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# Early stage detection and screening of ovarian cancer: A research opportunity and significant challenge for biosensor technology

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ABSTRACT

Women diagnosed with late-stage ovarian cancer suffer a very high rate of mortality. Accordingly, it is imperative to detect and diagnose the disease as early as possible in its development. Achievement of this aim implies relatively large scale screening of women at an age of clinically significance through assay of biomarkers for disease present in blood or serum. Biosensor detection offers an attractive technology for the automated detection of such species. Among several biomarkers that have been identified that are present in patients with ovarian cancer, the only one that is commonly tested for in clinical use is cancer antigen 125, which is considered to be poor biomarker for the disease. Here, we describe alternative biomarkers which overcome many of the problems associated with cancer antigen 125 such as increased sensitivity and specificity especially in the early stages of the disease and which could be employed successfully in a biosensor format. In particular, we discuss the chemistry of probes for the biomarkers, heat shock protein 10 and lysophosphatidic acid. The challenges presented by the fabrication of biosensor devices for the detection of the cancer, and the limited number of biosensors that have been developed for this purpose are discussed.

## 1. A précis of the disease

In women over the age of 50 cancer of various types constitutes one of the leading causes of death with a 15% mortality rate, increasing to 36% among women living in high income countries, rendering it a serious health problem around the world (Fig. 1, Stevens et al., 2013). Among cancers present in female patients one of the most dangerous is ovarian cancer, which is a general term used to identify any cancers that originate or find their main tumors in the ovaries or fallopian tubes of women (Vaughan et al., 2011). Though ovarian cancer is less common than several other female cancers, such as breast cancer, it has the highest fatality-to-case ratio of all gynecological cancers making it an especially serious issue for post-menopausal women (Jemal et al., 2011).

Specifically ovarian cancer accounts for over 225,000 new cases and over 140,000 deaths worldwide each year (Jemal et al., 2011). Though the majority of ovarian cancer cases are diagnosed in older women, the disease is also present to some extent in younger populations, with girls in their teens being diagnosed and even dying of the disease each year (Cancer Institute, 2011). Despite increased survival rates for other cancers over the last few decades the survival rate for ovarian cancer has remained roughly steady since 1995, with a 5 year survival rate of less than 40% of those women who survive their first year (Fig. 2, Vaughan et. al, 2011; Coleman et al., 2011). In fact, survival rates for the disease have improved little since the 1970s despite significant advances in cancer treatment and surgery (Cancer Institute, 2012).

The majority of women actually diagnosed with the disease are already displaying late-stage ovarian cancer (70% of cases). Unfortunately the five year survival rate for women diagnosed at a late stage, versus those diagnosed in stages I or II, is extremely low with less than 40% of late-stage women surviving 5 years compared to over 90% for those diagnosed in early stages (Cancer Institute, 2011). Given these medical facts it is crucial to improve diagnosis in the early stages of the disease in order to significantly increase the survival rate for those women who suffer from it. Indeed a USA-based report on ovarian cancer published in 2016 (Ovarian Cancers: Evolving Paradigms in Research and Care; National Academies of Sciences Committee on the State of the Science in Ovarian Cancer Research and Board on Health Care Services; Institute of Medicine stated the following recommendation 6 (of 11):

> *"Researchers and funding organizations should focus on the development and assessment of early detection strategies that extend beyond current imaging modalities and biomarkers and that reflect the pathobiology of each ovarian cancer subgroup".*

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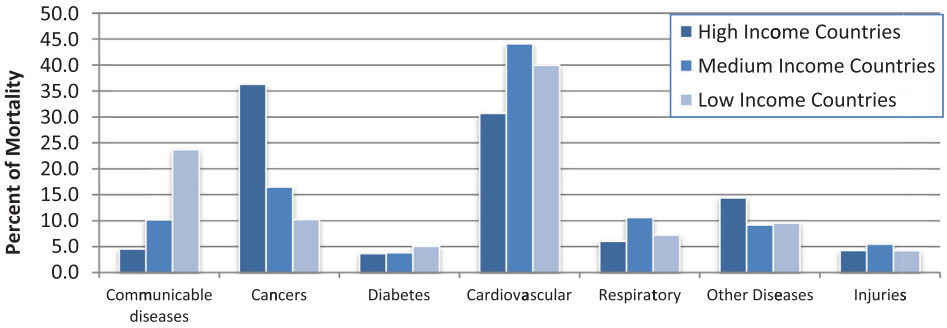


**Fig. 1.** Mortality of women over 50 by cause of death, and by relative income level of their home country. Data from Stevens et al. (2013).

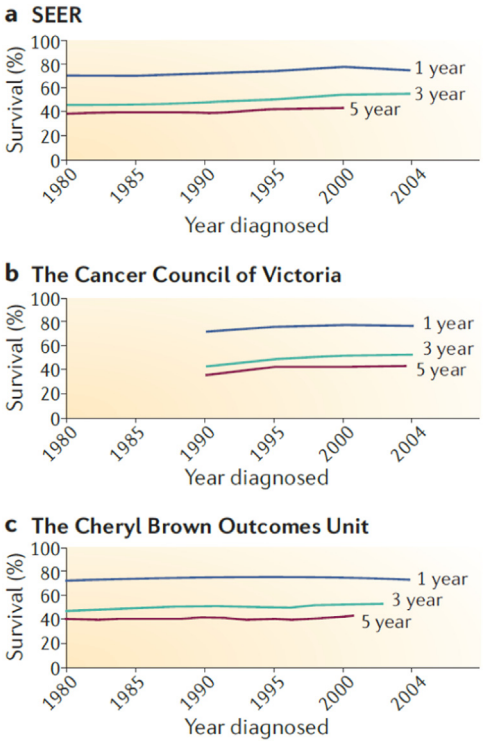


**Fig. 2.** Figure reproduced from Vaughn 2011. Survival rates of ovarian cancer patients at 1, 3 and 5 years. (Reprinted by kind permission of Springer Nature).

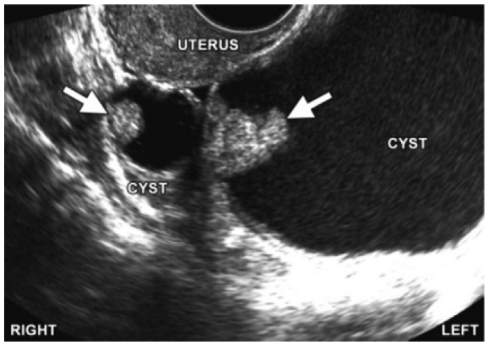


**Fig. 3.** Figure and caption reproduced from Mohagheg 2012. Low-grade serous carcinoma (left ovary) and borderline papillary serous tumor (right ovary) in a 25-year-old woman with nonspecific pelvic pain and CA- 125 level of 91 kU/L. Transvaginal US image shows bilateral complex adnexal cysts with midlevel echogenicity and posterior acoustic enhancement and irregular echogenic solid projections from the cyst walls (arrows), findings indicative of malignancy. According to the equation used to calculate the risk of malignancy index (RMI)—U (3) × M (1) × CA-125 level (91)—the patient had an RMI of 273, a value indicative of a high risk for malignancy. (Reprinted by kind permission of the Radiological Society of North America).

