A High Sensitivity Biosensor to detect the presence of Perfluorooctanoate in the environment

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Abstract

An optical biosensor system, based on an SPR optical fiber platform and a novel designed bio-receptor for Perfluorooctanoate detection in Sea Water, is realized and characterised. The SPR platform in optical fiber allows for a remote sensing and reduces the size and price of the sensor system. The work presents an analysis on the new bio-receptor, designed for this application, and the experimental results on the optical biosensor system, obtained in the detection of the analyte in buffer and real matrices. The performances of the developed biosensor system have shown that this new tool is suitable to monitor the presence of Perfluorooctanoate in the environment.

Keywords: Plastic optical fiber, Surface plasmon resonance Biosensors, Perfluorooctanoate

Introduction

Since 1950 perfluorooctanoate (PFOA; $C7F15COO^{-}$) and perfluorooctanesulfonate (PFOS; $C8F17SO^{-3}$), two of several perfluoroalkyl and poly-fluoroalkyl substances (PFASs), were used as surfactant and in polymer production for both commercial and industrial applications. Because of the important environmental presence and the potential toxicity of PFASs, in recent years the social and scientific interest in these compounds has notably increased. In particular, more and more attention has been devoted to PFASs and their effect on human health. PFOS and PFOA are the most extensively investigated PFASs because human exposition can occur through different pathways, even if the dietary intake seems to be their main route of exposure [1]. They are widely distributed in the environment and should be detected in the various kinds of micro-polluted water, such as river water and lake water. Also due to their remarkable chemical stability, they are inert and refractory to different chemical and microbiological treatments. Consequently, they are persistent, bio-accumulative and toxic to mammalian species.

In fact, the immune-toxic effects of PFASs in cellular systems and animals is largely demonstrated [2,3], and different epidemiologic researches have focused on possible effects of these chemicals on various immune related diseases in humans. Recently, in vivo and in vitro studies carried out on animal models have demonstrated that PFASs, such as PFOA and PFOS are weak environmental xenoestrogens [4].

Great efforts have been devoted by researchers to identify possible novel approaches for water treatment and analytes' detection in the environment. Presently, the conventional methods to PFOA determination include: high performance liquid chromatography (HPLC) [5,6], high performance liquid chromatography-mass spectrometry (HPLC-MS) [7], high performance liquid chromatography-mass spectrometry/mass spectrometry (HPLC-MS/MS) [8,9] and gas chromatograph-mass spectrometery (GC-MS) [10,11]. Furthermore, recently fluorous membrane based ion-selective electrodes [12] and a colorimetric sensor [13] for the detection of this class of compound have been described. All of the above methods require a complex pre-treatment and are noticeably time-consuming. In order to overcome these drawbacks, it is necessary to find a simple, rapid and sensitive method for the detection of PFASs. The biosensors have represented a valid tool to detect traces of specific molecules in different matrices. For example, an electrochemical biosensor for sensitive detection of PFOS was developed based on the PFOS inhibition influence on the bio-catalysis process of enzymatic biofuel cell (BFC) [14].

In PFOA/PFOS detection, a very interesting perspective is the to use a platform based on optical fibers for on-site fast detection, also exhibiting the possibility of remote control. On this line of argument, we exploited a low cost SPR sensor platform based on Plastic Optical Fibers (POFs) [15] to monitor a novel bio-receptor, developed for the detection of PFOA in aqueous medium.

The optical sensor platform has already been used, with good results, in different application fields with several kinds of receptors [16-18]. POFs systems are particularly advantageous due to the easy handling and installation procedures, large diameter of the fiber (a millimetre or more), low-cost and simplicity in manufacturing. The SPR sensor based on a D-shaped POF is especially interesting for bio/sensing application because it works with a planar gold surface, very simple to functionalize/derivatize, and with an external medium refractive index ranging from 1.33 to 1.42, typical of biosensors used for the selectivity detection of analytes in aqueous media.

