SPR Biosensor for Quantification of Fetuin-A as a Promising Multibiomarker

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Summary

Early diagnosis of ongoing malignant disease is crucial to improve survival rate and life quality of the patients and requires sensitive detection of specific biomarkers e.g. prostate-specific antigen (PSA), carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), etc. In spite of current technological advances, malignant diseases are still identified in rather late stages, which have detrimental effect on the prognosis and treatment of the disease. Here, we present a biosensor able to detect fetuin-A, a potential multibiomarker. The biosensing platform is based on polymer brush combining antifouling monomer units of *N*-(2-hydroxypropyl)methacrylamide (HPMA) and carboxybetaine methacrylamide (CBMAA), statistically copolymerized by surface-initiated atom transfer radical polymerization. The copolymer poly(HPMA-co-CBMAA) exhibits excellent non-fouling properties in the most relevant biological media (i.e. blood plasma) as well as antithrombogenic surface properties by preventing the adhesion of blood components (i.e. leukocytes; platelets; and erythrocytes). Moreover, the polymer brush can be easily functionalized with biorecognition elements maintaining high resistance to blood fouling and the binding capacity can be regulated by tuning the ratio between CBMAA and HPMA units. The superior antifouling properties of the copolymer even after biofunctionalization were exploited to fabricate a new plasmonic biosensor for the analysis of fetuin-A in real clinical blood plasma samples. The assay used in this work can be explored as label-free affinity biosensor for diagnostics of different biomarkers in real clinical plasma samples and to shift the early biomarker detection toward novel biosensor technologies allowing point of care analysis.

Introduction

Treatment of malignant diseases requires early and sensitive detection of specific biomarkers of the disease e.g. prostate-specific antigen (PSA), carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), cancer antigen 125 (CA-125). Early detection of ongoing malignant changes, even before onset of the symptoms, has been shown to improve survival rate and life quality of the patients (Neal *et al.* 2015). However, despite of our current technological advances, most malignant diseases are still being identified in rather late stages, which has detrimental effect on the prognosis and treatment of the disease, mostly due to lack of specific biomarkers with predictive value. Therefore, although challenging, the search for novel specific biomarkers is still a very promising approach for early detection of various malignant diseases (Konforte and Diamandis 2013).

Fetuin-A (also known as alpha-2-HS-glycoprotein) is a 59 kDa glycoprotein synthesized in liver participating in many physiological pathways (Denecke *et al.* 2003). Recently, a growing number of

studies indicates that fetuin-A also participates in various pathophysiological states, including vascular disorders (Naito *et al.* 2016), metabolic syndrome and insulin resistance (Malin *et al.* 2014), and chronic lymphocytic leukemia (Dalamaga *et al.* 2016). And in the last years, fetuin-A circulating in blood was proposed as a potential biomarker of various diseases such as diabetes (Yang *et al.* 2015, Yin *et al.* 2015), liver steatosis (von Loeffelholz *et al.* 2016), myelodysplastic syndrome (Majek *et al.* 2015, Majek *et al.* 2014, Májek *et al.* 2013), multiple sclerosis (Harris *et al.* 2013), spondyloarthropathies or rheumatoid arthritis (Harman *et al.* 2017), pre-eclampsia (Sanhal *et al.* 2016) and many others. Moreover, urinary fetuin-A was established as a sensitive biomarker of polycystic kidney disease (Piazzon *et al.* 2015). It is clear that fetuin-A becomes a potent multibiomarker in the last years. The plasma concentration of fetuin-A in healthy donors is usually in the range of 200-600 $\mu g \cdot ml^{-1}$ (Sun *et al.* 2014) and it changes in various pathophysiological states. Therefore, monitoring of fetuin-A plasma concentration might serve as an early marker of ongoing malignant diseases and pathological changes in the organisms.