## 2. Symptomology and imaging

Ovarian cancer often presents with few symptoms at an early stage and vague symptoms at later stages. These symptoms include bloating, nausea, difficulty eating, back pain, and urinary urgency among others, which tend to persist and worsen as the cancer advances (Torpy et al., 2011). As stated, these symptoms do not tend to appear until the later stages of the disease, rendering early detection more difficult as women with the disease have little to no physical indication of its presence.

Ovarian cancer presents as adnexal masses (Fig. 3), which are relatively common and rarely malignant (Mohaghegh and Rockall, 2012). Approximately 1% of women who have these adnexal masses do they turn out to be malignant, and for the remaining majority it is important to avoid unnecessary intervention (Sharma et al., 2012). As such it is important to establish the nature of these masses by imaging techniques before proceeding with biopsies and further testing.

The most common techniques for imaging ovarian tumors are transvaginal ultrasonography (TUS), computer tomography (CT) and magnetic resonance imaging (MRI) all of which are capable of basic imaging of the masses. Early stage and benign masses share different physical characteristics to later stage and malignant masses, which can be seen by TUS (Valentin et al., 2006). Differences also exist between benign and malignant masses when imaged by magnetic resonance imaging (Fig. 4, Mohaghegh and Rockall, 2012).

Ovarian cancer can be very difficult to detect due the depth of the tumors within the body, and in terms of the characteristic asymptomatic, or vague symptoms outlined earlier. Thus, it is often the case that it is not even looked for by women until their symptoms have progressed, which is generally when the cancer has reached later stages. In addition to imaging modalities, there is a blood test that is currently available, which assays the ovarian cancer biomarker, CA-125. This blood test is far from ideal for reasons that will be discussed in the next section, and is only useful when combined with imaging studies for the detection of ovarian cancer at any stage.

Based on physical differences between benign and malignant ovarian masses discussed earlier it is possible to determine the type of mass through imaging techniques. For ultrasound a simple scoring system has been developed to determine if the tumor is benign or malignant, and decide on future steps (Timmerman et al., 2008). If a mass is found to exhibit morphologies between benign and malignant then consultation with an expert sonographer and further imaging is required.

Further imaging can include MRI, which is a powerful imaging tool for determining if adnexal masses are malignant, due to the morphological differences outlined previously. It has a much higher specificity and accuracy for determining a masses nature than TUS, with a specificity of 84% versus 40% for TUS, and an accuracy of 89% versus 64% for TUS (Sohaib et al., 2005). It is however less accessible and more expensive than TUS, and is therefore generally used after a mass has

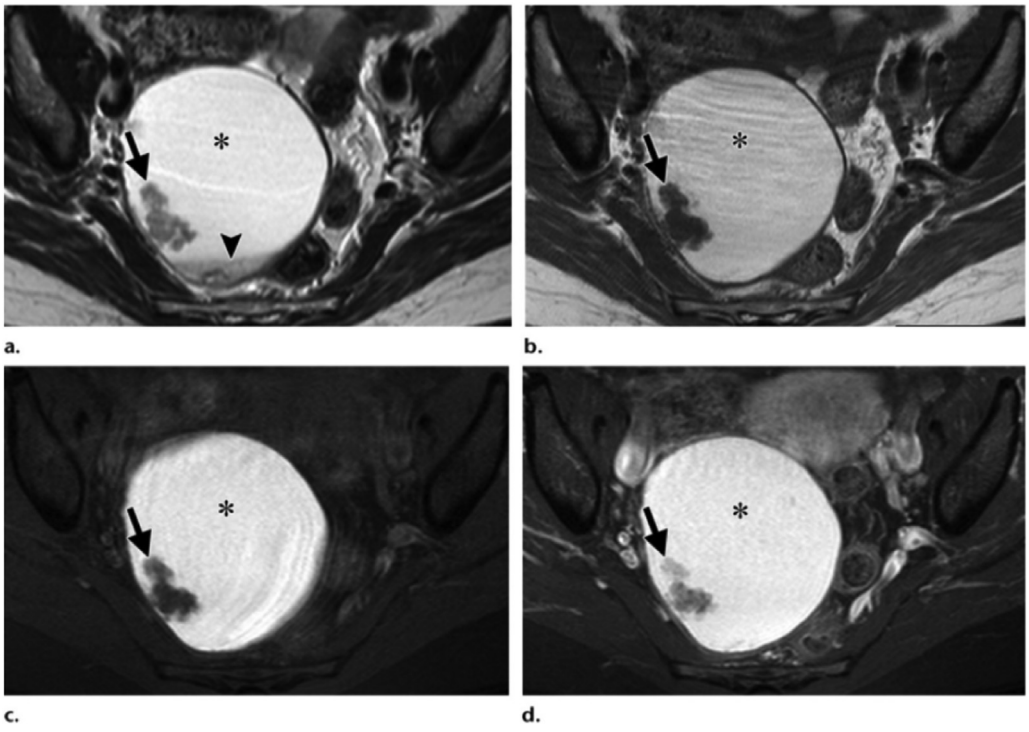


**Fig. 4.** Figure and caption reproduced from Mohagheg 2012. High-grade clear cell carcinoma (stage I) within an endometrioma in a 43-year-old woman with known endometriosis and normal CA-125 level. **(a–c)** Axial T2-weighted **(a)**, T1-weighted **(b)**, and T1-weighted fat-suppressed **(c)** MR images show a hyperintense 8 × 9-cm complex adnexal cyst (*) that demonstrates no loss of signal intensity at fat-suppressed imaging, a finding indicative of hemorrhagic content, and a basal layer with intermediate signal intensity on T2-weighted images (arrowhead in **a**), a finding indicative of blood of various ages. A solid papillary component (arrow) with intermediate signal intensity at T2-weighted imaging and low signal intensity at T1-weighted imaging is also seen projecting from the cyst wall, a finding indicative of debris or tumor. **(d)** Axial contrast-enhanced T1-weighted fat-suppressed MR image shows enhancement of the solid portion of the tumor (arrow), a finding that excludes debris and is indicative of malignancy. (Reprinted by kind permission of the Radiological Society of North America).

been located by ultrasound and determined not to be benign. As well as MRI, another imaging technique is computer tomography, which is useful in determining the malignancy of an adnexal mass, and may be preferable to MRI in certain cases due to its lower expense and greater availability (Bharwani et al., 2011; Zhang et al., 2008).

In general terms it is considered that TUS and other imaging techniques are expensive and time-consuming to perform and require a trained physician for the duration of the test. It has also been found that only 1% of screened women present with unusual ovarian morphology, of which only a fraction have ovarian cancer, making such imaging impractical in view of their high relative cost. In addition, the imaging modalities are not unduly effective for the detection of the presence of ovarian cancer in the *early* stages of the disease, with only 25% of women diagnosed in stages I and II. When detected at this point, the outcome for the patient is generally much better (Jelovac and Armstrong, 2011). Accordingly, a simple and inexpensive blood test, possibly a point-of-care variety, or biosensor-based detection strategy that can flag potential ovarian cancer patients for further imaging would be ideal, especially if this can be applied at an early stage of the disease. *A key component of both these approaches is the availability of ovarian cancer biomarkers.*

## 3. Detection via biomarkers

The term *biomarker* originated back in the 1980s and has been defined by The National Cancer Institute of the USA as “a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process or of a condition or disease”. The identification and, indeed, quantification of clinically important biomarkers is a rapidly growing area of research. With respect to cancer biomarkers, detection of the presence of such molecules is crucial in terms of both diagnosing the nature and stage of a particular disease condition and the monitoring of its progression as influenced by patient treatment. The wide variety of cancer biomarkers that have been studied range from those connected to genetic mutations and predisposition to disease to species released by primary or metastatic tumors. In addition to diagnosis and prognosis, detection of biomarkers in very important in terms of development of drugs to treat cancers, research which is included within the realm of pharmacology. As with all cancer biomarkers, the ideal criteria for detection of ovarian cancer-related entities are that the molecule should be present and directly connected with and reflect the specific stage of tumor-based processes, and that it should be detected with facility in, hopefully, an efficient and inexpensive manner. Several biomarkers for ovarian cancer have been characterized over relatively recent years (Table 1). It should be noted that different levels of characterization of each biomarker has been performed, with some having their specificity and selectivity for ovarian cancer determined, while others have been merely identified as associated with the disease.