In this work, we have developed and characterized a new SPR-POF biosensor to detect traces of PFOA in seawater samples. For this purpose, in the first step, the gold surface of the SPR-POF chip was chemically modified through the formation of a self-assembling monolayer (SAM) using the α-lipoic acid, as reported in Cennamo [19]. After this step, the functionalized gold surface was derivatized with *ad hoc* produced mono-specific antibody against the PFOA and the derivatization procedure was performed following the well-known carbodiimide method (EDC/NHS) [20]. The obtained results showed that the SPR-POF biosensor is able to sense PFOA at a concentration less than 0.25 ppb, that is considerably lower than the maximum residue limit of PFOA, fixed at 25 ppb by European Union regulations.

2. Materials and Methods

2.1 Materials

N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), and α-lipoic acid were purchased from Sigma-Aldrich (Sigma-Aldrich S.r.l. Milan, Italy). All other chemicals were commercial samples of the purest quality.

2.2 BSA-PFOA conjugates preparation

The antigen was prepared by reacting PFOA (0.8 mg/ml) with bovine serum albumin (8mg/ml) in the presence of 4 mg/ml 1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (ECD) in PBS buffer at pH 7.0. The reaction was run for 30 min at 37°C. Then, the mix was centrifuged at 350g for 10 min and the supernatant desalted using a PD10 column to remove un-bound PFOA. Mass spectrum shows that about 95% of the proteins have been derivatized with an average increase in the molecular mass of 2 kDa (66 to 68 kDa). As the molecular mass of PFOA is 414, this implies that, on average, about 5 PFOA molecules are bound to each BSA.

2.3 Antibody anti-PFOA production and purification

For the immunization, after extracting pre-immune serum, rabbits were intra-dermally injected with 200 μg antigen in complete Freund adjuvant, and again, after 21 and 28 days, with 100 μg antigen in incomplete Freund adjuvant. After 35 days, the animals were bled, the serum separated and subjected to the subsequent assays. The obtained serum was used for the purification of total IgGs anti-PFOA. A similar protocol used for the purification of the IgG anti-Naphthalene was used. In brief, 2.0 mL sample of anti-serum obtained from the two rabbits was diluted in 1:1 ratio with binding buffer (50 mM Tris-HCl, pH 7.4) and Protein A resin (Sigma) was applied. The wash step was done using binding buffer and the total fraction of IgGs from resin was eluted by using a strong pH change (50 mM Glycine, pH 3.0) and the purity of total IgGs was evaluated though the SDS-PAGE. In the following Figure we report the SDS-PAGE results from both rabbits' serum.

2.4 Affinity column preparation of PFOA–EAH Sepharose 4B and mono-specific antibody anti-PFOA purification

The PFOA-EAH affinity column for mono-specific anti-PFOA purification, was obtained by conjugating the PFOA to EAH Sepharose 4B. The protocol used is in accordance with the manufacturer's instructions. In brief, 1.2 mL sample of the resin was washed with H_2O at pH 4.5 (160 mL), with 0.5 M NaCl (100 mL), and again with H_2O at pH 4.5 (100 mL). The

Sepharose resin was finally packed into a polystyrene column (10 mL, BIORAD) suspended in 2.0 mL of H_2O at pH 4.5 and the resulting suspensions were gently shaken. In the meantime, 2.8 mM of PFOA were diluted in 2 mL of ethanol and EDC (in H_2O pH 4.5) to a final concentration of 0.1 M. The reaction solution was mixed and incubated for 10 min at room temperature and then overnight at 4°C. The solution of PFOA-EDC was added to the slurry resin and incubated with gentle shaking for 2 h at room temperature. The slurry resin solution was extensively washed with H_2O at pH 4.5, 0.5 M NaCl in 50% ethanol (15 mL). The slurry resin in 50% ethanol was previously treated with 15 mL of 0.1 M AcOH at pH 4.0, 0.5 M NaCl in 50% ethanol (blocking buffer) and later with 0.1 M Tris-HCl at pH 7.0, 0.5 M NaCl in 50% ethanol (wash buffer). After this step, the resin was washed with the blocking buffer and incubated for 30 minutes at room temperature. Afterward, the slurry resin solution was treated with 15 mL of the washing buffer and then with 15 mL of the blocking buffer. Finally, the resin was equilibrated with 10 mL of 20 mM sodium phosphate buffer, pH 7.4. The total IgGs obtained from the purification step of Protein A was loaded from the EAH-PFOA resin produced. The purification protocol used is similar to the one used in Varriale [20]. After the loading step, the column was washed extensively with sodium 20 mM phosphate buffer, pH 7.4 in order to remove un-specific binding of the IgGs with the EAH-PFOA resin. The mono-specific IgGs was eluted by strong pH changing (Glycine 0.1 M pH 3.0) and the purity of the obtained mono-specific antibody was evaluated through the SDS-PAGE test.

