Detection of Cancer Biomarkers from an Ionic-Liquid System using a Microfluidic Device

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Abstract

In recent years, cancer biomarkers have received remarkable attention in the biomedical field, since they are considered valuable indicators for the presence or absence of tumours, improving rapid and early cancer detection, efficacy of treatment, and allowing monitorization of disease progression. The development of a bead-based microfluidic system for the detection of prostate specific antigen (PSA) promises great potential for the creation of a portable, low-cost, rapid, and sensitive platform for prostate cancer diagnosis and prognosis. In the present work detection of PSA was performed not only in PBS but also in human serum and several ionic liquids. Ionic liquids are promising molten salts in extraction and concentration of analytes present in complex biological fluids and highly used in Aqueous Biphasic Systems. For this work, a sandwich-type immunoassay was selected, in which a capture antibody was immobilized on Protein G beads and fluorescence intensity was measured after the binding event between target analyte PSA and labelled detector antibody. Different protocol optimizations regarding microbeads choice, blocking method and capture antibody concentrations were performed. Calibration curves for different concentrations of PSA for both PBS and human serum were obtained. The detection of target concentration in ng/mL range was reached and detection limits of 10.8 ng/mL and 12.2 ng/mL were obtained for PBS and human serum samples, respectively, both close to clinically relevant range of 4-10 ng/mL.

KEWORDS: microfluidic system, beads, PSA, cancer biomarkers, aqueous biphasic systems, ionic liquids

1. Introduction

Cancer is a major public health problem worldwide, as it is commonly diagnosed at a later stage, when it is already metastasized to other sites of the body[1,2]. Therefore, early-stage cancer diagnosis is extremely important in timely and effective treatment and for monitoring disease progression[2]. In this context, cancer biomarkers have been receiving remarkable attention due to their high clinical significance[3], being considered indicators for tumour development, treatment response and disease recurrence. According to the National Cancer Institute, a biomarker is described as a "biological molecule found in blood, other body fluids or tissues that is a sign of normal or abnormal process, or of a condition or disease". Biomarkers can either be directly produced by a tumor itself or secreted by other tissues as a response to the presence of a malignancy[1,4]. There is a wide variety of cancer biomarkers, including proteins, nucleic acid-based biomarkers, tumor suppressor genes, oncogenes, and tumor specific antigens[4].

1.1. PSA and Prostate Cancer

The first cancer biomarker ever approved by the United States Food and Drug Administration (FDA)[5] for patient screening and diagnosis was Prostate Specific Antigen (PSA), a prostate-specific biomarker routinely used for early detection of prostate cancer (PC). PSA is a glycoprotein that belongs to the family of kallikrein proteases, also called kallikrein 3 (hK3). It is expressed by prostate epithelium and secreted into seminal plasma, in both cancerous and normal prostatic tissue and can be found both free (freePSA) or complexed with protease inhibitors (complexed PSA). The standard procedure to diagnose PC is to test the levels of total PSA (free plus complexed PSA). When these levels stand above 10 ng/mL, the patient is regarded as suspicious for cancer, thus indicating the need for further biopsy procedures. When the level of total PSA is included in the diagnostic "grey zone", between 4 ng/mL and 10 ng/mL, the freePSA to total PSA ratio (%fPSA) can be advantageous in the discrimination of PC from benign prostatic disease, even though it is associated with many false positives. Catalona and co-workers[6] studied the relation between the freePSA to totalPSA regarding differentiation of benign prostate disease and prostate cancer. The study concluded that man with PSA levels within the "grey zone" present a risk of PC of less than 8% when %fPSA is greater than 25%, whereas a %fPSA of less than 10% is associated with a risk of PC greater than 56%. Therefore, the risk of having prostate cancer increases as the %fPSA decreases. However, in patients with prostate cancer, prostate increased volume is associated with higher %fPSA, due to a dilution effect caused by prostate enlargement. Consequently, patients with BPH might receive false-negative results, increasing the risk of delayed diagnosis[7]. Therefore, %fPSA can be misleading and the assessment of total PSA values is still regarded as the gold standard because it is capable of detecting clinically insignificant prostate disease, being able to reduce the amount of unnecessary biopsies[8].

1.2. ABS and ILs

Generally, cancer biomarkers are primarily found in biological fluids, which consist of complexed matrices with a miscellaneous of proteins present at high abundance. These proteins can affect the detection and quantification of cancer biomarkers, which are usually present at limited concentrations in these biological fluids. Therefore, an appropriate sample pre-treatment is usually required to prevent non-specific interactions and a allow a reliable marker quantification. Even though conventional methods for purification and extraction of active biomolecules have well established protocols, are easily obtained and present good immiscibility properties, in recent years, there has been an increasing tendency to search for more ecological and "greener" methods, while maintaining a high degree of efficiency. In that context, Aqueous Biphasic Systems (ABS), originally presented by Albertsson in 1958[9], are considered a highly biocompatible method, essentially due to their aqueous composition in both phases, eliminating the need for toxic solvents. They are formed by two phases, which can be based on two polymers, a polymer and a salt or two salts, dissolved in aqueous media. Above a certain concentration of the polymer and/or the salt, these aqueous solutions separate into two immiscible phases, promoting partitioning of target bioactive substances between them[10]. Ionic liquid-based systems have been gaining ground since Gutowsky *et al.* [11] induced aqueous solution of ionic liquids to form ABS by adding kosmotropic salts. Ionic liquids (ILs) are salts liquid at temperature below 100ºC, constituted by organic cations and organic/inorganic anions[12]. ILs are recognized by their good thermal and chemical stability, negligible volatility, non-flammability and for being easily recyclable. Additionally, ILs present an elevated number of possible combinations of their anions and cations and strong solvation ability for a large selection of compounds. Due to these excellent properties, ILs are being considered the best eco-friendly alternatives for replacing polymers in formation of ABS[13,14], with several studies demonstrating their applicability in extraction of different biomolecules through ABS[15–17]. In 2010, Freire *et al*.[18] reported for the first time the usage of several imidazolium-based ionic liquids as constituents of ABS for the extraction of alkaloids. The group work concluded that is possible to extract caffeine and nicotine from aqueous or urine-type solutions in a single step procedure, with the advantage of using green and recyclable solvents instead of toxic components. The study also proved this IL-based ABS are promising in the obtention of concentrated samples of nicotine and caffeine for a posterior analysis.

1.3. Microfluidics

Despite the recent efforts in developing reliable techniques for early diagnosis, most of the conventional methods applied for the detection of cancer biomarkers present some limitations, namely the consumption of large amounts of reagents, slowness in detection and the fact that skilled personnel and bulky equipment is a mandatory requirement[3,19]. Therefore, it is crucial to invest in fully integrated and automated strategies capable of performing specific molecular recognition of multiple target cancer biomarkers in a simultaneous, simple, and cost-effective manner for an accurate diagnosis. Within this framework, biomarker detection in microfluidic devices have been studied as a promising alternative to traditional methods. In fact, microfluidic systems allow for low-cost and fast *in situ* diagnosis, with increased sensitivity and efficiency and smaller reagents volume[20]. Therefore, these are potentially good candidates for on-chip point-of-care (POC) testing near patient site in environments with low-resource settings, including undeveloped countries and in situations of emergency. Due to these remarkable characteristics, several microfluidic devices have been created in recent years for the detection of several diseases, including not only cancer, but also infectious diseases, cardiac diseases, Alzheimer, among others[20].

