution: red line – control sample with no CA 125, green line – 10 U/ml, blue line – 50 U/ml, light blue line - 250 U/ml, pink line – 1000 U/ml.

Figure 6 shows the experimental results of the ovarian biomarker CA 125 detection by the two-element (Fe and Si) Tag-LIBS assay in a blood mimicking PBS buffer. The curve on Fig. 6 represents the CA 125 concentration dependence of the intensity of Si emission line at about 288.1 nm obtained by Tag-LIBS assay. The experimental curve has a maximum at about 10 U/ml and will be discussed later in comparison with results of other experiments.

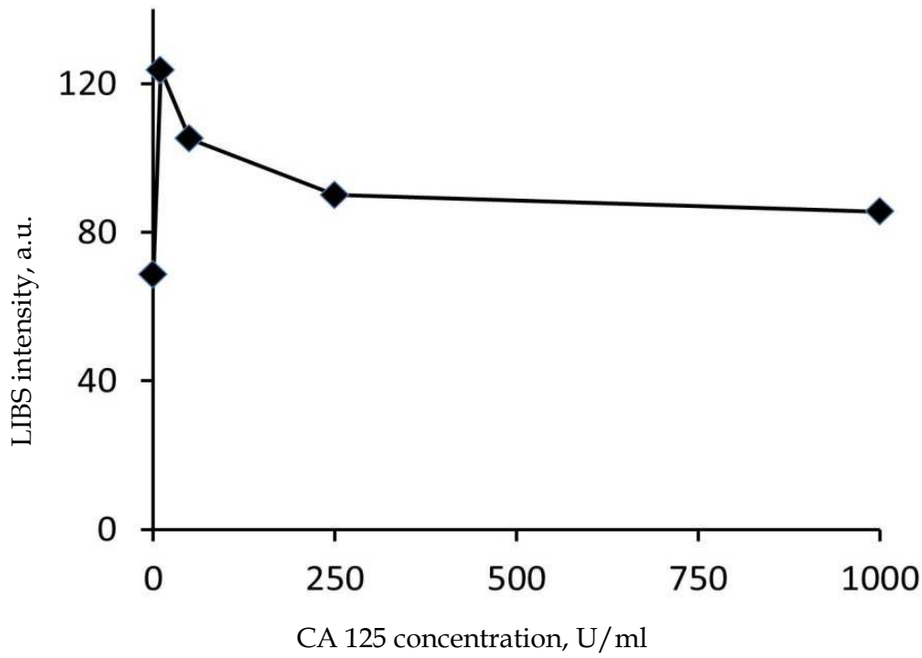


Fig. 6. Detection of ovarian cancer biomarker CA 125 by two-element (Si and Fe) micro-particle Tag-LIBS assay. The curve represents the LIBS intensity of Si emission line in the two-element Tag-LIBS assay at the various concentrations of CA 125.

The two-element (Si-Fe) coded Tag-LIBS assay has been used to analyze the ovarian cancer biomarker Leptin. Leptin, IgG monoclonal antibodies H86901M (Group A) and IgG H86412M (Group B) monoclonal antibodies to Leptin were purchased from Biodesign International. Monoclonal antibodies H86901M and H86412M were biotinylated prior to doing assay. EZ-Link Sulfo-NHS-Biotinylation Kit (Pierce Biotechnology, Inc.) was used for this purpose.

Equal amount of 3 μm silicon oxide particles (Kisker Biotech GmbH) and 1.5 μm iron oxide particles (Bangs Laboratories, Inc.) were added to pre-mixed solution of defined concentration of Leptin and equal concentrations of IgG monoclonal antibodies H86901M and IgG monoclonal antibodies H86412M for 3 hour incubation at room temperature and overnight at 4° C. Using strong magnets and residual particles on filters assayed, single and aggregated particles were separated. We monitored the amount of aggregates in a similar way like it was described earlier.

The intensity of the silicon emission line at about 288.1 nm as a function of the concentration of Leptin is shown in Fig. 7 for two independent Tag-LIBS experiments (marked with squares and diamonds) performed over with a one-month interval. The reproducibility is about 20-30%. The trend line of linear least-square approximation is shown on the figure and so is the R-squared coefficient, which is about 0.949. The ascending part of the Tag-LIBS assay curve was from 10 to ~ 400 μg/ml. In contrast with CA 125 Tag-LIBS assay (Fig. 6) the local maximum feature of the calibration curve for Leptin assay has not been determined in the range of concentrations investigated. The presence of Leptin at the level of 11μg/ml has been detected by Tag-LIBS approach.