Currently, fetuin-A plasma level is clinically determined to monitor vessel calcifications and metabolic syndrome (Koos *et al.* 2009) using enzyme-linked immunosorbent assay (ELISA) or immunoturbidimetry (using nephelometry method) from plasma (Wigger *et al.* 2009) or cerebrospinal fluid (Harris *et al.* 2013). Despite the tradition and wide use, such assays have their pitfalls (Tighe *et al.* 2015). Cost-effectiveness, the need for trained personnel and the time delay to obtain the results are some of the biggest obstacles; consequently they are not suitable for point-of-care diagnostics. Therefore, the clinical detection of biomarkers requires new and more effective techniques. Especially, it is of high interest to develop detection techniques that provide a fast response, high sensitivity, specificity, and low sample consumption to analyze blood plasma/serum samples as well as the ability to analyze samples obtained non-invasively such as urine or saliva.

Affinity optical biosensors are a promising and fast developing platform that enables fast and multiple analyses of various disease markers even outside of centralized laboratories, thus bringing the analysis closer to the patient. In particular, biosensors based on surface plasmon resonance (SPR) are potent analytical devices allowing the real-time observation of interactions of biomolecules with very low detection limits (Homola

2008, Riedel *et al.* 2014). Moreover, they do not require complex sample pre-treatment; usually a simple plasma (or blood) sample dilution is the only necessary step in the sample preparation process. Nevertheless, SPR-based assays have to cope with specific issues as well. Arguably, the key technical problem that has so far prevented the spreading of this analytical device into clinical praxis is fouling, i.e. non-specific protein interaction with the sensing surface (Thompson *et al.* 2013). In order to solve this problem, research in surface chemistries and architectures was pursued to decrease or completely eliminate the adverse effect of fouling and thereby maximize the efficiency of biosensors (Emmenegger *et al.* 2009). The most widely used surface modifications are based on grafting of preformed poly(ethylene glycol) (PEG) chains to the surface or self-assembled monolayers (SAM) terminated with short oligo(ethylene glycol) chains (OEG). However, it has been shown by many authors that such surface modifications fail when complex biological samples (e.g. blood plasma/serum, cerebrospinal fluid, saliva, urine) are analysed (Rodriguez-Emmenegger *et al.* 2012). On the other hand, surface modifications based on polymer brushes grown by surface-initiated radical polymerizations such as poly(2-hydroxyethyl methacrylate) and poly[oligo(ethylene glycol) methacrylate] are able to significantly reduce the fouling from complex biological media (De Los Santos Pereira *et al.* 2014, Karczmarczyk *et al.* 2016). Nevertheless, so far the only surfaces presented that can prevent the fouling from blood plasma are polymer brushes of poly[*N*-(2-hydroxypropyl)methacrylamide] (HPMA) and poly(carboxybetaine acrylamide) (CBAA) while the polymer side groups allow surface functionalization with bioactive recognition elements (Riedel *et al.* 2017, Riedel *et al.* 2016). Excellent resistance to fouling is one of the first requirements of an affinity biosensor. In addition, the presence of functional groups along the polymer chain and its facile biofunctionalization with desired biorecognition element is another limiting factor. It has been shown recently that the functionalization can lead to irreversible changes in the polymer brush and to increased fouling (Lísalová *et al.* 2017). Therefore, surface modifications that are able to resist the fouling from complex biological media even after its biofunctionalization are desired.

In this report, we developed a biosensing platform able to detect fetuin-A, a marker associated with various pathological conditions. The biosensing platform

consists of two antifouling monomer units HPMA and CBMA statistically copolymerized by surface-initiated atom transfer radical polymerization (SI-ATRP). The superior antifouling properties of the copolymer even after biofunctionalization were exploited to fabricate a new plasmonic biosensor for the analysis of fetuin-A in real clinical blood plasma samples. The copolymer combines the antifouling properties of both monomeric units, while eliminating the negative effects of activation and biofunctionalization of the copolymer brush on the fouling.