1.4. Microbead-based Immunoassays

The performance of microfluidic immunoassays can be enhanced using microbeads as a solid surface for the immobilization of biomolecules. The exploitation of microbead-based immunoaffinity assays have been growing interest among the scientific community, with several publications describing the use of these platforms for biomolecules detection[21–24]. Microbeads provide a significantly higher surface for immobilization in comparison with planar platforms. Consequently, there is a better interaction between target and reagents, which translates into a higher sensitivity. The surface of microbeads can be functionalized, which improves their binding affinity[25]. The other aspect to consider is the transport of analytes. When, instead of a planar configuration, microbeads are used, molecules can be easily transported by means of electric fields or pressure-driven systems, and gaps between closed beads are smaller, thereby diffusion distances are minimized[26].

In the present work, a microfluidic platform capable of detecting PSA, in PBS and human serum, with a limit of detection close to clinically relevant range was developed. The influence of several ILs in the detection of PSA was also analysed, since they are considered promising in sample pre-treatment procedures using ABS, which tend to increase sensitivity and improve detection limits. Therefore, the developed strategy is expected to work as basis for a future microfluidic device with fully integrated IL-based ABS and detection system capable of accurate diagnosis of PC from complex biological fluids.

2. Materials and Methods

2.1. Reagents

Phosphate Buffer Saline (PBS) (ThermoFisher Scientific) was prepared by diluting a PBS 10x stock solution in Milli-Q water to a final working solution of 1x (pH 7.4, 25°C). PBS

was used as main buffer. Different blocking agents were tested, namely 4% (w/v) Bovine Serum Albumin (BSA) (Sigma Aldrich) and ready-to-use Blocker™ Casein in PBS 1% (w/v) (ThermoFisher Scientific), and 5 mg/mL Human Normal Immunoglobulin (Gammanorm®) (Octapharma). 30% (w/w) Polyethylene glycol (PEG) 8000 (ThermoFisher Scientific) was used as suspension solution to help pack the beads in microchannels, since it promotes beads dispersion throughout the solution. Human Serum (Sigma Aldrich) was used as biological matrix. The salts, polymers and ionic liquids used in the present work were kindly prepared and supplied by Universidade de Aveiro/CICECO and are described in **Table 1**. Protein A agarose beads (MabSelect™) and Protein G agarose beads (Protein G Sepharose® 4 Fast Flow) were both purchased from Cytiva. Capture (MABX5532) and detector (MABX5523) anti-PSA antibodies and Prostate Specific Antigen were purchased from Sigma Aldrich and Alexa Fluor®430 NHS ester was purchased from ThermoFisher Scientific. Amicon Ultra-0.5 Centrifugal Filters (MWCO of 10KDa) were purchased from Merck Millipore.

Table 1 - Summary of all salts, polymers and ionic liquids tested.

Sample	Reagents	Mass percentage %(w/w)	pH
Citrate buffer	Potassium citrate tribasic monohydrate, 99%, extra pure (Acros Organics) Citric acid 1-hydrate for analysis, ACS, ISO (99.5-102.0%) (Panreac)	49.8652	6.87
Phosphate buffer	di-potassium hydrogen phosphate trihydrate, extra pure (98-102%) (Sharlau) potassium dihydrogen orthophosphate, analytical reagent grade (99.95%) (Fisher Chemical)	39.2675	7.13
PPG400	Polypropylene glycol (P 400) (Sigma)	59.7523	6.25
PEG1000	Polyethylene glycol (1000) (Alfa Aesar)	60.2984	8.48
Dex500k	Dextran from Leuconostoc spp. (Mr 450000-650000) (Sigma)	29.7945	7.18
UCON	Poly(ethylene glycol-ran-propylene glycol) (Mn≈2500)	60.2443	7.45
PL35	Poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (Mn ≈1900) (Sigma)	60.5815	4.30
NaPA8000	Poly(acrylic acid, sodium salt) solution (Mw ≈8000, 45 % (w/w) in H_2O) (Sigma)	30.7770	7.52
PEG2000	Polyethylene glycol (2000) (Alfa Aesar)	59.6514	8.15
[C_4C_1im] Cl	1-butyl-3-methylimidazolium chloride, 99% (Iolitec)	60.2358	8.54
[C_4C_1pyrr] Cl	1-butyl-1-methylpyrrolidinium chloride, 99% (Iolitec)	60.5261	8.62
[N_{4444}] Cl	Tetrabutylammonium chloride, ≥97% (Sigma)	60.0418	7.99
[P_{4444}] Cl	Tetrabutylphosphonium chloride, >95% (Iolitec)	60.2343	3.82
[Ch]Cl	Choline chloride, 99% (Acros Organics)	38.9467	7.91
[P_{4444}] Br	Tetrabutylphosphonium bromide, >95% (Iolitec)	59.8961	0.53

2.2. Microfabrication

2.2.1. Hard mask fabrication

For the hard mask fabrication, a glass substrate was previously washed with acetone, isopropanol (IPA) and deionized (DI) water before being immersed in an Alconox solution at 65℃ for 30 minutes, followed by another washing step with DI water and finally a drying step. Subsequently, using a Nordiko 7000, a 200 µm layer of Aluminium was poured on the substrate by magnetron sputtering. Afterwards, a layer of 1.5 µm of positive photoresist was spin-coated on top of the Aluminium layer, using a SVG track/automatic coating track. The substrate was then baked for 60 seconds at 85℃. The mask design obtained in AutoCAD software according to the dimensions desired, was transferred to the photoresist through a Heidelberg DWL (direct write laser) lithography equipment, using a laser wavelength of 405 nm. After baking the photoresist for 60 seconds at 110℃ and letting it cool down, the photoresist was developed for 60 seconds, which exposes selected areas of the aluminium layer. The exposed areas were then etched with a proper aluminium etchant until they were completely dissolved. To conclude the hard mask fabrication process, a final washing step with acetone and isopropanol was performed to ensure that the remaining photoresists was completely removed. All these steps were performed under class 100 cleanroom conditions, except photolithography step which was performed in a class 10 cleanroom. In the present work, the structure was composed of columns with 2 different heights, a 100 µm height chamber and another 20 µm height channel connected to the former. Therefore, two different hard masks (Figure 1) were fabricated.