The only current widely used such biomarker for ovarian cancer is the well-known cancer antigen 125 (CA-125) assay, which has been employed in the monitoring of patents for over three decades. CA-125 immunoassays are commercially produced and can be purchased (Fujirebio, 2018). These assays measure CA-125 levels from blood samples using $^{125}$I-radiolabelled monoclonal mouse antibody for CA-125 in a one-step sandwich assay on solid beads (Fig. 5). The radioactivity of the bound molecules is measured, and is proportional to the concentration of CA-125 in the sample. Assays such as this are used both clinically, and in research (Aktas et al., 2013).

The CA-125 assay is not viable on its own for the detection of ovarian cancer, since the antigen is only present at elevated levels in approximately half of patients at an early stage, although it is present in 92% of late stage patients (Kobayashi et al., 2012). So on its own the protocol cannot be used to identify most early stage ovarian cancer patients when detection is most useful, and it isn’t applicable for monitoring of all patients, as 8% do not show elevated levels. It is also present at elevated levels in benign conditions such as pregnancy, or menstruation giving it a high rate of false positives when used for detection (Kobayashi et al., 2012).

Given the fact that the CA-125 assay is not a catch-all biomarker, since the antigen is not expressed in all ovarian cancer patients at all stages, there has been a considerable effort from researchers to characterize other potential markers. One such marker is HE4, which is found to be elevated in two thirds of early and late stage ovarian cancer

**Table 1**
A selection of identified ovarian cancer biomarkers.

| Biomarker | Cut-off | Method | SE | SP | Down/Up Regulated | Reference/s |
|---|---|---|---|---|---|---|
| **CA-125** | > 35 U/mL | CA-125 immunoassay | 82.2 | 67.3 | Up | Kobayashi et al., 2012 |
| **CA-125 with ApoA-I, transferin and TTR** | N/A | Chemiluminescence, and immunoturbimetry | 91% | 92% | N/A | Su et al., 2007 |
| **OVA 1 Panel** | N/A | ELISA and Chemiluminescence | 91% | 69% | N/A | Kumari, 2018 |
| **leptin, prolactin, osteopontin, insulin-like growth factor II, macrophage inhibitory factor,and CA-125** | N/A | bead-based immunoassay | 95% | 99% | N/A | Visintin et al., 2008 |
| **TTR, Hb, ApoAI, TF** | N/A | SELDI-TOF-MS | 86% | 86% | N/A | Kozak et al., 2005 |
| **HE4** | > 70 pM | PCR and DNA sequencing | 72.9% | 95% | Up | Peters et al., 2005 |
| **HE4 with CA-125** | N/A | ELISA Assays | 89% | 75% | Up | Moore et al., 2009 |
| **Mesothelin** | > 2 nM | ELISA | 60% | 98% | Up | Aktas et al., 2013 |
| **Osteopontin** | > 260 ng/mL | PCR and immunohistochemistry | 81% | 34% | Up | Kim et al., 2002; Zhang et al., 2012 |
| **HSP-27** | > 0.25 ng/μg cytosolic protein | glycotranscriptome comparative analysis | | | Up | Langdon et al., 1995; Abbott et al., 2010 |
| **HSP-60** | | Gel electrophoresis and mass spectrometry | | | Up | Li et al., 2009 |
| **Calreticulin** | | Gel electrophoresis and mass spectrometry | | | Up | Bengtsson et al., 2007 |
| **Vimentin** | | Gel electrophoresis and mass spectrometry | | | Down | Lim et al., 2011 |
| **Fibrinogen-γ** | > 400 ng/dL | Clauss method | | | Up | Polterauer et al., 2009 |
| **miRNAs** | N/A | PCR and Taqman Open Array | | | Up/Down | Shapira et al., 2014 |
| **HSP-10** | > 0 | Western immunoassay | | | Up | Akyol et al., 2006 |
| **LPA** | 1.3 μM | Capillary electrophoresis | 98% | 90% | Up | Xu et al., 1998; Sedláková et al., 2011 |

patients, has very high specificity, and is even present in a third of non CA-125 producing tumors (Peters et al., 2005). However studies have found that only serous and endometrioid epithelial ovarian cancers (EOCs) overexpressed HE4, and that other EOCs rarely overexpressed this protein (Zhang et al., 2011).

As mentioned above, the CA-125 method alone is not ideal for the detection for ovarian cancer, but studies combining CA-125 screening with other biomarkers has shown a positive increase in detection ability. Su and colleagues used a multiple logistic regression model (MLRM), with values for CA-125, ApoA-I, transferrin (TF), and TTR (which are all prospective biomarkers for ovarian cancer) for early detection of ovarian cancer. This model provided a sensitivity of 89% and a specificity of 97% for detection of early-stage ovarian cancer. This test however uses multiple screening methods, and is therefore expensive to perform (Su et al., 2007).

Recently a screening panel called OVA-1 gained FDA approval for ovarian cancer diagnosis (Kumari et al., 2018). This panel uses multivariate analysis of multiple biomarkers including CA 125-II, HE4, apolipoprotein A-1, FSH, and transferin, allowing for 91% selectivity towards ovarian cancer detection. However the test does little to improve the specificity of detection, with a maximum specificity of 69%, similar to that of CA-125 alone. As such it has limited use as a diagnostic tool. Another immunoassay that similarly looks at a panel of biomarkers was published in 2008 (Visintin et al., 2008). This panel has a remarkably high sensitivity and specificity of 95% and 99% respectively, and was able to identify 90% of stage 1 cancer patients. Unfortunately this assay is not currently being used for ovarian cancer screening, despite being first published in 2008. Another screening panel was also proposed in 2005 (Kozak et al., 2005), produced sensitivity and specificity of 86% each. Unfortunately no screening platform has been developed for this panel, preventing it from being used in clinical diagnostics.

Another biomarker, which has been mentioned in the media, is mesothelin. Mesothelin is found to be elevated in three quarters of ovarian cancer patients, but it is also found to be elevated for those suffering from other cancers and those with mesothelioma (Aktas et al., 2013). As a result, mesothelin is again not an ideal marker, since as is often the case, it is necessary to combine it with other biomarker tests such as CA-125. For now mesothelin is most useful in monitoring disease progress for ovarian cancer patients currently undergoing treatment (Aktas et al., 2013). Though several other potential markers have been identified, such as osteopontin (Zhang et al., 2012), HSP-27 (Langdon et al., 1995), HSP-60 (Li et al., 2009), calreticulin (Bengtsson et al., 2007), vimentin (Lim et al., 2011), and fibrinogen-γ (Polterauer et al., 2009), none of these species are expressed in most if not all of ovarian cancer patients. They all suffer from the same limitation that they are not consistently overexpressed until later stages of the disease, when detection is less important.