Methods

Materials

1,4,8,11-tetramethyl-1,4,8,11-tetraazacyclotetradecane (Me₄Cyclam, 98 %), CuCl (≥99.995 %), CuCl₂ (99.999 %) were purchased from Sigma-Aldrich and used as received. Initiator ω-mercaptoundecyl bromoisobutyrate was synthesized by the reaction of ω-bromoisobutyryl bromide (Sigma-Aldrich, 98 %) with 11-mercapto-1-undecanol (Sigma-Aldrich, 99 %) according to the literature procedure (Jones *et al.* 2002). *N*-[3-(Dimethylamino)propyl] methacrylamide (DMAPMA, 98 %) and β-propiolactone (90 %) were purchased from TCI Europe and Serva Electrophoresis GmbH, respectively. Water was purified by Millipore device (Milli-Q). THF, dichloromethane, diethylether, ethanol (spectroscopy grade) were purchased from LachNer, Czech Republic. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. The monomers 3-methacryloylaminopropyl-2-carboxyethyl-dimethylammonium betaine (carboxybetaine methacryl-amide, CBMAA) and *N*-(2-hydroxypropyl)methacrylami-de (HPMA) were synthesized according to the literature (Lidický *et al.* 2016, Ulbrich *et al.* 2000). The buffers used were: phosphate buffered saline (PBS, 10 mM disodium hydrogen phosphate, 2 mM potassium phosphate, 137 mM sodium chloride, 2.7 mM potassium chloride, pH 7.4); sodium borate buffer (SB, 10 mM, pH 8.5); sodium acetate buffer (SA, 10 mM, pH 5). Polyclonal chicken anti-human fetuin-A IgY and human fetuin-A were purchased from Abcam.

Preparation of SPR sensor surface

We used a recently developed poly(HPMA-*co*-CBMAA) brushes grown by surface initiated atom transfer radical polymerization on SPR chips, prepared form microscopy glass slides coated with an adhesion titanium layer (2 nm) and gold layer (thickness 50 nm) (Riedel *et al.* 2016). Briefly, the brushes were prepared as follows: 7 ml of methanol was degassed and transferred under argon atmosphere to a Schlenk tube containing CuCl (35 mg, 354 µmol), CuCl₂ (10.5 mg, 78 µmol) and Me₄Cyclam (121 mg, 472 µmol) and all solids were dissolved. In the second Schlenk tube HPMA (2.4 g, 16.6 mmol) and CBMAA (0.7 g, 2.9 mmol) were dissolved in 12 ml of degassed water and 5 ml of degassed methanol. Both solutions were mixed and the homogenized polymerization mixture was brought into the reactor containing the substrates coated with the initiator SAM. The polymerization was carried out at 30 °C for 2 h. The ratio 85/15 % of HPMA and CBMAA monomer was used in order to achieve non-fouling polymer coating that enable postfunctionalization.

Fourier-transform infrared spectroscopy

Chemical characterization of the obtained polymer brushes was carried out by Fourier-transform infrared spectroscopy in the grazing angle specular reflectance mode (FTIR-GASR) on samples prepared on gold-coated SPR chips, identically as for the biosensing experiments, using a Nicolet Nexus 870 spectrometer equipped with a SAGA attachment (256 scans, resolution of 2 cm⁻¹).

Surface plasmon resonance

Surface plasmon resonance (SPR) was performed using an instrument based on the Kretschmann geometry and spectral interrogation custom-built at the Institute of Photonics and Electronics of the Academy of Science of the Czech Republic (Kumorek *et al.* 2016, Riedelová-Reicheltová *et al.* 2016). The incident polychromatic light beam excited surface plasmons at a wavelength close to 750 nm, and the reflected signal was collected into four optical fibers coupled to an Ocean Optics spectrophotometer. The binding of the analyte induces changes in the resonant wavelength λ_{res} that are recorded in real time using dedicated software. The limit of detection of the SPR set-up is $\Delta\lambda = 0.02$ nm (Filová *et al.* 2014). A four channel flow cell (volume 1 µl) was used. The flow cell was equipped with a temperature controller (stability 0.01 °C). All measurements were performed at 25 °C. The flow of liquids over the sensing surface of the SPR chip was controlled by a peristaltic pump and the flow rate was kept at 25 µl·min⁻¹.

Immobilization of anti-fetuin-A

Chicken polyclonal anti-fetuin-A IgY antibody was immobilized using EDC/NHS coupling chemistry *via* formation of amide bond between carboxylic groups of the polymer brush and primary amines of the anti-fetuin-A antibody. Briefly, the polymer surface was contacted with SA buffer pH 5. Subsequently, the surface was reacted with a freshly prepared solution 1:1 v/v of EDC (0.4 M) and NHS (0.1 M) for 30 min to form active succinimidyl esters. The surface was subsequently rinsed with SA and SB buffers for one minute each and then anti-fetuin-A antibody (10 μg$\cdot$ml^{-1} in SB buffer) was flowed over surface for 10 min. Finally, the unreacted active ester groups were allowed to hydrolyze by flowing PBS for 90 min.