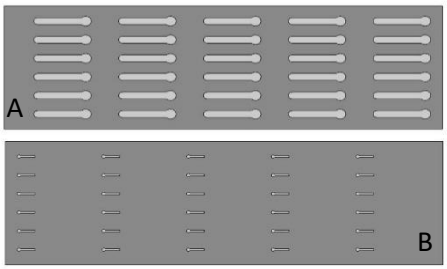

Figure 1 *- Schematic representation of the two hard masks fabricated and used in the present work. Mask for a height of 100 µm (A) and 20 µm (B).*

2.2.2. Master mold fabrication

The first step of the mold fabrication consists of cleaning a silicon substrate with acetone, IPA and DI water, to remove any residues on the surface and then immerse it in an Alcanox™ solution in a hot water bath at 65℃ for 20 minutes. Afterwards, the substrate was cleaned with DI water, then dried with compressed air and placed for 15 minutes in a UVO cleaner to degrade any contaminants. To prepare the 20 µm layer, a negative photoresist (SU-8 2015) was poured onto the substrate and spin coated in a two-step process: first the substrate was spin-coated at 500 rpm and 100 rpm/min for 10 seconds followed by 34 seconds at 1700 rpm and 300 rpm/min. The substrate was then placed in a hot plate at 95℃ for 4 minutes and cooled down for 1 minute (pre-exposure baking). Subsequently, the 20 µm height hard mask was placed on top of the SU-8 layer, with the aluminium surface facing down, and exposed to an UV light for 30 seconds. After the exposure, the substrate was baked in a hot plate at 95℃ for 5 minutes. After cooling down for 2 minutes, the stack was immersed in a PGMEA 99% solution for 2 minutes with manual agitation, which allows the non-exposed photoresist to develop. Then the substrate was cleaned with IPA and dried with compressed air. The second layer with 100 µm height was defined by spin-coating a SU-8 50 film on top of the previous layer at 10 s at 500 rpm with an acceleration of 100 rpm/s, followed by 30 s at 1000 rpm with an acceleration of 300 rpm/s. A pre-exposure bake process

was then performed in a hot plate starting with 10 minutes at 65℃, followed by 30 minutes at 95℃. After cooling down for 1 min, the 100 µm hard mask was placed over the SU-8 layer, ensuring the aluminium surface was facing down and it was aligned with a low power AmScope microscope. Subsequently, the hard mask together with the silicon substrate was exposed to an UV light for 70 seconds and then baked for 1 minute at 65℃ in a hotplate. The temperature of the hotplate was increased until 95℃ and the substrate was left there for further baking for 10 minutes, followed by a cooling down step for 2 minutes at room temperature. The substrate with the photoresist was then developed in a PGMEA 99% solution with manual agitation for 10 minutes, cleaned with IPA and dried with a compressed air gun. A final baking step can be added, in which the substrate can be place in the hotplate for 15 minutes at 150℃, to ensure that the SU-8 properties remain unchanged.

2.2.3. PDMS structures fabrication

The process started with mixing a curing agent with polydimethylsiloxane (PDMS) base (Dow Cornig) in a 1:10 weight ratio. After homogenisation, the mixture was placed in the desiccator for 45 minutes to degas. Subsequently, PDMS was poured on top of the SU-8 mold and placed in the oven for 90 min at 70℃ to cure. The cured PDMS was then cut with a scalp and separated from the mold with the help of tweezers. In order to open the inlets and outlets in the structure, needles of 20Ga and 18Ga (Instech), respectively, were used to punch the holes in the PDMS. To seal the PDMS structure a 500 µm PDMS membrane was prepared. For this, the PDMS mixture was poured on top of a cleaned silicon wafer and spin coated at 250 rpm with an acceleration of 100 rpm/min for 25 seconds. Afterwards, the wafer was placed in the oven for 90 minutes at 70℃ and then cut in pieces slightly bigger than the PDMS structure containing the microchannels. To seal the PDMS structure against the membrane, both surfaces were oxidized with a plasma cleaner (Harrick Plasma) at high power for 60 seconds, which allows the combination of both surfaces, forming a covalent bound between them.

2.2.4. Microchannel packing, fluid handling and image acquisition

During experimental assays, a double syringe pump (NE-1002X, New Era Pump Systems) was used for the fluid manipulation, assembled with two 1 mL syringes (Codan), filled with PBS. Each syringe was connected to a luer stub adapter (LS20, Instech) and a polyethylene tube (BTPE-90, Instech), with an open stainless-steel adapter (SC20/15, Instech) at the other end. This metallic adapter is connected to the outlet of microfluidic channel, where a negative pressure is applied pulling the required solutions from the inlet towards the outlet. The solutions were inserted in the microchannel through a pipette tip placed at the inlet. The microchannels used in all experiments of the present work has two sections, the first is composed of a column with 100 µm height and 700 µm width, and a smaller portion presents 20 µm height and 200 µm width. The channel length is 1 cm (**Figure 2**).

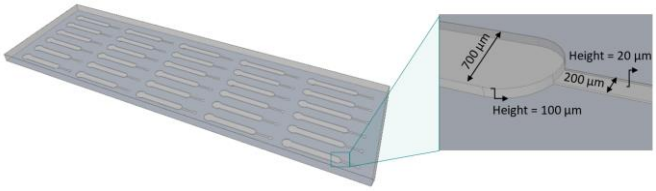

Figure 2 *- Microfluidic structure obtained after peeling the PDMS from master mold. The microfluidic structure is composed of 30 microchannels with two sections, in which the first consists of a column with 700 µm width and100 µm height, and the second has 200 µm.*

To prepare the beads, the first step is the homogenisation of bead suspension. For Protein A or Protein G beads, 3 µL of bead stock solution was added to 20 µL of anti-PSA antibody solution prepared in PBS with a concentration of 100 µg/mL. The mixture was then incubated for 15 min using a rotator mixer at 250 rpm. After incubation, 110 µL of PEG 30% (w/w) in PBS was added to each solution, to ensure a homogeneous dispersion of beads, providing a more viscous media, thus facilitating beads packing. The beads were packed in microchannel by applying a negative pressure at the outlet, after washing the channel with PBS. The packing was always performed until the microchannel was ¾ full (**Figure 3**).

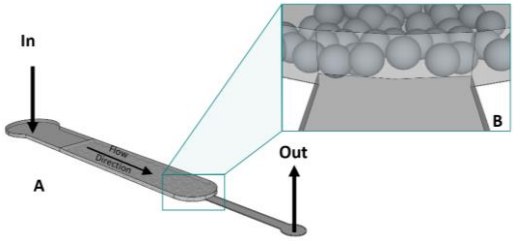

Figure 3 *- Schematic representation of microchannel used for all experiments. (A) The beads are inserted in the inlet through a pipette tip and a negative pressure is applied at the outlet forcing the bead solution to enter the microchannel. (B) Beads packed in microchannel.*

Fluorescence measurements are performed using an inverted fluorescence microscope (Olympus CKX41) coupled to a CCD colour camera (Olympus XC30). Images are analysed using ImageJ software (National Institutes of Health, USA). The measurements obtained correspond to the mean values from independent experiments, considering a certain exterior area of the microchannels (background signal) and subtracting that value from the one obtained by selecting an interior area of the microchannel.

2.3. PSA detection assays

2.3.1. Preparation of anti-PSA-Alexa 430 conjugates

To do the labelling procedure, 100 µL of anti-PSA antibody was diluted in 400 µL of 100mM sodium bicarbonate and then conjugated with 10 µL of the Alexa Fluor® (A430) dye,

previously dissolved in DMSO at 10 mg/mL. The mixture was incubated in the dark for 1 hour at room temperature. The excess non-conjugated dye was then removed through a series of washing steps using a 10KDa Amicon Ultra-0.5 centrifugal filter unit, previously washed with 500 µL of PBS and centrifuged at 14,000 g for 10 minutes. For this, the incubated antibody-dye solution was added to the Amicon centrifugal unit and centrifuged at 14,000 g for 10 minutes. After centrifugation was complete, the permeate at the bottom of the tube was discarded, then 500 µL of PBS were added to initiate the washing process and the solution was centrifuged for 14,000 g for 10 min. This latter step was repeated a series of 6-7 times until the permeant becomes totally colourless. Then, the labelled antibody solution was collected by reverse centrifugation at 2,000 g for 2 minutes and the remaining volume measured and made up to initial volume with PBS.