One potential biomarker that may overcome the limitations of those mentioned previously is heat shock protein 10 (HSP-10), which is a protein that has been subjected to an initial study in terms of its potential as an ovarian cancer biomarker (Akyol et al., 2006). Using Western immunoblotting the study analyzed levels of HSP-10 in the sera of 10 patients with stage III ovarian cancer, and 9 healthy controls. They found HSP-10 was present in all ovarian cancer patients, and no controls, suggesting HSP-10 would make for a suitable biomarker for ovarian cancer detection. However, it would be ideal to detect ovarian cancer at an early stage, and at this point it is still unknown if HSP-10 is found in the sera of patients with stage I ovarian cancer. Further studies need to be performed before HSP-10 can be confirmed as a prospective biomarker for the detection of ovarian cancer. In this connection, an additional interesting feature of the biochemistry of HSP-10 is its multiplicity of roles. It is up-regulated in cancer cells and is known to be involved with other molecules in protein -folding processes (Czarnecka et al., 2006), but also is associated with the cell signalling network and in the inhibition of apoptosis. These other potential roles for the protein

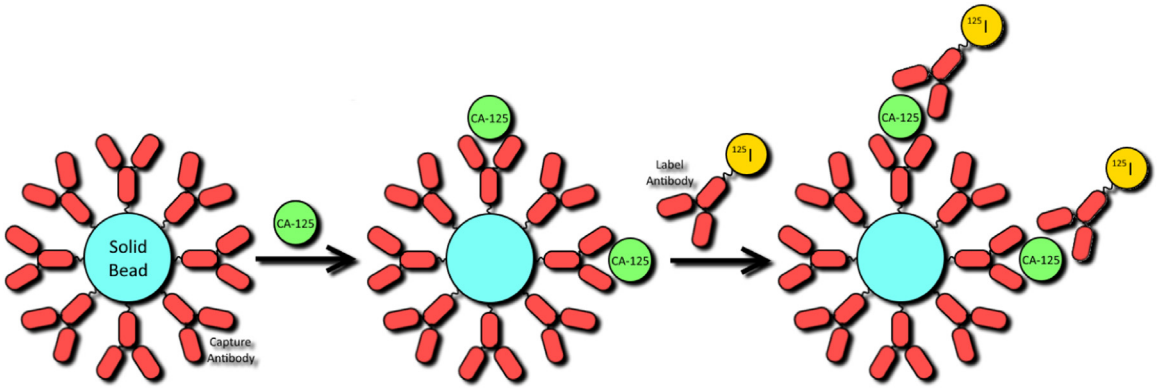


**Fig. 5.** Operating principle of CA-125 immunoassay.

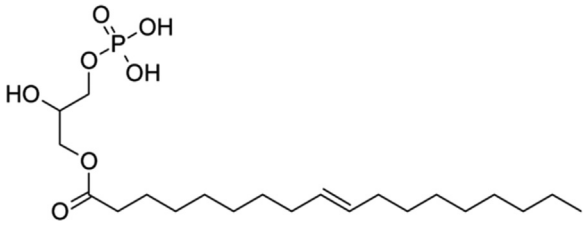


**Fig. 6.** Structure of lysophosphatidic acid (LPA).

in the etiology of cancer led the aforementioned authors to use the phrase "capsula eburnean" in connection with HSP-10 and carcinogenesis.

Lysophosphatidic acid (LPA, Fig. 6), which is a signalling lipid (Pagès et al., 2001), is another potential ovarian cancer biomarker. In two separate studies, this signalling lipid was found to be elevated in 90% of stage I ovarian cancer patients, and 100% of later stage patients (Xu et al., 1998; Sedláková et al., 2011). It was also found that LPA elevation correlated to the stage of the disease with stage III and IV patients presenting higher LPA serum concentrations than stage I and II patients (Sedláková et al., 2011). These elevated levels render LPA a promising marker in the early detection of ovarian cancer. However, as with the other biomarkers discussed above, LPA levels were found to be somewhat elevated in a subset of healthy controls and patients with benign gynecological diseases, approximately 10% and 25% respectively (Xu et al., 1998). A detection method for ovarian cancer utilizing LPA as a marker would be useful in identifying ovarian cancer patients in at-risk populations, such as women over 50 and women with family histories of ovarian cancer, but would not necessarily be useful as a screening tool for the entire population of women given the number of false positives it may produce. Much further research is required to establish the efficacy of LPA detection in terms of such a wide-ranging screening protocol.

## 4. Potential for biosensor-based detection of ovarian cancer

### 4.1. General criteria

For the purpose of this review we take the perhaps more restrictive view that a biosensor for detection of cancer biomarkers should involve the relatively direct conversion of the presence (and concentration) of a marker into a device-generated electrical signal. Such a strategy stands in contrast with multi-step methodologies that are often required in order to measure marker concentrations. An example of this approach would be those that have been designed to assay LPA in biological fluids, the biomarker introduced above. These are generally time-consuming and expensive to operate.

The particular biosensor format that is appropriate for detecting any form of cancer, including the ovarian version, obviously depends on the specific goal of the assay. Potential biosensor measurements of markers of clinical interest in biological fluids can be divided up into two distinct categories, those performed in the central clinical laboratory and those determined by point-of-care devices (Orth et al., 2017). The latter approach is largely intended to serve the needs of assays at home, at the hospital bedside or facility of a general practitioner. Although potentially very useful, this methodology does not lend itself to the "mass" screening of large numbers of patient samples. As implied above, an attractive scenario would be the cost-effective, general screening the blood samples of females for ovarian cancer biomarkers, *especially* if such an assay was applicable in the earlier stages of the disease. Clearly, the central clinical biochemistry laboratory is the most appropriate vehicle for conducting such assays, given the scale of robot-oriented, automated equipment available in such a facility (Horvath et al., 2014). Incorporated into this type of laboratory are rapid methods for the production of sera within an automated analytical train. Although an assay that could be performed on a whole blood sample would be highly attractive, it is more often the case in a central facility that sera samples are those that are subject to analysis.

Given the slate of biomarkers described above it will be necessary to choose probes for these that can be incorporated into the anatomy of a chosen device (here we use the term "probe" to represent a selective binding entity for a particular biomarker). Ideally, the design and fabrication of devices for the detection of several markers would constitute an ideal approach for obvious reasons. These probes must retain their capability for marker binding and be in the correct orientation for this purpose when attached to a device surface. Secondly, the choice of device will be crucial in terms of sensitivity since it is envisaged that, at least for early stage cancer samples, the concentration of marker in sera is expected to be very low (for some markers this is, approximately, nanomolar at best). Finally, the device must clearly be operable in media such as blood serum, where considerable surface fouling is expected. This phenomenon has certainly constituted something of an "Achilles Heel" for biosensor technology over a number of years.