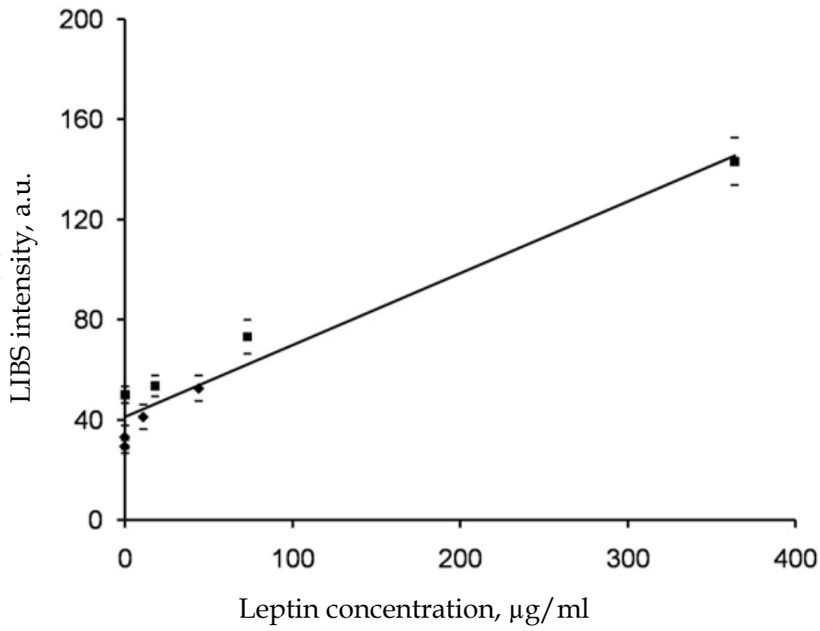


Fig. 7. Detection of ovarian cancer biomarker Leptin by two-element (Si and Fe) micro-particle Tag-LIBS assay in two independent experiments (squares and diamonds) with an interval of one month. Solid line is obtained by the linear least squares approximation.

To ensure the applicability of the Tag-LIBS approach to the clinical environment the human fluid with bio-molecules of interest has to be checked. Blood is an extremely complex solution composed of plasma with dissolved proteins and blood cells (Cohn, 1948). Therefore, human blood plasma and model molecular pair avidin-biotin were chosen to test the Tag-LIBS approach for future clinical applications. To maintain homogeneity and equivalent conditions for all samples the human blood plasma after thawing has been filtered over centrifugal filter with relatively big pore size about 5 µm.

Two types of particles were used for the assay, i.e. 50 nm gold nano-particles and 1.5 µm iron oxide micro-particles. Due to significant differences in size and taking into account the densities of gold and iron (we used density of iron instead of combined iron and iron oxide density of particle for simplicity), every microgram of gold particles counted for approximately $1.4 \cdot 10^9$ nano-particles and every microgram of iron oxide particles included about $1.8 \cdot 10^5$ micro-particles. Furthermore, the total surface area of 1 µg of gold particles was about $6.2 \cdot 10^6$ µm$^2$ in comparison with about $7.1 \cdot 10^5$ µm$^2$ total surface area of 1 µg of iron oxide particles. Thus, to balance the surface areas of the two types of particles, we took about 10 times more iron oxide particles (by weight) than gold particles. In addition, the concentration of biotin molecules attached to the nano-particles was chosen to be greater than the concentration of avidin. Under these conditions, the molecules of avidin are less likely to compete for the binding sites, which yields enhanced detection limit.

Results shown in Fig. 8 demonstrate the ability of the Tag-LIBS approach to detect model molecules avidin in human blood plasma. Tag-LIBS analysis has been performed with a series of dilutions with the following final concentrations of avidin: 0 ppb, 6 ppb, 64 ppb, 322 ppb, 644 ppb, 1483 ppb, 2321 ppb, 3224 ppb, and 6448 ppb (curves 0 – 8, Fig. 8). The

spectrum of the empty filter was subtracted from the sample spectra. To enhance the clarity and viewing of the gold emission peak intensities at 280.2 nm, the sample spectra are slightly shifted along the Y-axis (Fig. 8). Data of three Tag-LIBS experiments were averaged to plot the control curve (curve 0, Fig. 8). The lowest 6 ppb concentration of model protein avidin was measured by Tag-LIBS approach in human blood plasma with about 8:1 signal-to-noise ratio (curve 1, Fig. 8).