2.3.2. Detection of PSA

Several immunoassays were performed for detection of PSA and each of the immunoassays performed consisted of complete sandwich assay, unless stated otherwise. Each assay started with immobilization of anti-PSA antibody on agarose beads coated with Protein G and the beads were packed in microfluidic channels in a process described in section **2.2.4**. Briefly, after packing the beads in the channel, PBS is flowed through the column at 15 µL/ to remove remaining PEG solution. Then, blocking agent, which was BSA 4% (w/v) unless stated otherwise, was inserted in the channel to minimize non-specific signal and flowed for 10 minutes at 0.5 µL/min. In between every individual assay step, PBS was flowed through the microchannel to remove any unbound molecules for 1 minute at 5 µL/min, in a process known as intermittent washing. Subsequently, specific concentration of target analyte PSA spiked either in PBS, human serum or several ionic liquids or PEG was flowed through the channel for 10 minutes at 0.5 µL/min. Then the same procedure is followed for detector anti-PSA antibody labelled with Alexa430 fluorophore, flowed at concentration of 100 µg/mL. A final washing step of 1 minute with PBS at 5 µL/min was performed. The specific concentrations of PSA in either PBS, human serum or ILs ranged from 0 ng/mL (control sample) to 25 ng/mL.

3. Results and Discussion

3.1. Solid-support choice

The main goal of PSA detection studies is to achieve reasonable levels of sensitivity. Therefore, the first immunoassay performed aimed at identifying the best option for immobilization of capture anti-PSA antibodies, choosing between PDMS walls of a bare microchannel and microbeads. The protocol followed for the experiment performed in open channel was similar to the one described in section **2.2.4**, except anti-PSA antibodies were immobilized on PDMS walls at a flow rate of 0.5 µL/min for 10 min, after cleaning the channel with PBS. The fluorescence signal registered for both PSA concentrations tested (0 ng/mL of PSA – control sample – and 25 ng/mL of PSA) with and without microbeads are depicted in **Figure 4**.

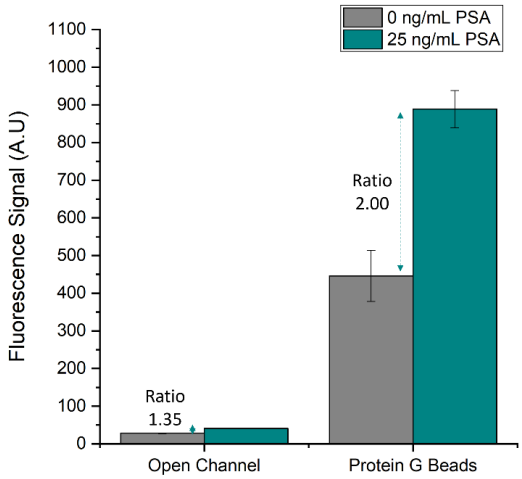

Figure 4 *- Study of fluorescence signal PSA detection using two different approaches: open channel and microchannel packed with protein G beads. Error bars represent the standard deviation of two repeated measurements, which were acquired with Olympus Microscope with an 4x objective, exposure time of 2 seconds and 0dB of gain.*

By analysing **Figure 4** it is possible to conclude that higher fluorescence signals are achieved when Protein G agarose beads are used to immobilize capture anti-PSA antibodies in comparison with the same experiment performed in open channel. In fact, microbeads bring several advantages over planar surfaces, such as higher surface-to-volume ratio, which consequently increases sensitivity, higher binding capacities, tunable surface functionalization and short diffusion distances in between beads. Some studies proved that, in addiction to higher surface-to-volume ratios, short diffusion distances between beads in microfluidic channels cause a reduction in analysis capture times and, consequently in overall immunoassay timeframes in relation to planar counterparts[27,28]. Therefore, the strategy chosen to continue the study of PSA detection in different solutions is agarose Protein G microbeads for the immobilization of capture antibodies.

3.2. Immobilization of capture anti-PSA antibody on different microbeads

The next immunoassay aimed at comparing agarose beads coated with Protein A or Protein G beads and their affinity to capture anti-PSA antibodies. In both cases the experiments were performed using a complete sandwich immunoassay already described in section **2.2.4**. Both set of experiments were performed using 0 ng/mL of PSA as control sample and 25 ng/mL of target analyte as sample under analysis. **Figure 5** illustrates these results.

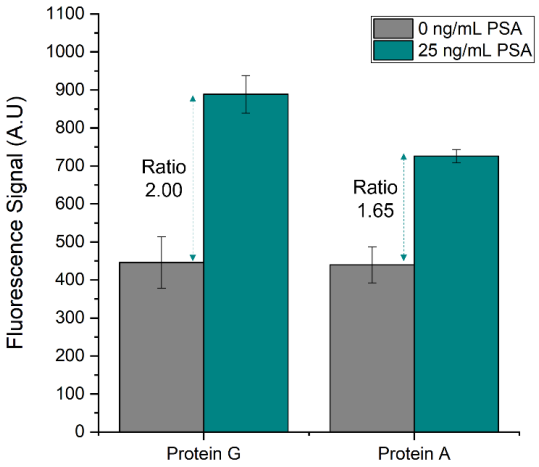

Figure 5 *-Study of fluorescence intensity for PSA detection, in which the capture anti-PSA antibodies were immobilized on Protein G agarose beads or Protein A agarose beads. Error bars represent the standard deviation of two repeated measurements acquired with Olympus Microscope with an 4x objective, exposure time of 2 seconds and 0dB of gain.*

As can be seen by the graph in **Figure 5**, protein G beads present higher affinity for capture anti-PSA antibodies in comparison with Protein A beads. In fact, each protein has a different binding strength for IgG molecules which varies according to their source species and immunoglobulin subtype. Protein A has a greater binding affinity towards various mammalian IgG subclasses, being generally preferred for rabbit, goat, and only IgG1, IgG2 and IgG4 human subclasses, while Protein G specifically binds a broader range of human and mouse subclasses[29]. The reduction in fluorescence signal when anti-PSA antibodies are immobilized on Protein A can be explained by the biological source of the capture antibodies used, which were derived from mouse species, specifically IgG1 isotype, since Protein A has a significantly lower binding affinity to this subclass of mouse IgG molecules. Thus, these results are in agreement with literature. Having this into consideration, the microbeads chosen to perform the remaining assays for PSA detection studies were agarose coated Protein G beads.

3.3. Blocking optimization

Working towards the detection of lower concentrations of target analyte PSA with minimum non-specific signal, several assays were performed in order to optimize surface-blocking, by testing different blocking agents. The blocking agents used included BSA 4% (w/v), Casein 1% (w/v), a mixture of both consisting of 50% BSA 4% (w/v) and 50% Casein 1% (w/v), and, finally, a mixture of human serum antibodies, commercially available as Gammanorm®, at a working concentration of 5 mg/mL. The assays performed consisted in a complete sandwich immunoassay described in section **2.2.4**. and set of experiments were performed using 0 ng/mL of PSA as control sample and 25 ng/mL of target analyte as sample under analysis. The results obtained are depicted in **Figure 6**.