### 4.2. Biosensor probes for ovarian cancer biomarkers

In the light of the fact that there are a very limited number of available probes for the markers described above, we begin with our own efforts to develop two of these with a biosensor end point in mind. First we discuss the biomarker HSP-10, which as outlined above displays multifaceted biochemical behaviour. In addition to the property of the protein as a suppressor of apoptosis of malignant cells, HSP-10 is also an immunosuppressant (Taylor et al., 2001; Akyol et al., 2006). Accordingly, the obvious choice of the development of an antibody probe for this molecule is not viable. This leads to the oligonucleotide alternative which involves the well-known SELEX procedure for the production of aptamers that can bind the target molecule, HSP-10 in

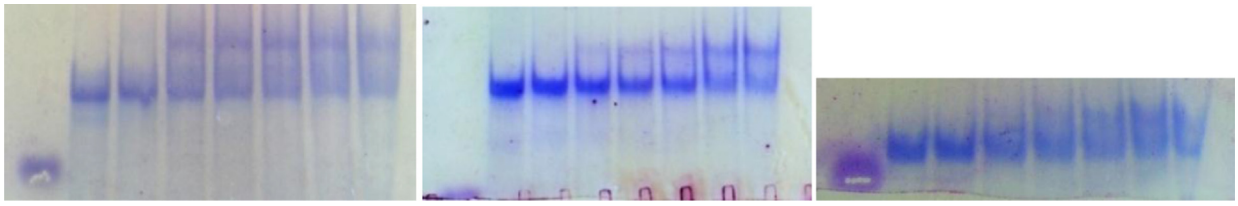

**Fig. 7.** DNA-Native PAGE for aptamer binding of constructs MNB-38, MNC-38, and MND-38 (left to right) binding to HSP10. Reproduced from Chen, 2016. (Reprinted by kind permission of the University of Toronto).

this case, hopefully, with a high degree of selectivity (Ellington and Szostak, 1992).

Such nucleic acid moieties, as compared with their protein-based counterparts, are considered to be chemically stable and can be produced in relatively large quantities once the SELEX protocol is deemed to be successful (Ellington and Szostak, 1992; Esposito et al., 2014). Using such a method we have initiated research on the HSP-10 binding of four promising constructs (Chen et al., 2016). This effort requires the generation of the protein since it is not readily available on a commercial basis. This is achieved by overexpression of the protein BL21 Rosetta E. coli cells, followed by purification via nickel affinity chromatography and cleavage fusion protein to obtain pure HSP-10. The basic binding characteristics are being obtained by conventional electrophoretic mobility shift assay (Fig. 7) and more detailed study by NMR spectroscopy and isothermal titration calorimetry. Preliminary results are that the constructs bind HSP-10 in the Kd regime of nanomolar concentration, confirming the promise of aptamer chemistry for ovarian cancer biomarker sensing. Additionally this method has also been used to develop aptamers for ovarian cancer cells themselves, potentially allowing for detection of metastasized cancer cells in patients' blood (Van Simaeys et al., 2010).

A number of probes are available for interaction with LPA, including several naturally occurring proteins, which are detailed below. There are several membrane proteins that bind to LPA and act as receptors for various cellular processes (Noguchi et al., 2009). These receptors include $LPA_1$ which is responsible for cellular proliferation, survival, stress fiber formation, and neurite retraction (Weiner and Chun, 1999; Estivill-Torrús et al., 2007). Additional membrane receptors are $LPA_2$ and $LPA_3$, responsible for cell rounding and neurite elongation respectively (Ishii et al., 2000) There are also $LPA_4$ and $LPA_5$ present, which are both responsible for neurite retraction and cell rounding (Lee et al., 2007, 2006; Yanagida et al., 2007).

Although these proteins could make for a suitable probe for LPA they may lose functionality when removed from a cellular membrane and deposited on a biosensor surface as a result of no longer being surrounded by the lipid membrane, which is required for maintaining most membrane proteins' structure (Popot and Engelman, 1990). As such it would be better to identify a non-membrane protein that can be used as a probe for LPA. An antibody for LPA has been developed (Goldshmit et al., 2012) which could be used for biosensing applications.

Another possible probe is the protein gelsolin (Goetzl et al., 2000), which binds to LPA through a small chain of amino acids known as the $PIP_2$-binding domain (Fig. 8, Mintzer et al., 2006). Gelsolin binds LPA with a high affinity measured by its $K_d$ of 6 nM, which on par with the previously mentioned receptors (Noguchi et al., 2009; Goetzl et al., 2000). The $PIP_2$-binding domain however only binds LPA with a $K_d$ of 920 nM, suggesting that the interactions of LPA and gelsolin are heavily dependent on the rest of the protein. The protein itself is a large 6 domain protein, with a molecular mass over 80 kDa (Goetzl et al., 2000). Of the six domains the protein essentially exists as two identical components comprised of domains 1–3 and domains 4–6, with these halves individually being able to bind to LPA (Nag et al., 2009). As such half of gelsolin, which shall be referred to as gelsolin 1–3, could alone be used as a probe in binding to LPA.

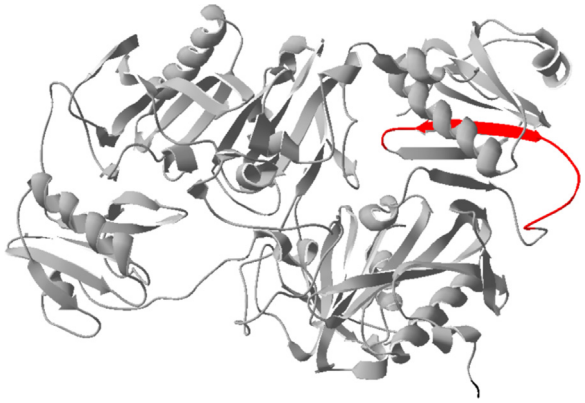

**Fig. 8.** Structure of Gelsolin with the PIP-2 domain highlighted in red. Protein data bank number 2FGH (Urosev et al., 2006).

Apart from developing and utilizing probes for LPA and HSP-10, one could also use such probes to analyze CA-125 using a biosensing platform as opposed to the available immunoassay. Alongside the available antibody for CA-125, which was mentioned in Section 3, SELEX could be used to develop an aptameric probe for CA-125. The probe for CA-125 could be added to a biosensor via the methods mentioned in the following section. Adapting the CA-125 assay could make measuring the biomarker faster or more cost effective than the current immunoassay.

The discussion outlined above applies to any ovarian cancer biomarker, with probes either existing naturally, or being developed either through aptamer technology or antibody production. However due to the large number of ovarian cancer biomarkers that have been discovered discussion has been limited to the most relevant biomarkers.

### 4.3. Probe surface attachment and anti-fouling chemistry

A myriad of methods are available for the attachment of protein and aptamer probes to the surface of various substrates and devices, both in the biosensor and biocompatibility literature. Here we include a concise look at potential chemistries for attaching the types of probe outlined above to gold and silica surfaces. The reason for the choice of these two substrates is that they frequently form the foundation of such binding to electrochemical, acoustic wave (AW) and SPR sensors. Gold figures prominently with respect to both the label-free acoustic wave and SPR-based detection systems and silica (quartz) with regard to AW sensing. A particularly crucial aspect of such probe chemistry is that it is necessary to embed the binding agent in a device coating that minimizes fouling of the device in use by the components of serum.