2.5 ELISA test

The antibody titer was determined using indirect ELISA assay. We used the general procedure reported by Pennacchio [21]. GlnBP-PFOA (2 mg/ml), diluted 1/200, was dissolved in coating buffer at pH 9.5 (25 mM carbonate/bicarbonate) and was deposited on coat 96-well micro-plates surface in a range of concentrations from 1.2 ng/mL to 1.7 ng/mL. GlnBP with same concentration was dissolved in coating buffer and used as control sample. The plate was incubated overnight at 4°C. After this incubation, it was washed three-times with PBS buffer (0.1 M) containing 0.05% Tween (PBS-T), pH 7.4 and blocked for 1 hour at room temperature with a solution of 1% milk in PBS-T buffer. The wells were washed several times with PBS-T after each step, incubated with mono-specific anti-PFOA antibodies at 25°C for 1 hour and subsequently with horseradish peroxidase-conjugated anti-rabbit IgG antibodies (diluted 1:12000). This solution was incubated for 1 hour at room temperature. The enzyme substrate TMB was added, and the colour reaction was quenched after 5 minutes by the addition of 2.5

M HCl. The absorbance value at 450 nm was measured, plotting the reciprocal of the antibody dilution against absorbance.

2.6 Optical Platform and experimental setup

The optical sensor platform is based on surface plasmon resonance (SPR) in a D–shaped POF, with a buffer layer between the exposed POF core and the thin gold film. This optical platform is realized by removing the cladding of POF (along half circumference), spin coating a buffer layer on the exposed core and finally sputtering a thin gold film. The sensing region is about 10 mm in length. The buffer layer proposed in this work is the photoresist Microposit S1813, with a refractive index greater than the one of the POF core. This buffer layer improves the performances of the SPR sensor [15].

In the visible range of interest, the refractive indices of the optical materials are about 1.49 RIU for POF core (PMMA), 1.41 RIU for cladding (fluorinated polymer) and 1.61 RIU for buffer layer (Microposit S1813). The size of the POF is 980 μm of core and 20 μm of cladding (1 mm in diameter), whereas the multilayer on D-shaped POF presents a thickness of the buffer layer of about 1.5 μm and a thin gold film of 60 nm. The planar gold surface can be employed for depositing a bio-receptor layer, as we will explain in the next section. In this case the selectivity detection of the analyte is possible. The experimental configuration based on simple and low-cost components is composed by a halogen lamp (HL–2000–LL, manufactured by Ocean Optics, Dunedin, FL, USA) exhibiting a wavelength emission range from 360 nm to 1700 nm, as the light source, the SPR-POF biosensor and a spectrometer (FLAME-S-VIS-NIR-ES, manufactured by Ocean Optics, Dunedin, FL, USA), with a detection range from 350 nm to 1023 nm, connected to a PC.

The SPR transmission spectra, normalized to the reference spectrum achieved with air as the surrounding medium, are obtained using the Matlab software and the resonance wavelength was extracted for the analytical [15].