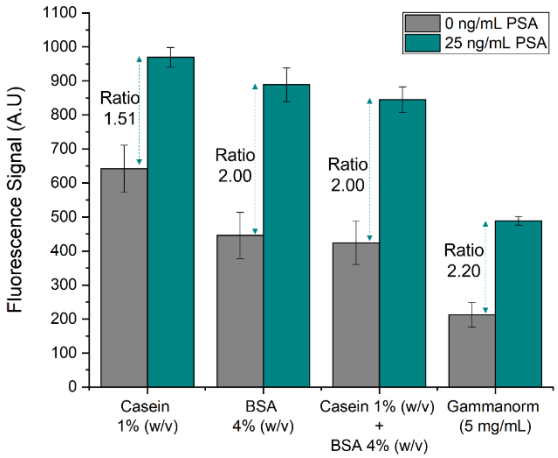

Figure 6 *- Blocking optimization for PSA detection, in which different blocking agents were tested - Casein 1% (w/v), BSA 4% (w/v), a mixture of 50% Casein 1% (w/v) with 50% BSA 4% (w/v) and Gammanorn at 5 mg/mL. A complete sandwich immunoassay was performed to evaluate the efficacy of each blocking agent for sample (25 ng/mL of PSA) and control (0 ng/mL of PSA). The dashed arrows represent the ratio calculated between sample and control. Error bars represent the standard deviation of two repeated measurements acquired with Olympus Microscope with an 4x objective, exposure time of 2 seconds and 0dB of gain.*

By analysing **Figure 6** it is possible to verify that both BSA 4% (w/v) and the mixture of equal volumes of BSA 4% (w/v) and Casein 1% (w/v) presented the same signal-to-noise ratio. The mixture of Casein and BSA was therefore excluded from the blocking agents considered, since the reduction of reagents used in each experiment is one of the major goals of microfluidic applications and because the fluorescence signal obtained in the experiment performed with 25 ng/mL of PSA in which this blocking mixture was used was slightly lower in comparison with the same experiment performed with BSA 4% (w/v) as blocking agent. Regarding Casein 1% (w/v), even though the experiments with 25 ng/mL of target analyte presented a slightly superior fluorescence signal in comparison with the experiments performed with remaining blockers, this blocker was excluded since the signal-to-noise ratio was lower, indicating an increased control signal resulting from non-specific absorption, which translates in a less efficient blocking performance. As it is possible to observe in **Figure 6**, even though signal-to-noise ratio is higher when the Gammanorm® is used as blocking agent in comparison with BSA 4%(w/v), this former blocker also causes a noticeable decrease in the overall fluorescence signal of the system. This might be explained by the composition of this blocker, consisting of a mixture of antibodies in which about 95% are human IgGs, possibly leading to interspecies cross-reactivity, which may decrease specific fluorescence signal. Hence, BSA 4% (w/v) was chosen as the appropriate blocking agent for the microfluidic immunoassays, even though there was still some non-specific signal that was not possible to eliminate.

3.4. Concentration of capture antibody

In order to allow a complete saturation of capture antibody on microbeads surface and simultaneously ensure an efficient antigen capture, two different approaches were used: (I) a complete sandwich assay performed as described in detail in section 2.2.4, where capture anti-PSA antibodies were used at concentrations ranging from 0 µg/mL to 1 mg/mL, and (II) an assay where Alexa430-labelled anti-PSA antibodies were immobilized on Protein G beads at same concentrations and incubated in the microchannel, followed by a washing step to remove unbound molecules. The results for both approaches are shown in **Figure 7**.

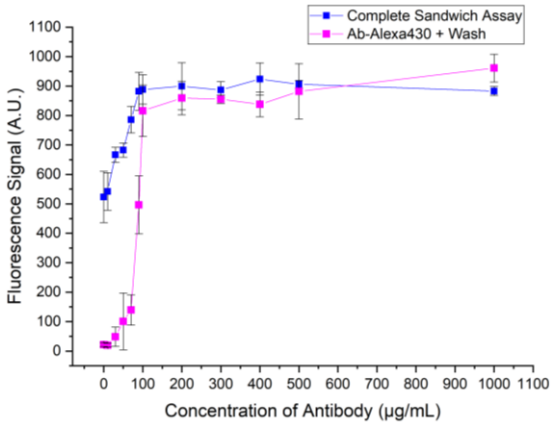

Figure 7 *- Optimization studies for capture anti-PSA antibody concentration. Fluorescence signal obtained with increasing concentration antibody concentration from 0 µg/mL to 1 mg/mL using two different approaches: (I) a complete sandwich immunoassay and (II) by incubation anti-PSA antibodies conjugated with Alexa430 fluorophore in Protein G beads in microfluidic channel, followed by a washing step to remove unbound molecules. Error bars represent the standard deviation between two repeated measurements acquired with Olympus Microscope with an 4x objective, exposure time of 2 seconds and 0dB of gain.*

There is an increase in fluorescence signal with increasing concentrations of anti-PSA antibodies (I), reaching a plateau regime around 100 µg/mL of antibody. This same behaviour is confirmed by the second assay in which only labelled anti-PSA antibodies immobilized on Protein G beads were incubated in the microchannel (II). Therefore, a concentration of 100 µg/mL of anti-PSA antibody would guarantee a complete saturation of microbeads surface since the use of concentrations above the reached plateau would possibly lead to an excess antibody coverage. Some studies suggest that these excessive layer of antibodies would lead to these antibodies to build up and block other available antibody molecules, possibly due to antibody-antibody interactions and aggregation, thus rendering their binding sites unavailable[30]. Therefore, to prevent this masking effect and simultaneously allow an efficient bead surface coverage and capture while accounting for reagent saving the concentration of antibody chosen to continue the experiments for PSA detection was 100 µg/mL.

3.5. Simulation of PSA detection in biological matrix

After completing the optimizations studies for PSA detection, the effect of a biological matrix in the detection of PSA was evaluated and specific concentrations of PSA were detected in spiked solutions of either PBS or human serum. The results obtained are a result of a simultaneous measurement of a control sample, with no PSA, i.e., sample with plain PBS or non-spiked human serum and 25 ng/mL of PSA. The main goal of this experiments was to bring them closer to real-life POC applications, considering that most cancer biomarkers can be found in biological fluids. The results obtained are depicted in **Figure 8**

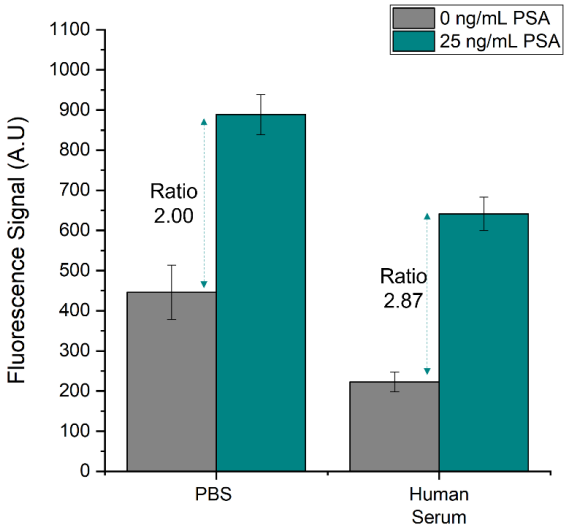

Figure 8 *- Biological matrix effect in PSA detection. Fluorescence intensity of complete sandwich immunoassays performed with spiked solutions of PSA in PBS and human serum for sample (25 ng/mL of PSA) and control (0 ng/mL of PSA). The dashed arrows represent the ratio of the sample to the control. Error bars represent the standard deviation between two repeated experiments acquired with Olympus Microscope with an 4x objective, exposure time of 2 seconds and 0dB of gain.*