Biological samples

Human blood plasma was withdrawn from seven different healthy donors at the Institute of Hematology and Blood Transfusion (Czech Republic). Blood was collected by venipuncture into tubes coated with EDTA. Plasma samples were obtained by centrifugation (15 min, 3,000 x g). All samples were obtained in accordance with the Ethical Committee regulations of the Institute of Hematology and Blood Transfusion. The level of fetuin-A in the samples was estimated with an ELISA kit (ab108855; Abcam) according to the manufacturer's instructions.

SPR biosensor assay

The biosensor was based on polymer brushes of poly(HPMA-*co*-CBMAA) with covalently immobilized chicken polyclonal anti-fetuin-A antibody. The detection of fetuin-A was performed both in model PBS solutions as well as in real (non-spiked) plasma samples. After establishing stable baseline in the SPR system in the PBS buffer, the samples were flown through the individual chambers for 5 min followed by rinsing with PBS. The calibration curve for fetuin-A was measured using model solutions of fetuin-A in PBS at different concentrations (10 ng$\cdot$ml^{-1}-10 μg$\cdot$ml^{-1}). Blood plasma samples were diluted in PBS at 1:500 volumetric ratio and immediately measured. The interaction of the target fetuin-A with the anti-fetuin-A antibody was observed in real time by measuring the changes in the sensor signal. The sensor response was defined as a difference in the SPR wavelength before the sample injection and after the rinsing with PBS. All measurements were carried out in triplicate.

Results

The detection of fetuin-A in real human plasma samples was based on the synthetic interface of copolymer brush poly(HPMA-*co*-CBMAA) grown by SI-ATRP on gold-coated SPR chips with an immobilized bioactive element, chicken polyclonal anti-human fetuin-A IgY, by using an amino-coupling surface chemistry (Fig. 1). The poly(HPMA-*co*-CBMAA) copolymer is based on two well-known monomers HPMA and CBMAA, the homopolymer of which are able to suppress the protein fouling from blood plasma, achieving complete prevention in the case of poly(HPMA) (Kostina *et al.* 2012, Rodriguez-Emmenegger *et al.* 2011). Both homopolymer offers the presence of function groups that can be used for postmodification with biorecognition element, hydroxyl groups in HPMA and carboxyl groups in CBMAA. However, it has been shown in the past that activation of all functional groups along the polymer chain leads inevitably to increased protein fouling of these brushes, thus lowering their potential for real applications (Vaisocherová *et al.* 2014). The main advantage of this interface is the combination of two excellent antifouling units with different side chain chemistries, where HPMA units serve as an antifouling layer and CBMAA units are used for immobilization of bioreceptors *via* formation of amide bond between the carboxyl group of the CBMAA and primary amine group of the bioreceptor.
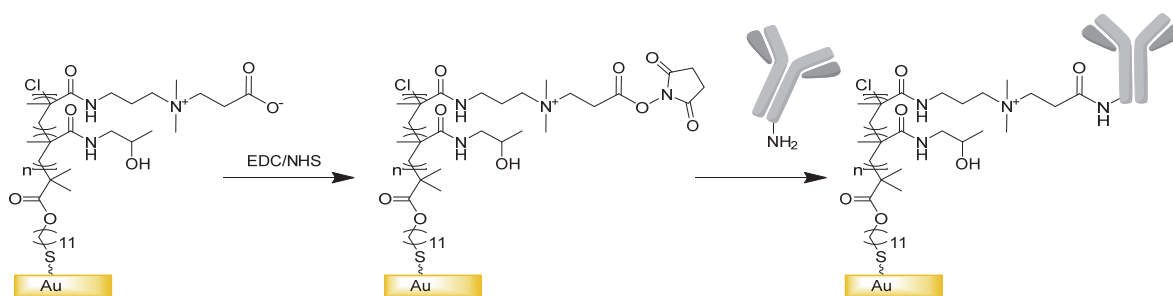

Fig. 1. Chemical structure of poly(HPMA-*co*-CBMAA), activation and anti-fetuin-A immobilization procedure.