2.7 Immobilization process on the chip surface

The surface of the POF chip was sequentially cleaned with: (1) milli-Q water (3 times for 5 minutes) and (2) 10 % of ethanol solution in milli-Q water (3 times for 5 min). The surface of the chip was pre-treated before the covalently immobilization of butyric acid and the procedure consists of three different steps: (1) thiol film production, (2) derivatization of the surface by EDC/NHS (3) antibody against PFOA/PFOS immobilization.

In the first step the gold chip was immersed in freshly prepared solution of α-lipoic acid

dissolved in a solution of pure ethanol 10 % in water at the final concentration of 40 mM and incubated at 25°C for 18 h. After this period of incubation, the gold-coated substrate surface was washed with milli-Q water and incubated 20 minutes at room temperature with a mixture of EDC/NHS at the final concentration of 20 mM and 50 mM respectively and dissolved in 100 mM potassium phosphate buffer, pH 5.5. The final step was the incubation of the surface with a solution 2 mg/ml (100 µl) for 2 h at room temperature in sodium phosphate buffer 20 mM at pH 7.5. The chips were at the end of this treatment washed with 20 mM potassium phosphate buffer sodium phosphate buffer, pH 7.5 and finally dried with nitrogen.

2.8 Binding experiments

Experimental results were collected by the SPR-POF biosensor and the setup previously illustrated. After each addition of the sample (solution with different concentration of the analyte), we have used a standard measuring protocol based on these three steps: first, incubation step for bio-interaction between analytes and receptor (for 10 minutes at room temperature); second, washing step with PBS (buffer); third, recording step for the spectrum (when buffer is present as bulk).

This protocol is necessary in order to measure the shift of the resonance determined by the specific binding (analyte/receptor interaction) on the sensing surface, and not by bulk refractive index changes or by non-specific binding between gold surface and analyte. We have tested the binding between the SPR-POF-biosensor's receptor and the PFOA in the range from 0 to 100 ppb, in 20 mM sodium phosphate buffer pH 7.4 (PBS). A similar experiment was performed in real matrices, a solution of water with 460 mM NaCl and PFOS. Finally we have tested, in the same experimental condition, the interaction between PFOA and the non-functionalizated gold surface (bare surface), with the aim of verifying that non-specific binding occurs and the effectiveness of the washing step.

3. Results and Discussion

3.1 The novel bio-receptor: Preliminary investigation and selectivity

PFOA is a low molecular weight compound (**Figure 1A**) used together with other perfluoroalkyl and poly-fluoroalkyl compounds as surfactant in polymer production for both commercial and industrial applications.

PFOA compound is too small to elicit any immunological response, and to overcome this problem we have developed a strategy to produce high-affinity polyclonal antibody against this compound. In brief, the strategy adopted in the antibody development and production was the following: PFOA compounds were covalently attached to an immunological protein carrier (BSA). In **Figure 2** is reported the schematic conjugation reaction between BSA and the PFOA. The reaction between the carboxyl group of PFOA and amino reactive groups of the carrier was performed at room temperature and pH 6.0, using the EDC/NHS conjugation protocol. The obtained BSA-PFOA conjugate was used to produce a high-affinity antibody, using a standard protocol of immunization. Then the mono-specific antibodies against PFOA (msAb-PFOA) were purified from the total IgGs fraction by an affinity chromatography PFOA-EAH-Sepharose-4B resin as previously described. The purity of different samples, obtained from the chromatography step, was analyzed by SDS-PAGE, their molar concentration was determined spectrophotometrically and the pooled samples of antibodies were tested, according to Di Giovanni [22] by Dot blot experiments. The results show a response to antibody binding. It was observed only for the conjugate GlnBP-PFOA and negative response was registered for GlnBP and BSA confirming the specificity and selectivity of antibodies versus the PFOA compound and not against the carrier used in the immunization processes (These data are not shown, for simplicity). To estimate the titer of the msAb against the PFOA, an indirect ELISA test was performed. In order to avoid interference by the carrier protein in the msAb detection process, the PFOA molecule was conjugated to the Glutamine binding protein (GlnBP) isolated from *E. coli.* Microplate wells were coated with different concentrations of antigens GlnBP-PFOA and reacted with serially diluted mono-specific antibodies against PFOA. **Figure 3** shows the results of the ELISA tests as a bar histogram in which the absorbance value at 450 nm is plotted against different concentrations of coated GlnBP-PFOA. No signal was registered from non-coated wells. The results show a positive signal when the msAb dilution is up to 1 in 12000.