There is an overall decrease in fluorescence intensity when samples of PSA are spiked in human serum in comparison with its PBS counterpart. This reduction might be explained by the effect of several proteins which constitute human serum, possibly interfering with antibody-antigen complex formation, creating a blocking effect noticeable not only in the experiment with PSA concentration of 25 ng/mL, but also with the control sample. The other factor might be related to the viscosity inherent to increased concentrations of proteins in solution, which possibly affects the transport of molecules inside the channel, consequently hindering capture of PSA molecules. Furthermore, there is still a considerable amount of fluorescence signal in the absence of PSA (control samples) even with the current blocking strategy, due to non-specific molecular interactions for both PBS and human serum tests. Therefore, further work is in need to optimize the detection of PSA, minimizing non-specific interactions. However, there is still a significant amount of specific signal obtained for 25 ng/mL of PSA for both PBS and human serum samples.

3.6. Detection of PSA within clinically relevant range

After analysing the effect of biological matrix in the detection of PSA, the capacity to detect this cancer biomarker within a clinically relevant range was further analysed, using the microfluidic sandwich immunoassay described. Therefore, different concentrations of PSA were prepared in spiked solution of either PBS or human serum, ranging from 0 ng/mL to 25ng/mL. For this purpose, calibration curves for PSA were obtained and the results of this approach are depicted in **Figure 9**.

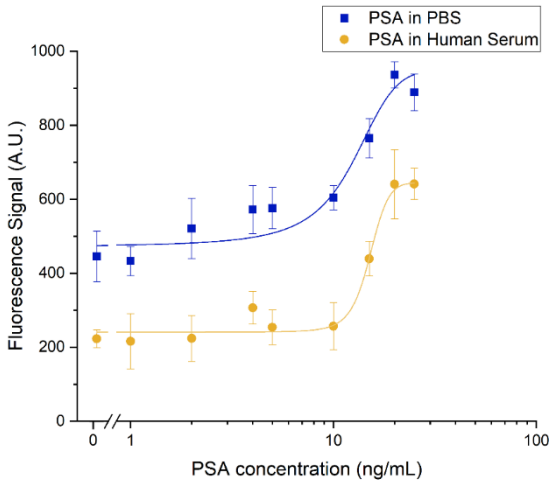

Figure 9 *- Calibration curves for quantification of PSA spiked in PBS and human serum. Error bars represent the standard deviation of two repeated measurements. Data acquired in Olympus Microscope with an 4x objective, exposure time of 2 seconds and 0dB of gain.*

The calibration curves obtained show that overall fluorescence signal increases with increased concentrations of PSA for both conditions and the value of all concentrations of PSA in buffer are higher than that of PSA in human serum, which is in agreement with the latter results presented. Moreover, for experiments performed with PSA spiked in PBS it is possible to detect and distinguish from blank samples concentrations of nearly 10 ng/mL and clearly 15, 20 and 25 ng/mL of PSA spiked in PBS solutions. For experiments performed with PSA spiked in human serum solution, the concentrations of target analyte that were possible to undoubtedly detect ranged from 15 to 25 ng/mL, with little significant difference detected between lower concentrations of PSA in solution. By performing a non-linear fit to the data, the LoD was calculated for both calibration curves depicted, being considered as the concentration that corresponds to a signal of three times the standard deviation of the blank sample. For PSA samples spiked in buffer solution, the LoD was found to be 10.8 ng/mL, slightly above the "grey zone" of 4 to 10 ng/mL considered clinically relevant. This sensitivity represented a ~ 2-fold improvement in comparison to a previous study in which Madaboosi et al.[31] reported a LoD of 21.4 ng/mL using a similar sandwich immunoassay system in a bare microfluidic channel with target PSA spiked in buffer solutions. These results prove, once again, the higher sensitivity that microbeads bring to immunoassays. However, the LoD achieved for spiked solution of PSA in human serum, was slightly higher, with a LoD of 12.2 ng/mL, a sensitivity value comparable to that reported in 2017 by Pinto and co-workers[21], which achieved a LoD of at least 10 ng/mL of PSA in human serum samples, in a sandwich immunoassay performed with Protein A beads used to immobilize anti-PSA antibodies. These results indicate that the study performed in buffer solution is more sensitive in comparison with the assay performed with human serum spiked samples, possibly due to the interference of the several protein constituents of this latter biological matrix, which might interfere with specific molecular recognition.

Despite these promising results and their approximation to the clinical window for PSA detection, novel strategies are in need to further push down the values of LoD, allowing the use of this system for PC diagnosis. In that context, an amplification strategy based on streptavidin-biotin complex can be employed to enhance assay sensitivity. The interaction between streptavidin and biotin has been considered the strongest, more stable non-covalent interaction ever known[32], which makes this strategy an extensively used system for the detection of biomarkers in several immunoassays. Madaboosi *et al*[31], reported a 10-fold reduction in LoD of PSA from 21.4 ng/mL to 2.7 ng/mL thanks to the biotin-streptavidin amplification strategy implemented in the sandwich immunoassay performed in a microfluidic device, successfully reaching the clinically relevant "grey zone" for PC diagnosis. Therefore, streptavidin-biotin complex it is a promising signal amplification strategy, that can be applied in future experiments within the present work context, in order to lower the LoD herein reported.

3.7. Influence of ILs and PEG in PSA detection

The influence of all ILs and two different molecular weights of PEG in the detection of prostate cancer biomarker PSA was analysed based on the sandwich immunoassay described in detail section **2.2.4**. For the purpose of this analysis, a specific concentration above LoD of PSA in PBS was used, namely 25 ng/mL, ensuring the fluorescence signal obtained was not influenced by a possible lack of sensitivity of the system if lower concentrations of PSA were used. The samples tested consisted in buffer, ILs or PEGs non-spiked solution of PSA (control sample with no PSA) and spiked solution with 25 ng/mL of PSA. The ILs and PEGs were prepared at 5% (w/w), since a lower interference in PSA detection would be expected when the solutions are used in lower concentrations. The results obtained are depicted in **Figure 10**. It is possible to conclude that the ILs showing higher signal-to-noise ratios and higher fluorescence signals for PSA detection are [C_4C_1pyrr]Cl, [N_{4444}]Cl, [P_{4444}]Cl and [P_{4444}]Br. However, despite the high fluorescence intensity registered by PSA spiked solution of [P_{4444}]Cl, there is an increase in non-