Surface chemical characterization

To assess the success in the preparation of the targeted interface architecture, the presence of the polymer brush was confirmed and its chemical structure characterized by Fourier-transform infrared spectroscopy in the grazing angle specular reflectance mode (FTIR-GASR). The obtained spectrum is presented in Figure 2. The dominating features of the spectrum are the bands at 1,653 and 1,525 cm^{-1}, which result from the amide I and II vibrations of the methacrylamide groups, respectively. A shoulder band at 1,617 cm^{-1} and a band at 1,375 cm^{-1} have their origins in the asymmetric and symmetric stretching modes of the ionized carboxylate groups, present only in the CBMAA units. The bands at 2,972, 2,927, and 2,878 cm^{-1} originate from various CH2 and CH3 stretching modes. The broad band around 3,400 cm^{-1} is a result of the HPMA hydroxy groups, as well as N-H stretching and residual water bound to the hydrophilic polymer layer.

Resistance to non-specific adsorption

The resistance to non-specific adsorption, i.e. fouling, was evaluated after contact of the polymer brush-coated sensor with undiluted blood plasma for 30 min using SPR and the changes in the resonant wavelength λ_{res} were recorded. As displayed in Figure 3, no detectable fouling was observed from undiluted blood plasma. The pristine copolymer coating shows superior antifouling properties.

Immobilization of anti-fetuin-A

The activation of the poly(HPMA-*co*-CBMAA) brush and immobilization of anti-fetuin-A were carried out *in situ* and monitored by the SPR (Fig. 4). The polymer brush was activated by 30 min flow of an aqueous solution of EDC/NHS. The reaction of EDC/NHS with carboxy groups present in the CBMAA units of the polymer brush chains leads to formation of amine-reactive NHS ester. Subsequently, a solution of anti-fetuin-A in sodium borate buffer (SB, pH 8.5) was flowed over the surface. After 10 min the surface was rinsed with borate buffer and PBS. The amount of immobilized anti-fetuin-A was estimated from the SPR wavelength shift before and after the immobilization. Saturation of the SPR response to anti-fetuin-A immobilization was obtained after 10 min flow when the SPR response reached its plateau of $\Delta\lambda_{SPR}$=11.27±0.68 nm. The surface mass coverage can be calculated as 169±10 ng·cm^{-2}, based on our previous

calibration ($\Delta\lambda_{SPR}$ of 1 nm corresponds to 15 ng·cm^{-2}) (Emmenegger *et al.* 2009).
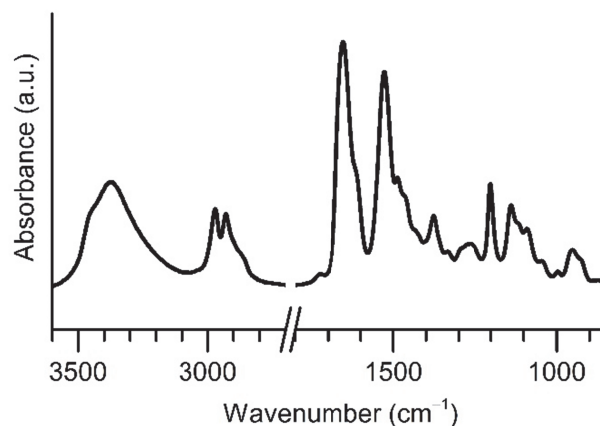