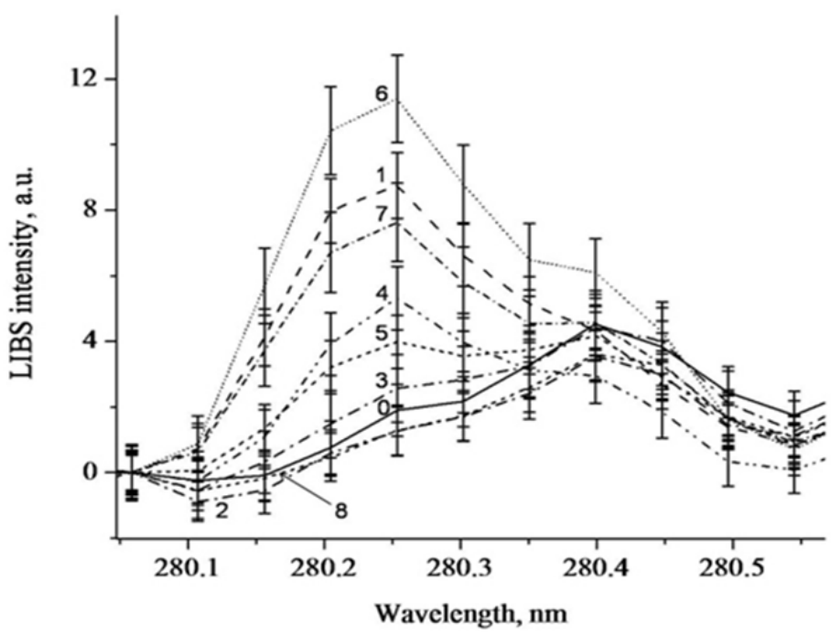


Fig. 8. The fragment of background subtracted LIBS spectra around 280.2 nm Gold emission line in the two-element (Au and Fe) Tag-LIBS assay for detection of avidin in human blood plasma. Concentration of avidin: 0 – 0 ppb (control sample), 1 – 6 ppb, 2 – 64 ppb, 3 – 322 ppb, 4 – 644 ppb, 5 – 1483 ppb, 6 – 2321 ppb, 7 – 3224 ppb, 8 – 6448 ppb.

Clearly it is important to maintain, and possibly enhance, the analytical sensitivity of an assay while at the same time obtain detection limits that are comparable or better than the current attainable. For the CA 125 biomarker the normal level has been previously determined, e.g. Niloff *et al.* (Niloff *et al.*, 1985) have demonstrated that the elevation of serum CA 125 concentration over 35 Units/ml (the upper limit of normal) correlates with cancer disease progression. For the Leptin biomarker the level below 2.5 ng/ml (the lower limit of normal) typically correlates with the presence of cancer (Mor *et al.*, 2005).
In the proof-of-principle experiments with ovarian cancer biomarkers we **achieved about 1 U/ml detection limit for CA 125 and about 11μg/ml for Leptin**. The typical minimum detectable concentration of C A125 for solid phase enzyme-linked immunosorbent assays (ELISA) was estimated to be from 5 U/ml (Alpha Diagnostic Intl. instruction manual, n.d.) to 7 U/ml (Thomas *et al.*, 1995). Therefore, the current Tag-LIBS sensitivity for CA 125 is comparable to existing commercial ELISA assays. For ovarian biomarker Leptin more work has to be done to improve sensitivity of the current method up to necessary for clinical

applications level 2.5 ng/ml. To achieve the goal we plan to use more sensitive gated spectrometer (Andor ME 5000 Echelle Spectrograph) with a better spectral resolution. Preliminary experiments (data not shown) demonstrated about 20 times improved sensitivity for detection of iron oxide micro-particles. Thus, we can expect significant improvement of the Tag-LIBS detection limits for biomarkers.

Figures 4, 6 and 7 show that the binding curves have a local maximum on the calibration curves for avidin (Fig. 4) and CA 125 (Fig. 6), but has not been observed for Leptin (Fig. 7). In these experiments, three different proteins and protein molecules were investigated. These have different molecular weights (mw). The molecule avidin is a tetrameric biotin-binding protein with molecular weight about 67 KD (UniProt protein database, n.d., a), ovarian cancer biomarker CA 125 (also known as mucin 16 or MUC16 (HUGO Gene Nomenclature Committee database, n.d.)) has a molecular weight about 2,353 KD (UniProt protein database, n.d., b), and Leptin has molecular weight about 18.6 KD (UniProt protein database, n.d., c). Proteins with higher molecular weight usually have bigger size (i.e. hydrodynamic radius) (Armstrong *et al.*, 2004). Therefore, during the cross-linking process the bigger proteins may occupy larger areas and steric effects may take place especially for larger proteins (Connolly *et al.*, 2001). This is in agreement with observed Tag-LIBS data (Fig. 4, 6, and 7). The presence of local maximum on the calibration curves detected for molecules with bigger molecular weight avidin and CA 125 but not for Leptin might be in part the result of steric effects and constraints of bio-molecule access to unbound binding sites on micro-particles (Connolly *et al.*, 2001) or due to the cross linking critical concentration of protein molecules in a solution (Heidelberger, 1933). It is also possible that the differences in the experimental protocols (see description above) used for different proteins played some role. It is important to mention that the matrix effects accompanying LIBS may play a role (Cremers & Radziemski, 2006, B). Further studies are currently ongoing.