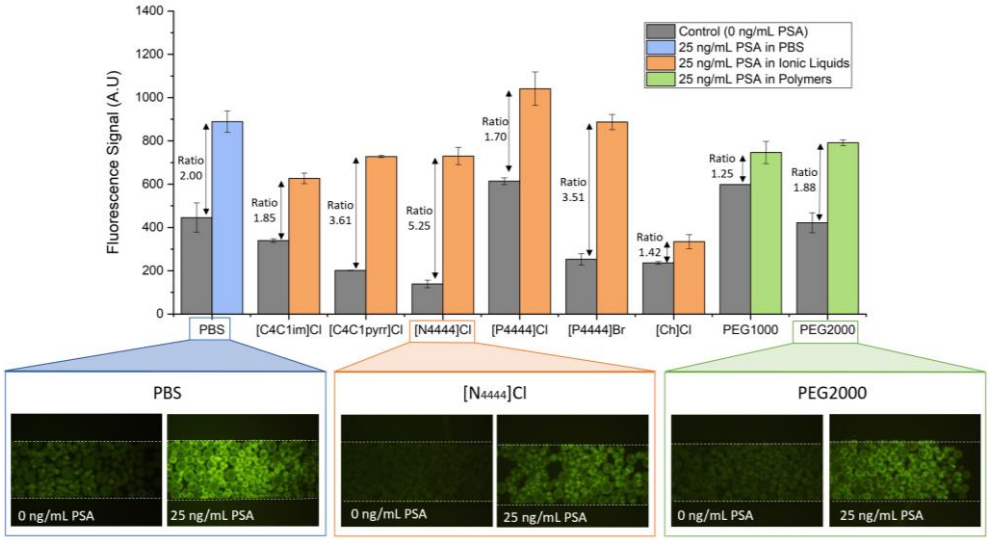

Figure 10 *- Influence of ILs and PEG in PSA detection studies. (Top) Fluorescent signal obtained by performing sandwich immunoassays for the detection of PSA spiked in PBS, six ILs ([C4C1im]Cl, [C4C1pyrr]CL, [N4444]Cl, [P4444]Cl, [P4444]Cl and [Ch]Cl) and PEG with two different molecular weights (PEG1000 and PEG2000) for both sample (25 ng/mL) and control (0 ng/mL). The arrows represent the ratio of sample to control and error bars represent the standard deviation between two repeated measurements. (Bottom) The set of experimental images from left to right represent the fluorescence signal of PSA detection in PBS, [N4444]Cl and PEG2000. All data was acquired with Olympus Microscope with an 4x objective, exposure time of 2 seconds and 0dB of gain.*

specific interactions, reducing the ratio between sample and control experiments to a value even lower than that of the experiments performed in PBS. Therefore, $[P_{4444}]Cl$ will probably not be considered for the formation of ABS for the extraction of PSA from complex biological matrices. The experiments for the detection of PSA spiked in $[N_{4444}]Cl$ was the one which registered a higher ratio from sample with 25 ng/mL of PSA to that of the control with a significant decrease of non-specific signal and slightly lower fluorescent specific signal in comparison with PBS experiments. Regarding $[C_4C_1pyrr]Cl$ and $[P_{4444}]Br$, both registered similar signal-to-noise ratios, but the latter showed a better specific fluorescent signal, comparable with that of the experiments performed in PBS. Interestingly, ammonium and phosphonium based ILs, such as $[N_{4444}]Cl$ and $[P_{4444}]Br$ are commonly used as constituents of ABS for extraction and concentration of several target biomolecules and stand out in terms of cost and thermal and chemical stability[33]. Therefore, and considering that PSA, similarly to other biomarkers, is only present in trace amounts in a sample, these results attest the potential of these ILs as promising candidates for the formation of ABS for extraction of PSA biomarker from complex biological fluids and subsequent pre-concentration, without jeopardizing their detection using a sandwich-type of immunoassay, as the one presently employed. In addition to ILs, two different molecular weights of PEG were tested at 5% (w/w) concentration, namely PEG1000 and PEG2000, since it is one of the most used polymers for the formation of polymer-based and IL-based ABS. In **Figure 10**, it is possible to observe that PEG2000 presented lower interference in the detection of PSA in comparison with PEG1000, achieving a slightly higher ratio between 25 ng/mL of PSA sample and control sample than that of PEG1000. Moreover, the signal-to-noise ratio achieved with PSA spiked in PEG2000 is similar to that of the assay performed in PBS. Therefore, PEG2000 also proved to be a reliable choice for the formation of ABS for sample pre-treatment, prior to PSA detection, with minimum interference during target measurement and could be used together with one of the referred ILs for the formation of IL-based ABS.

3.8. Future development of fully integrated microfluidic device

Based on the results obtained, it is possible to assume the use of some ILs, such as $[P_{4444}]Br$ and $[N_{4444}]Cl$, as constituents of ABS for the extraction and concentration of PSA, to overcome the matrix interference effects faced by immunoassays. Therefore, the developed strategy is expected to work as basis for a fully integrated microfluidic device capable of simultaneously performing extraction and detection of target analyte. The system would consist of a two-module device, in which the first module would be dedicated to extraction and concentration through an IL-based ABS and the second to the detection of target analyte through a sandwich immunoassay. The idea would consist of converging a serum sample spiked with salt (containing a certain concentration of PSA) with an IL-rich phase. In the extraction module, the partitioning of PSA is expected to occur to the IL-rich phase. Then, PSA would diffuse to the detection module, where it would be capture by anti-PSA antibodies immobilized on protein G beads. Afterwards, through a third inlet, detector anti-PSA antibodies would be inserted in the channel, binding to captured target PSA, and completing the sandwich immunoassay. This way it would be possible to create a fully integrated system capable of accurate diagnosis of PC from biological samples. **Figure**

11 represents the schematics of the integrated microfluidic system described.

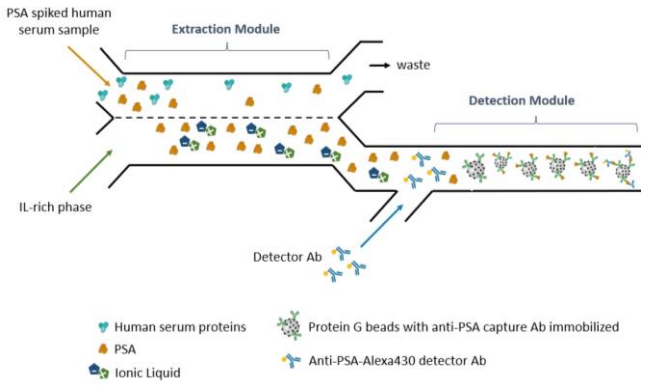

Figure 11 *- Conceptual schematic of integrated microfluidic platform for simultaneous extraction, concentration, and detection of PSA in biological samples to increase system sensitivity and lower detection limits.*

4. Conclusions and Future Prospects

In the present work a bead-based sandwich immunoassay was implemented for the detection of PSA within clinically relevant range. The capacity to detect PSA in different ILs and PEG was also studied, mainly because these solutions can be applied for extraction and concentration of PSA using ABS prior to its detection, improving system sensitivity, and lowering detection limits. The developed system was able to reach a LoD of 10.8 ng/mL of samples spiked in buffer and a LoD of 12.2 ng/mL of PSA in human serum, which are close to the upper limit of the clinically relevant range (4-10 ng/mL) and comparable to other published studies. Despite these promising results, further work is in need to overcome a high level of non-specific interaction obtained, which can be achieved by using streptavidin-biotin complex or aptamer-based immunoassays. Nevertheless, the developed system has potential to be applied to other types of cancer biomarkers and works as basis for future development of a microfluidic device with fully integrated IL-based ABS and detection system capable of accurate diagnosis of prostate cancer from complex biological fluids.