Fig. 2. FTIR-GASR spectrum of the poly(HPMA-*co*-CBMAA) brush prepared on a gold-coated SPR chip.
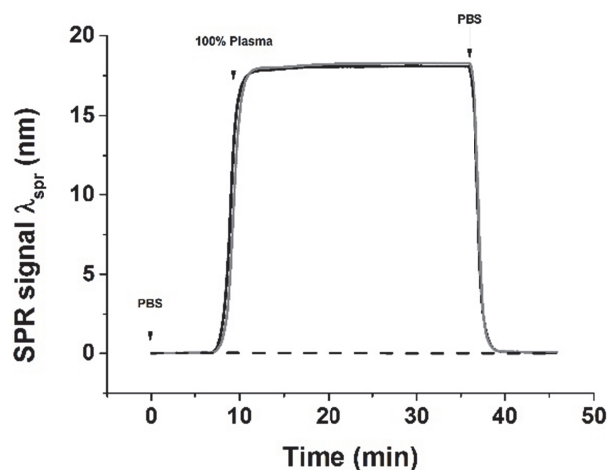

Fig. 3. Fouling from 100 % plasma after 30 min on poly(HPMA-co-CBMAA) observed by SPR. Arrows shows the time when individual solutions were exchanged (PBS – phosphate buffered saline).
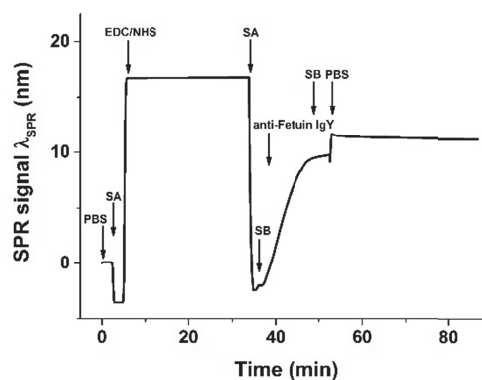

Fig. 4. SPR sensogram of the immobilization of anti-fetuin-A onto poly(HPMA-*co*-CBMAA) brush. The arrows indicate solutions changes (PBS – phosphate buffered saline buffer, pH 7.4; SA – sodium acetate buffer, pH 5; SB – sodium borate buffer, pH 8.5; 0.2 M EDC and 0.05 M NHS; anti-fetuin-A 10 µg·ml^{-1} in PBS).

Calibration curve

To assess the sensitivity of the functionalized polymer brush a series of samples containing human fetuin-A in PBS with a concentration range of 3-15,000 ng·ml^{-1} were injected over the sensing surface for 5 min followed by washing with PBS buffer. The amount of captured fetuin-A was evaluated from the resonant wavelength shift before and after the analysis. Each point was measured in triplicate always using a freshly functionalized SPR chip. Figure 5 presents the standard curve for the detection of fetuin-A in PBS. The calibration curve could be fitted with an exponential function, exhibiting saturation behavior. With increasing concentrations of fetuin-A in the sample the $\Delta\lambda_{SPR}$ increases and the sensor response becomes saturated at a concentration of approximately 5 µg·ml^{-1}. This behavior can probably be attributed to the immobilized anti-fetuin-A antibodies reaching their maximum binding capacity at the higher value end of the concentration range used. The calibration curve was later employed to estimate the concentration of the fetuin-A in real plasma samples. Due to the endogenous concentration of fetuin-A in plasma being in the range of 200 to 600 µg·ml^{-1}, the plasma samples were diluted to 1:500 to achieve precise quantification in the working range of the proposed sensor (3-1,500 ng·ml^{-1}).
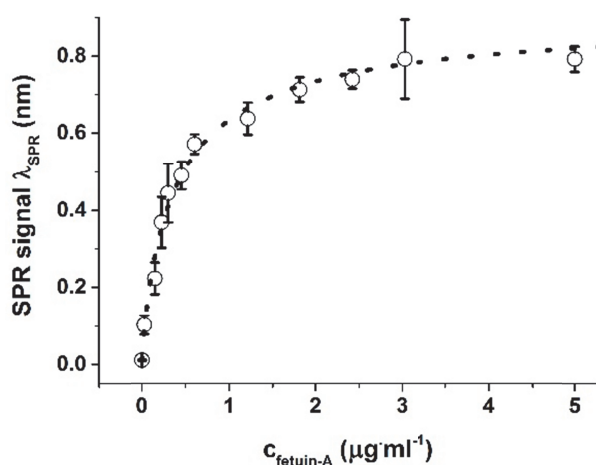

Fig. 5. Calibration curve of fetuin-A in PBS after 5 min contact with the sensor surface measured by SPR. Each point represents an average of three individual measurements and a standard deviation. The dotted line represents an exponential fit.