In **Figure 4** is shown the SPR-POF biosensor system (Figure 4A) and the strategy adopted for derivatization of the gold surface of the SPR-POF platform (Figure 4B). We have applied a modified version protocol used in Cennamo [19]. In particular, as shown in **Figure 4B**, in order to obtain a smart surface able to bind the PFOA compound, the gold surface was treated

sequentially with a solution of α-lipoic acid (a), EDC/NHS (b) and finally with mono-specific antibodies against the PFOA (c). The immobilization of the antibody on the sensor surface is confirmed by SPR curves obtained by using the SPR-POF platform, directly. **Figure 4C** shows the SPR transmission spectra (normalized to the reference spectrum) when is present the PBS buffer solution, before and after the functionalization with antibody anti-PFOA. This experimental result shows a shift in the SPR transmission spectrum (the resonance wavelength increases) when is present the same bulk refractive index, before and after the functionalization procedure. The shift of resonance wavelength indicates that the refractive index in contact with the gold surface is increased, in other words that the antibodies were immobilized on the gold surface. The shift due to the antibody immobilization is about 25 nm.

3.2 PFOA detection

Figure 5A shows the transmission spectra of the SPR biosensor, normalized to the reference spectrum, obtained by contacting solutions at increasing concentrations of PFOA in buffer solution in the range 0-100 ppb. The resonance wavelength is shifted to smaller values by increasing the concentration of PFOA in buffer solution, which demonstrates that the analyte is actually adsorbed at the derivatized gold surface, with the consequent decrease of the refractive index of the receptor layer (see Figure 5A inset). This effect is related to the chemical composition of the per-fluorinated compounds. In fact, these compounds are used, for example, to decrease the refractive index of PMMA, to obtain the cladding in the PMMA POFs.

On the other hand, in **Figure 5B** are shown the SPR spectra obtained with different concentrations of PFOA in real matrices (460 mM NaCl). In Figure 6 is reported the resonance wavelength shift versus the log of PFOA concentration. The data show different binding effect for PFOA in buffer solution and in real matrices (dips in Figure 5 A and 5B). Each experimental value is the average of 5 subsequent measurements and the respective standard deviations (error bars) are also shown. The experimental values, reported in Figure 6, have been fitted by Hill's equation, in order to determine the kinetic parameters of the interaction. In table 1 are reported the values obtained by OriginPro8.5, Origin Lab. Corp. (Northampton, MA, USA). As shown in Table 2, at low concentration of the analyte (c), much lower than K_{Hill}, the Hill equation is linear, when $n \approx 1$, with sensitivity $\Delta\lambda max/K_{Hill}$, defined as the sensitivity at low concentration.

3.3 PFOS detection

The same procedure was used to study the interaction between the produced antibodies (msAb-PFOA) and PFOS. **Figure 7** shows the transmission spectra, normalized to the reference

spectrum, obtained by contacting buffer solutions at increasing concentrations of PFOS compounds (0-100 ppb). When the concentration of the PFOS increases the resonance wavelength decreases. In this case the SPR biosensor presents the same response obtained with PFOA. This effect should be due to the similar structure of both molecules that were exposed on the protein carrier during the immunization procedure. In other words, this receptor can be used to monitor both PFOA and PFOS molecules. This aspect is very significant because both analytes are interesting for several applications.