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6. References

1. Wagner, P. D., Verma, M. & Srivastava, S. Challenges for biomarkers in cancer detection. *Ann. N. Y. Acad. Sci.* **1022**, 9–16 (2004).
2. Wu, L. & Qu, X. Cancer biomarker detection: Recent achievements and challenges. *Chem. Soc. Rev.* **44**, 2963–2997 (2015).
3. Jayanthi, V. S. P. K. S. A., Das, A. B. & Saxena, U. Recent advances in biosensor development for the detection of cancer biomarkers. *Biosens. Bioelectron.* **91**, 15–23 (2017).
4. Henry, N. L. & Hayes, D. F. Cancer biomarkers. *Molecular Oncology* vol. 6 140–146 (2012).
5. Pérez-Ibave, D. C., Burciaga-Flores, C. H. & Elizondo-Riojas, M. Á. Prostate-specific antigen (PSA) as a possible biomarker in non-prostatic cancer: A review. *Cancer Epidemiol.* **54**, 48–55 (2018).
6. Catalona, W. J. *et al.* Use of the percentage of free prostate-specific antigen to enhance differentiation of prostate cancer from benign prostatic disease: A prospective multicenter clinical trial. *J. Am. Med. Assoc.* **279**, 1542–1547 (1998).
7. Stephan, C., Lein, M., Jung, K., Schnorr, D. & Loening, S. A. The influence of prostate volume on the ratio of free to total prostate specific antigen in serum of patients with prostate carcinoma and benign prostate hyperplasia. *Cancer* **79**, 104–109 (1997).
8. Adhyam, M. & Gupta, A. K. A Review on the Clinical Utility of PSA in Cancer Prostate. *Indian J. Surg. Oncol. 2012 32* **3**, 120–129 (2012).
9. Albertsson, P. Å. A. Partition of proteins in liquid polymer-polymer two-phase systems (Nature (1958) 182, 4640). *Nature* **182**, 918 (1958).
10. Pereira, J. F. B., Lima, A. S., Freire, M. G. & Coutinho, J. A. P. Ionic liquids as adjuvants for the tailored extraction of biomolecules in aqueous biphasic systems †. *R. Soc. Chem.* **12**, 1661–1669 (2010).
11. Gutowski, K. *et al.* Controlling the aqueous miscibility of ionic liquids: aqueous biphasic systems of water-miscible ionic liquids and water-structuring salts for recycle, metathesis, and separations. *J. Am. Chem. Soc.* **125**, 6632–6633 (2003).
12. Ventura, S. P. M. *et al.* Ionic-Liquid-Mediated Extraction and Separation Processes for Bioactive Compounds: Past, Present, and Future Trends. *Am. Chem. Soc.* **117**, 6984–7052 (2017).
13. Freire, M. G. *et al.* Aqueous biphasic systems: A boost brought about by using ionic liquids. *Chem. Soc. Rev.* **41**, 4966–4995 (2012).
14. Louros, C. L. S. *et al.* Extraction of biomolecules using phosphonium-based ionic liquids + K3PO4 aqueous biphasic systems. *Int. J. Mol. Sci.* **11**, 1777–1791 (2010).
15. Li, Z., Liu, X., Pei, Y., Wang, J. & He, M. Design of environmentally friendly ionic liquid aqueous two-phase systems for the efficient and high activity extraction of proteins. *Green Chem.* **14**, 2941–2950 (2012).
16. Lin, X., Wang, Y., Zeng, Q., Ding, X. & Chen, J. Extraction and separation of proteins by ionic liquid aqueous two-phase system. *Analyst* **138**, 6445–6453 (2013).
17. Neves, C. M. S. S. *et al.* Simultaneous separation of antioxidants and carbohydrates from food wastes using aqueous biphasic systems formed by cholinium-derived ionic liquids. *Front. Chem.* **7**, 1–12 (2019).
18. Freire, M. G. *et al.* High-performance extraction of alkaloids using aqueous two-phase systems with ionic liquids. *Green Chem.* **12**, 1715–1718 (2010).
19. Wu, J. *et al.* Lab-on-a-Chip Platforms for Detection of Cardiovascular Disease and Cancer Biomarkers. *Sensors* **17**, 2934 (2017).
20. Sanjay, S. T. *et al.* Biomarker detection for disease diagnosis using cost-effective microfluidic platforms. *Analyst* **140**, 7062 (2015).
21. Pinto, I. F. *et al.* The application of microbeads to microfluidic systems for enhanced detection and purification of biomolecules. *Methods* **116**, 112–124 (2017).
22. Caneira, C. R. F. *et al.* Development of a rapid bead-based microfluidic platform for DNA hybridization using single- and multi-mode interactions for probe immobilization. *Sensors Actuators, B Chem.* **286**, 328–336 (2019).
23. Brás, E. J. S., Fortes, A. M., Chu, V., Fernandes, P. & Conde, J. P. Microfluidic device for the point of need detection of a pathogen infection biomarker in grapes. *Analyst* **144**, 4871–4879 (2019).
24. Soares, R. R. G. *et al.* Multiplexed microfluidic platform coupled with photodetector array for point-of-need and sub-minute detection of food contaminants. *IEEE Micro Electro Mech. Syst.* 6–9 (2018).
25. Lim, C. T. & Zhang, Y. Bead-based microfluidic immunoassays: The next generation. *Biosens. Bioelectron.* **22**, 1197–1204 (2007).
26. Verpoorte, E. Beads and chips: new recipes for analysis. *Lab Chip* **3**, 60N–68N (2003).
27. Sato, K. *et al.* Integration of an Immunosorbent Assay System: Analysis of Secretory Human Immunoglobulin A on Polystyrene Beads in a Microchip. *Am. Chem. Soc.* **72**, 1444–1147 (2000).
28. Zammatteo, N. *et al.* Comparison between microwell and bead supports for the detection of human cytomegalovirus amplicons by sandwich hybridization. *Anal. Biochem.* **253**, 180–189 (1997).
29. Choe, W., Durgannavar, T. A. & Chung, S. J. Fc-Binding Ligands of Immunoglobulin G: An Overview of High Affinity Proteins and Peptides. *Materials (Basel).* **9**, 994 (2016).
30. Wiseman, M. E. & Frank, C. W. Antibody Adsorption and Orientation on Hydrophobic Surfaces. *Am. Chem. Soc.* **28**, 1765–1774 (2012).
31. Madaboosi, N., Soares, R. R. G., Chu, V. & Conde, J. P. A microfluidic immunoassay platform for the detection of free prostate specific antigen: A systematic and quantitative approach. *Analyst* **140**, 4423–4433 (2015).
32. Holmberg, A. *et al.* The biotin-streptavidin interaction can be reversibly broken using water at elevated temperatures. *Electrophoresis* **26**, 501–510 (2005).
33. Pereira, M. M. *et al.* Enhanced extraction of bovine serum albumin with aqueous biphasic systems of phosphonium- and ammonium-based ionic liquids. *J. Biotechnol.* **206**, 17–25 (2015).