In our research we have demonstrated that probes can be successfully attached to quartz, via thiosulfonate-to-disulphide chemistry and rather than the use of the more conventional amino functionalities (Sheikh et al., 2010; Blaszykowski et al., 2012a; De La Franier et al., 2017). Coupled to this work we have shown by systematic study that among various surface modifying species produced by silanization, the MEG-OH entity dramatically reduces fouling of surfaces when placed in biological fluids (Fig. 9). This molecule also serves as a diluent to space

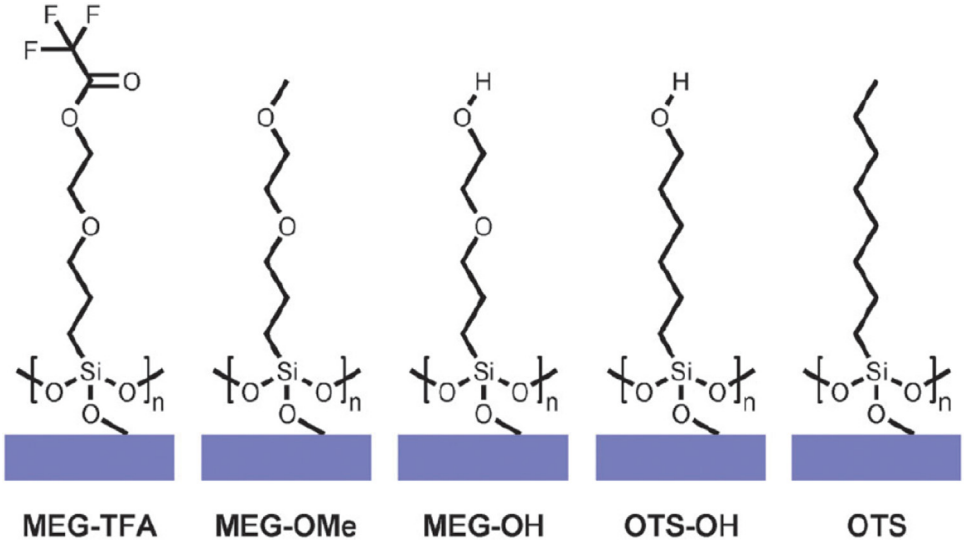


**Fig. 9.** Trichlorosilane surface linkers from Blaszykowski et al. (2012a). (Reprinted by kind permission of American Chemical Society).

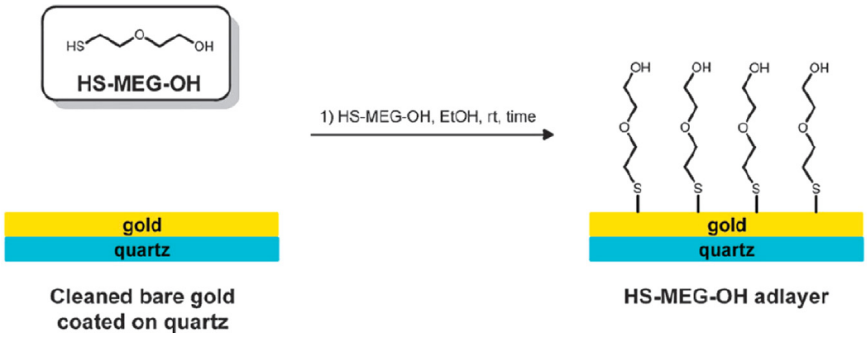


**Fig. 10.** Thiol surface linker from Avci et al. (2013). (Reprinted by kind permission of The Royal Society of Chemistry).

**Table 2**
A summary of ovarian cancer biosensors that have been developed and are discussed in this section.

| Biomarker | Method | Reference |
|---|---|---|
| **HE4** | Localized surface plasmon resonance | Yuan et al. (2012) |
| **HE4** | Electrochemical impedance spectroscopy | Whited (2012) |
| **HE4** | Double-gate field effect transistor-based nano biosensor | Sajjad et al. (2015) |
| **Be3l-2** | Ultrasonic MEMS-based biosensor | Onen et al. (2012) |
| **HE4 and CA125** | Optical nano-sensor implant | Williams et al. (2018) |
| **HSP-10** | Acoustic wave biosensor | Chen et al. (2016) |
| **LPA** | Fluorescence spectroscopy | De La Franier and Thompson (2016) |

out a chosen linker on a substrate surface. The modifier-linker configuration is designed to produce a surface monolayer, but *not* necessarily a close-packed conventional SAM. The key issue is that the silane backbone (modifier or linker can also be employed to introduce an ether moiety which, with reference to polyethylene glycol chemistry, has for a considerable period of time been proven to be one approach for the avoidance of surface fouling. Accordingly, an attached probe can be embedded in a "background" of an anti-fouling agent, MEG-OH, which, as mentioned above, reduces adsorption of unwanted components to the device surface (Blaszykowski et al., 2012b; Sheikh et al., 2010; Fedorov et al., 2014). Detailed research on the nature of the surface-bound structure of MEG-OH by neutron reflection spectrometry and molecular dynamic calculations has confirmed that a *non-close packed* SAM with penetration by a film of hydration is critical in terms of the anti-fouling behaviour (Pawlowska et al., 2014; Sheikh et al., 2015). Interestingly, the analogous chemistry involving thiol attachment to a gold surface (with the ether moiety in place, Fig. 10)) is not nearly as effective as the trichlorosilane method on silica (Avci et al., 2013). This result is attributed to the fact that a film of hydration is more likely to penetrate the trichlorosilane monolayer than the thiol on gold.

## 5. Biosensor detection

To date there have been relatively few works that describe the fabrication of biosensors for the detection and assay of biomarkers for ovarian cancer. A number that have appeared deal with attempts to place the CA125 assay described above into a sensor format, despite the doubtful potential offered *for early stage* detection by this biomarker. Furthermore, some of the technologies described do not provide the possibility for the sensor to actually function in biological fluids. Selected examples of devices employed for the detection of ovarian cancer follow (Table 2).