3.4 Non-specific Binding

In order to verify the non-specific binding between sensing surface and analyte, the response of SPR-POF sensor without the antibodies layer was checked. **Figure 8** shows the SPR curves at different concentrations of PFOA (0-100 ppb). When the PFOA concentration increases, from the analysis of data it is clear that in absence of antibodies on the gold surface, a smaller shift of the resonance wavelength, in the same direction of the binding's shift, was registered. This should be due to the PFOA - "bare surface" interaction. This effect should be related to the chemical composition of the per-fluorinated compounds that are used to decrease the refractive index of PMMA, to obtain the cladding in the PMMA POFs.

Conclusion

We have designed and realized a novel biosensor system based on optical fiber to remote monitor the presence of the Perfluorooctanoate in Sea Water. The new bio-receptor has been immobilized on the gold surface of the SPR-POF platform by a specific designed procedure. The biosensor chip has been characterised and the obtained experimental results have shown that this SPR-POF biosensor system is able to sense PFOA at a very low concentrations (a concentration less than 0.25 ppb).

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Table

PFOA detection					Hill Equation $\lambda_c = \lambda_0 + \Delta\lambda max \cdot (c^n / (K_{Hill}^n + c^n))$			
Matrices	λ_0 [nm]		$\Delta\lambda$max [nm]		K_{Hill} [ppb]		n	
	Value	Std. dev	Value	Std. dev	Value	Std. dev	Value	Std. dev
Buffer	0.24	2.34	8.05	0.19	0.27	0.38	1.32	0.75
Real	0.88	2.42	6.10	0.64	0.27	0.99	1.24	3.50

Table 1. Analytical parameters of the *Hill* equation relative to PFOA detection in buffer and real matrices

Low concentration Hypothesis: ($c << K_{Hill}$)	Hill Equation at low concentration (with $n \approx 1$) $\lambda_c - \lambda_0 \approx (\Delta\lambda max / (K_{Hill}) \cdot c$	
Matrices	Sensitivity at low c ($\Delta\lambda max / (K_{Hill})$) [nm/ppb]	LOD [ppb] (2*standard deviation of blank / sensitivity at low c)
	Value	Value
Buffer	29.82	0.16
Real	22.59	0.21

Table 2. Analytical Parameters at low concentration of analyte.

Figure legends

Figure 1. Structure of (A) perfluorooctanoate acid (PFOA) and (B) perfluorooctanesulfonic acid (PFOS) compounds.

Figure 2. Conjugation production between BSA and PFOA compounds. The reaction was performed at RT, at pH 6.0 for 2 h.

Figure 3. Indirect ELISA test results of msAb anti-PFOA. The assay was performed in the Tris-borate buffer in the presence of 0.005% Tween and 1% milk. Temperature was set at 25°C. The dilution of anti PFOA used was 1:12000.

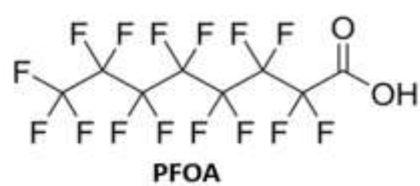
Figure 4. (A) Optical biosensor system based on POF-SPR platform. (B) Functionalization process of the gold surface. (C) Resonance spectra in buffer solution obtained before and after the functionalization process with msAb anti PFOA. Both measurements are obtained by dropping 50 μl of 20 Sodium phosphate buffer pH7.4 over the sensing surface, with and without receptor layer.

Figure 5. Experimental SPR spectra relative to PFOA detection. (A) SPR spectra obtained with different concentrations of PFOA in buffer solution (0–100 ppb). (B) SPR spectra for the same PFOA concentrations in real matrices. Inset (A) and (B): zoom of the resonance wavelengths region.

Figure 6. Plasmon resonance wavelength shift ($\Delta\lambda$), with respect to the resonance wavelength at 0 ppb, versus log of concentration of PFOA (ppb) and Hill Fitting of the experimental values.

Figure 7. SPR spectra obtained with different concentrations (0-100 ppb) of PFOS in buffer solution. The experiments were performed at room temperature and each sample was incubated 10 minutes before acquire the signal.