Laser-induced breakdown spectroscopy yields integrated data about atomic composition of a sample. LIBS can yield more than ten thousand spectral data points for each sample. Several methods do exist to reduce the massive amount of data up to the reasonable point. The traditional way of analysis involves taking into account the emission spectral amplitudes (or areas under the spectral lines) related to few chosen elements of interest omitting all other spectral data (Cremers & Radziemski, 2006, B). This leads to plotting standard calibration curves to be compared with unknown sample. Another way to reduce the amount of experimental data involves the use of the principal component analysis (PCA) (Labbe *et al.*, 2008). PCA converts the multi-dimensional set of experimental data into the new typically less dimensional set of principal components with higher variance. Several classification algorithms such as k-Nearest Neighbor and Support vector machines can be further employed (Vance *et al.*, 2010).

## 4. Conclusions

Clearly, it is important to develop novel diagnostic methods for higher throughput screening of human samples. Known types of assays for detection of the disease biomarkers include enzyme, fluorescence, chemiluminescence, nephelometric and radio- immunoassays (Koivunen & Krogsrud, 2006). Encoded particle assays are attracting more attention due to their inherent ability for easier scaling-up from the single analyte to highly parallel multi-analyte systems (Rubenstein, 2010). Such particle based assays are sometimes called virtual

arrays to be distinguished from more conventional positional arrays (Rubenstein, 2010). For capture entities, the currently employed encoded particle assays use typically antibodies or oligo-nucleotides coded by nucleotide sequences or set of dyes (Rubenstein, 2010). Metals, non-metals, their alloys and composites have not been used extensively despite the fact that they can offer some advantages over traditional coding entities. First, they are chemically and biologically inert. They are stable, not harmful for patients, have longer shelf-time and relatively easier to handle.

The choice of proteins to detect is paramount to the diagnostic of cancers (Petricoin *et al.*, 2002; Mor *et al.*, 2005; Wagner, 2003; Garber, 2004). By employing the multi-element coded technique, we offer a novel approach to **detect many proteins in serum simultaneously**. The fundamental question that needs careful attention is: which proteins should be detected and monitored? Therefore, the screening for the potential cancer markers becomes crucial for the successful diagnostic development. The use of multi-element micro- and nano-particles labels for possible biomarkers can help in the multiplex biomarker panel development.

Compared with the numerous elemental analytical techniques available, LIBS provides many advantages. LIBS method requires much smaller sample volumes and minimal sample preparation. It provides real-time spectra, does not require the use of time-of-flight devices and is easy to implement. In addition, elements analyzed by LIBS have extremely narrow emission bandwidths and characterization of each chemical element, as defined by a unique series of emission lines, is highly specific. As a result, LIBS is one of the most effective techniques for multi-element analysis of samples. For the Tag-LIBS application the identification code is the multi-elemental composition of nano- and micro-particles.

Using micro-particles for the detection of biomarkers has several advantages compared to ELISA micro-titer plates type assays. First, the throughput of the particle assay can be greater because of the larger surface-to-volume ratio. Second, the use of mixtures particles coated by individual capture molecules allows development of multi-analyte assays. Nontoxic and inexpensive labels with minimal interference of background signal and improved specific activity, with unique signature appeared to be the most desirable label technologies from the viewpoint of industry and academia (Harma, 2002).

We suggest that the use of LIBS after tagging specific proteins provides a novel approach for inexpensive, robust and accurate diagnosis of EOC. In principle, the proposed method is applicable for different types of cancer (i.e. ovarian, prostate or lung cancer (Brambilla *et al.*, 2003)) provided we can identify specific biomarkers.

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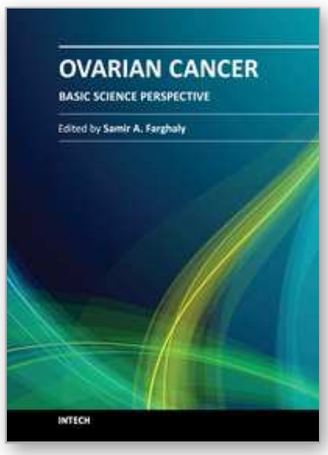


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