One of the earlier reports described a biosensor for the detection of

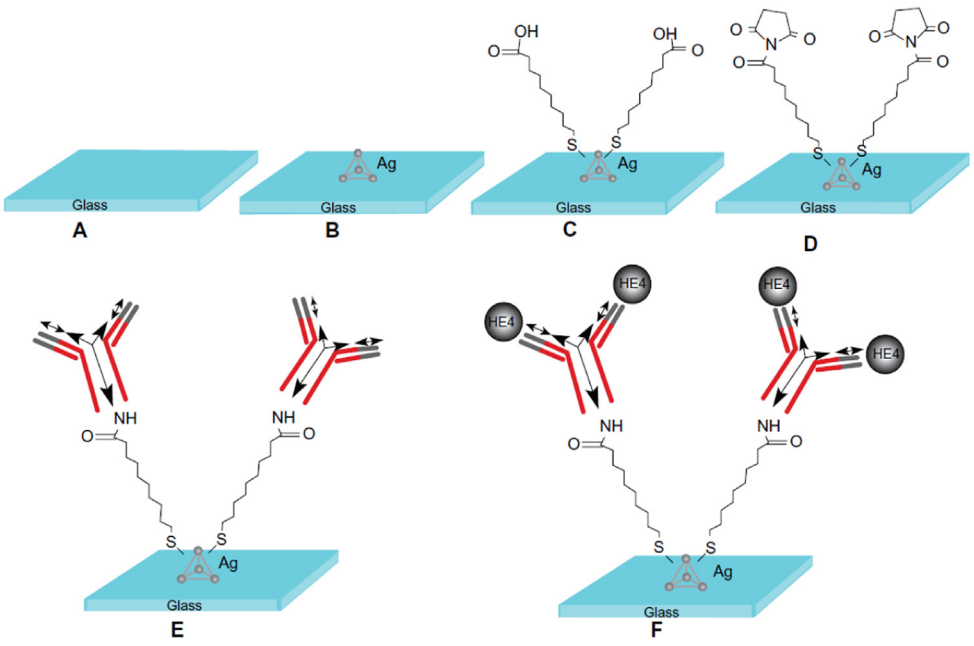


**Fig. 11.** Reproduced from Yuan et al. (2012) (Reprinted by kind permission of The PubMed Central). Design of the localized surface plasmon resonance biosensor for HE4 detection using a direct assay format. (**A**) Glass substrate, (**B**) silver nanoparticles synthesized through NSL technology, (**C**) A self-assembled monolayer layer formed by incubation in 1 mM 11-mercaptoundecanoic acid, (**D**) incubation in 75 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/ 15 mM N-hydroxysuccinimide, (**E**) anti-HE4 antibody (10 μg/mL) covalently attached to the nanoparticles, and (**F**) different concentrations of the HE4 both in buffer and serum samples reacted with the anti-HE4.

the biomarker human epididymis secretory protein 4 (HE4) based on a localized surface plasmon resonance (Yuan et al., 2012). In this case the probe was an antibody species for HE4 which was attached to a silver nano-chip via standard EDC-NHS chemistry (Fig. 11). The device was subjected to different concentrations of standard HE4 (1 pM to 0.1 μM) incubated on the functionalized LSPR chip for 40 min, followed by a thorough rinsing with phosphate-buffered solution containing 0.05% Tween-20 to dissociate the nonspecific binding. The peak wavelength of the LSPR extinction spectrum (λmax) excited by the silver nanoparticles was measured and recorded for each experiment using an ultraviolet-visible spectroscope with a charge-coupled device detector. Evidence that HE4 in a *buffer solution* was detected successfully by the LSPR biosensor was that following incubation in 500 pM HE4, the LSPR wavelength shifted to + 14.48 nm, showing a λmax of 645.45 nm. The limit-of-detection for the method was said to be 4 pM and in overall terms it compared favourably with a standard ELISA assay. There was concern expressed about the viability of a calibration curve for concentration when serum was present because of the ubiquitous nonspecific adsorption issue.

Unsurprisingly, there have been attempts various attempts have been made to produce electrochemical-type biosensors for biomarkers. One example of this is the development of an electrochemical impedance spectroscopic method for the detection of HE4 and CA125 (Whited, 2012). This involved the use of a device with micron-scale interdigitate electrodes (IDEs) in an SD card format. The probe for HE4 in this case was a protein-enzyme conjugated label. It was unclear in this work if the device was employed directly on serum or blood samples containing the markers, although the sensor was aid to measure the molecules at serum type concentrations. A second electrochemistry example is that of a double-gate field effect transistor-based nano biosensor (DGFET) (Sajjad et al., 2015). The advantages of this device were extolled in this work through various plots of signal versus HE4 concentration but curiously there was no mention in the work of experiments conducted with biological fluids or even the employment of a selective probe for the marker!

Another example of the use of a microelectronic device is the work on an ultrasonic MEMS-based biosensor of the detection of urinary anti-apoptotic protein B-cell (Be3l-2) (Onen et al., 2012). In this study, the design, fabrication, and surface functionalization of antibodies for the marker were described resulting in an experimental sensitivity for detection around the level of sub ng/mL. The sensor is based on shear horizontal (SH) surface acoustic wave technology using ST-quartz surface functionalized to quantify the mass loading change by protein adhesion to the delay path. SH-SAWs were generated and received by a pair of micro-fabricated interdigital transducers (IDTs) separated by a judiciously designed delay path. The surface chemistry employed was considered to avoid fouling issues from species present in urine. The argument was made that the configuration could be best employed in a point-of-care operation.

Recently, non-invasive ovarian cancer biomarker detection based on an optical nano-sensor implant was introduced (Williams et al., 2018). In this work there was a tacit recognition that both HE4 and CA125 are marginal biomarkers for the early stage detection of ovarian cancer. Accordingly, the sound argument was made that detection of these markers proximal to where the disease occurs such as the fallopian tube, ovary, uterine cavity, or peritoneal cavity, where marker concentrations are higher, would be highly desirable. The device used in this work was composed of single-walled carbon nanotubes (SWCNTs) which have electronic and optical properties that are well suited for in vivo signal transduction. Semiconducting carbon nanotubes yield near-infrared (NIR) bandgap photoluminescence between 800 and 1600 nm, which can penetrate living tissues to a distance of centimetres. Practically, an antibody for HE4 was attached to a nano-probe tip via a stepwise process involving ssDNA, standard EDC-NHS chemistry followed by the antibody (Fig. 12). Following considerable ex-vivo characterization the sensor was implanted in animals for photo-luminescent measurement of HE4. In conclusion, the authors comment that an implantable system portends clinical translation for use in patients with risk factors to detect disease onset, recurrence, or to monitor treatment response. It remains to be seen if relatively large scale screening of a population could be effected using this approach.

In our own work, we have studied the interaction of HSP10, alluded to above, with various chosen aptamers by acoustic wave biosensor (Chen et al., 2016) The purpose of this investigation was to examine the nature of this interaction rather than develop an ovarian cancer biosensor per se. The hexa-histidine-tagged protein was attached to the device (quartz) surface via nickel- NTAL chemistry for acoustic experiments conducted at 940 MHz frequency. Interestingly, regarding the binding of the DNA aptamer to the surface-immobilized protein exhibited a significantly smaller signal compared to surfaces without protein being present, which comparatively yielded much larger frequency shifts. The smaller shift in frequency was attributed to the