Detection of fetuin-A in real plasma samples

Owing to the observed concentration dependence of the sensor as well as the excellent resistance to fouling, the proposed SPR sensor was applied for the in-real-time detection of human fetuin-A in clinical plasma samples. Samples from 7 healthy donors were collected and analysed. The total amount of sample needed for one analysis was minuscule (less than 0.3 µl). The sensor assay was based on poly(HPMA-co-CBMAA) brush grafted from the surface with immobilized polyclonal chicken anti-human fetuin-A onto the brush. The SPR wavelength shift was tracked upon sequential flow of analyzed sample followed by rinsing with PBS. The sensor response was determined as the shift of the SPR wavelength $\Delta\lambda_{SPR}$ due to the binding of fetuin-A after 5 min flow of the sample followed by rinsing with a buffer, identically as in the assay performed to obtain the calibration curve in PBS. The SPR wavelength shift $\Delta\lambda_{SPR}$ measured in the assay was used to calculate the fetuin-A concentration value in the plasma samples with the help of the calibration curve (presented above). As a reference, the fetuin-A concentration in the samples was measured using commercial ELISA kits (Table 1). Figure 6 shows a comparison of the fetuin-A concentration in each sample as measured by ELISA and by SPR. The line shown in the graph indicates the expected tendency of equality between the concentrations determined by both methods. The concentrations obtained by herein presented SPR assay agree closely with values measured by ELISA. This indicates that the effect of plasma fouling on the SPR assay is completely suppressed, highlighting the benefit of the polymer brush interface.

Importantly, only 5 min of contact between the surface and the sample were sufficient for fetuin-A quantification with the SPR assay, while the only sample preparation procedure required was dilution with PBS. In contrast, the commercial ELISA kit available for fetuin-A determination involves a multistep procedure, which takes approx. 5 h between beginning of the measurement and readout of the results. Thus, the rapid availability of the readout is a significant advantage of the SPR assay, which is a consequence of the direct and label-free nature of the method.

Table 1. Concentration of fetuin-A in the blood plasma from individual donors measured by ELISA kit. Each value represents an average of three individual measurements and a standard deviation.

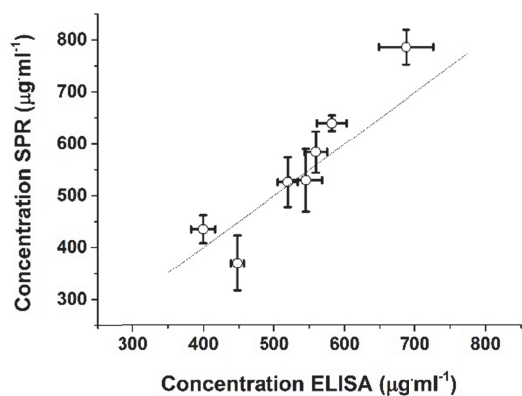| Sample No.	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7
Fetuin (µg·ml^{-1})	399 ± 17	448 ± 9	520 ± 14	545 ± 23	559 ± 16	582 ± 21	648 ± 39

Fig. 6. Concentration of fetuin-A as measured by SPR plotted a function of fetuin-A concentration in plasma determined by ELISA. Each point represents an average of three individual measurements and a standard deviation both for SPR and ELISA measurement. The line indicates the expected tendency (y=x).

Conclusions

We report an SPR sensor for in real time quantification of human fetuin-A in clinical plasma samples. This label-free direct assay is made possible by the superior antifouling properties of the poly(HPMA-*co*-CBMAA) brush interface and its functionalization *via* amine-coupling surface chemistry. Importantly, the obtained results showed good agreement with reference measurements carried out by ELISA. Unlike ELISA, the SPR assay took only 5 min and only a negligible amount (0.3 µl) of plasma sample was necessary for the analysis. The procedure used in this work can be explored for development of label-free affinity biosensors for diagnostics of various different markers in real clinical plasma samples by selecting appropriate biorecognition elements. Application of this novel biosensor technology will bring early biomarker detection and point-of-care analysis closer to clinical application.

Conflict of Interest
There is no conflict of interest.

Acknowledgements

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