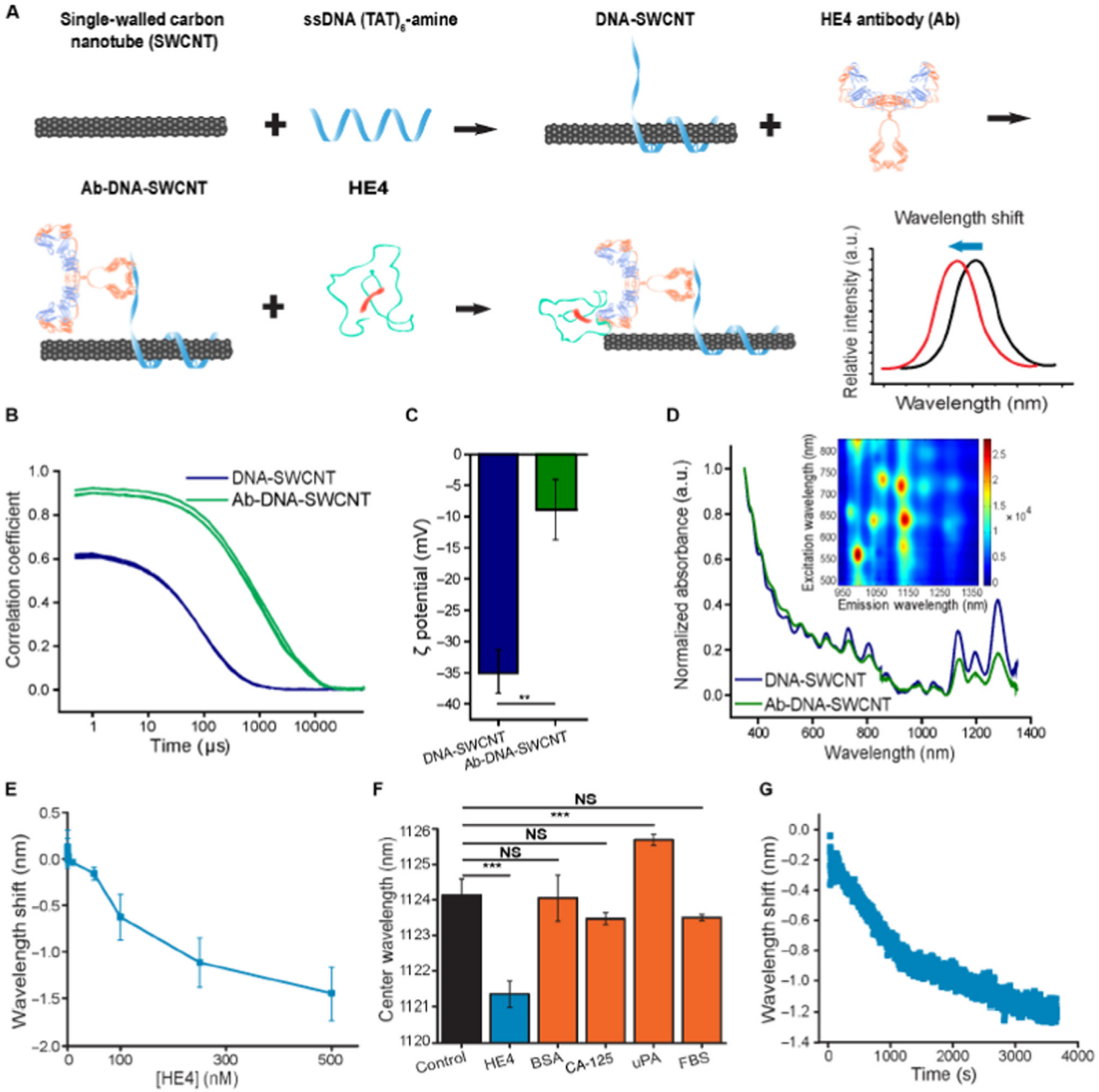


**Fig. 12.** Reproduced from Williams et al. (2018) (Reprinted with kind permission from Science). Design and in vitro characterization of optical nanosensor for HE4. (A) Scheme of Ab-DNA-SWCNT complex synthesis and proposed nanosensor function. a.u., arbitrary units. (B) Correlograms from a dynamic light scattering instrument showing correlation coefficient of pre-Ab– and post-Ab–conjugated ssDNA-SWCNT samples. n = 3 for each complex. (C) Electrophoretic light scattering of ssDNA-SWCNT before and after anti-HE4 antibody conjugation. n = 3, mean ± SD; **P < 0.01, *t*-test. (D) Representative absorbance spectra of the hybridized ssDNA-SWCNT before and after conjugation of the anti-HE4 antibody. Inset: Representative PL excitation/emission plot of the Ab-DNA-SWCNT sensor. (E) Dose-response curve of the Ab-DNA-SWCNT sensor emission [of the (9,4) nanotube species] as a function of HE4 concentration in 10% fetal bovine serum (FBS). Each point is the mean of three experiments ± SD. (F) Response of the Ab-DNA-SWCNT complex to interferent proteins. n = 3, mean ± SD; control and HE4, $P = 1.0 \times 10 - 4$; control and bovine serum albumin (BSA), P = 0.998; control and CA-125, P = 0.163; control and urokinase plasminogen activator (uPA), $P = 1.0 \times 10 - 3$; control and FBS, P = 0.64 [two-sided one-way analysis of variance (ANOVA) with Dunnett's post hoc analysis]. NS, not significant. (G) Representative kinetic response of nanotube emission upon introducing recombinant HE4.

rigidification of the monolayer upon interaction between probe and ligand as the overwhelming factor. This rigidification is counteracted by mass loading effects, in which a balance between these two factors contributes to the overall smaller frequency shift. Without the binding interaction, the bulk layer does not rigidify, resulting in mass loading being the main contributor to the signal.

Given the high promise exhibited by LPA as a biomarker for ovarian cancer in comparison with CA125 and HE4 we have developed a prototype biosensor for this molecule, however it is not yet at the stage of clinical use (De La Franier and Thompson, 2016). This test relies on the duel protein system of gelsolin and actin. In the presence of LPA fluorescently labelled actin is released into solution, and can be measured. At the present time, this test works in serum, and presents a low limit of detection for LPA. Research is underway to reach the quantifiable the ovarian cancer LPA cut-off level of 1.3 μM.

## 6. Summary of challenges

It is evident from the research summarized in this review that the detection of ovarian tumors present in the very early stages of the development of the disease could potentially result in the saving of numerous lives. Although the disease is notoriously dangerous in stage 4 of development, evidenced by the low 20% 5-year survival rate, stage 1 or 2 detection within the context of a large scale screening protocol could be extraordinarily effective in terms of very significantly increasing percentage survival rate. If the assumption is made that literally millions of patients of a certain age (over 40 years old?) need to be screened annually, as mentioned above, a highly reliable device will be required for operation within a robotized train configuration typically employed in a hospital clinical biochemistry facility. (This sort of system is conventionally used for magnetic-bead-chemiluminescence based ELISA assays in such laboratories.) As well, many of the optimal biomarker systems for detecting ovarian cancer rely on measuring multiple different biomarkers. As such a single device that can perform the proteomic or genomic analysis of all required markers would be incredibly useful. For proteomic biosensing a CMOS device where each individual sensor measures one of the desired biomarkers (MacKay and Chen, 2014).

The sensor would obviously need to function in a reversible, flow injection fashion in contrast with the conventional one-shot end-point spectroscopic assays. There is no doubt that this approach presents considerable challenges which are reflected in the fact that it is uncommon in modern clinical biochemistry facilities, despite the considerable promise offered by biosensor technology over many years. The dearth of flow-injection style employment of such devices is clearly associated with difficulties caused by a number of factors including interference caused surface fouling in biological fluid (serum), lack of specificity to the biomarker involved, biomarker concentration measurement at anticipated low levels, and sensor stability and reversibility over significant periods of operational time. From the biological perspective it would be desirable to produce a system that would be capable of assay of a common marker connected to the various cell lines of ovarian tumors. For example, with respect to ovarian cancer pathobiology there are at least 5 ovarian epithelial carcinomas of which the dangerous, high grade serous carcinoma accounts for 75% of cases.

In conclusion, the genuine invocation of biosensor technology into a large-scale screening protocol for the detection of ovarian cancer represents great opportunities in terms of the saving of women's lives. Solving the difficult challenges outlined in this review will obviously require intimate collaborative input from a highly disparate range of expertises such as electronic device engineering, surface physical chemistry, oncology, molecular biology, and clinical biochemistry.

## CRediT authorship contribution statement

**Brian De La Franier:** Writing - original draft, Writing - review & editing. **Michael Thompson:** Writing - original draft, Writing - review & editing.

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## Declaration of interests

None.

## CRediT authorship contribution statement

**Brian De La Franier:** Writing - original draft, Writing - review & editing. **Michael Thompson:** Writing - original draft, Writing - review & editing

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