Figure 8. SPR spectra for different PFOA concentrations in the case of the "bare sensor" (gold film without bio-receptor layer). The experiments were performed at room temperature and each sample was incubated 10 minutes before acquire the signal.



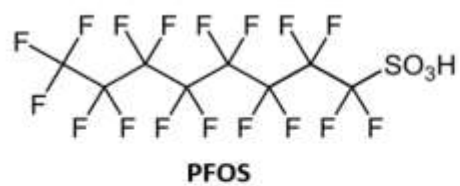


Fig. 1

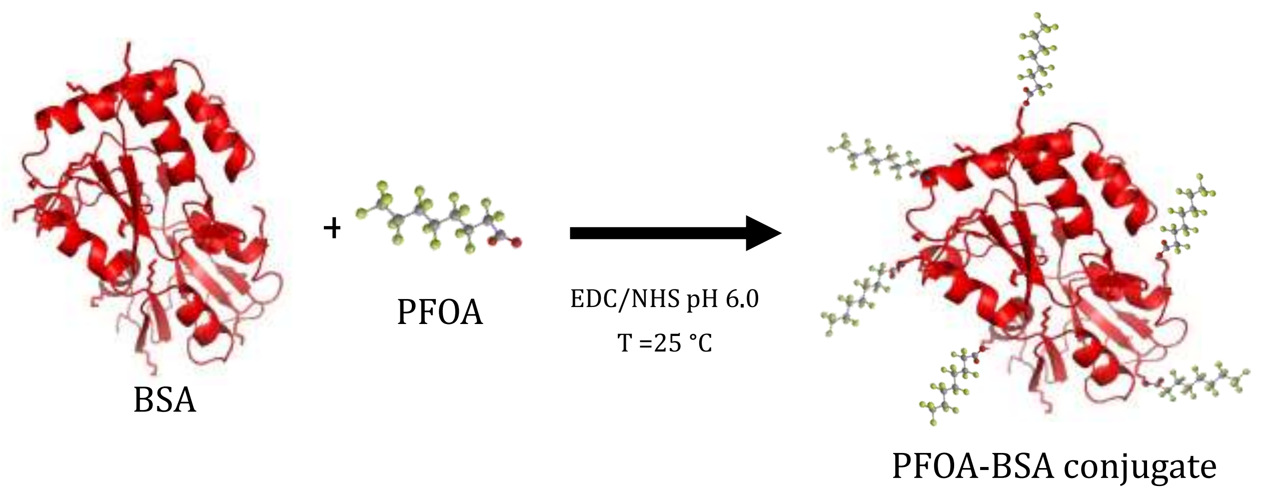


Fig. 2

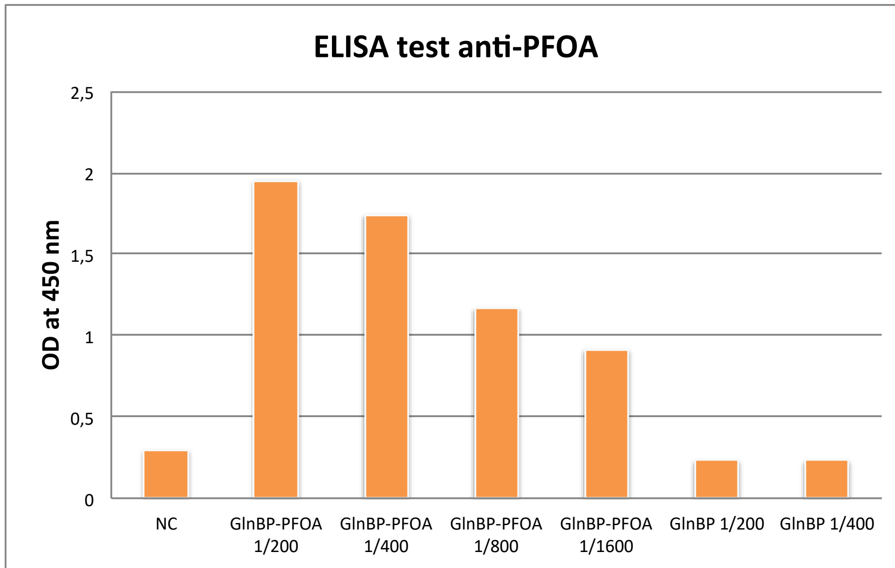


Fig. 3

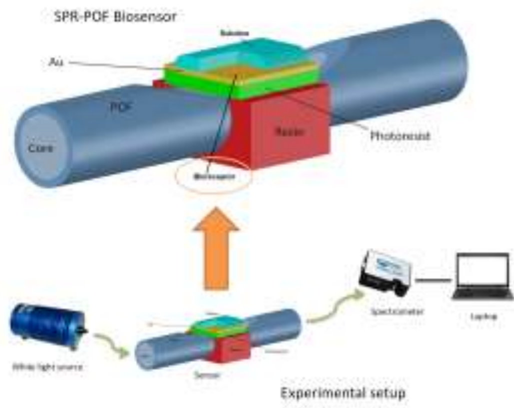


Fig. 4A

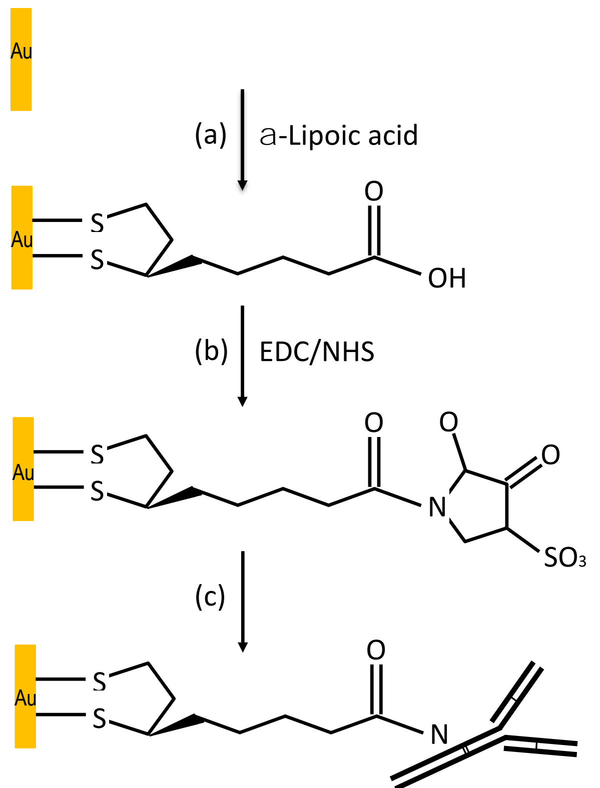


Fig. 4B

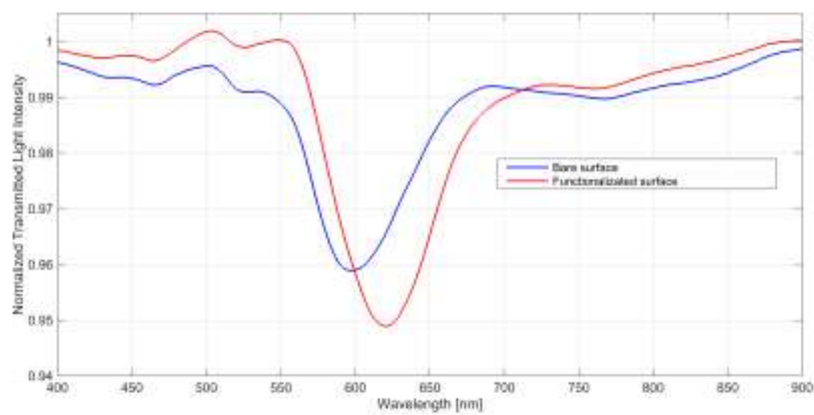


Fig. 4 C

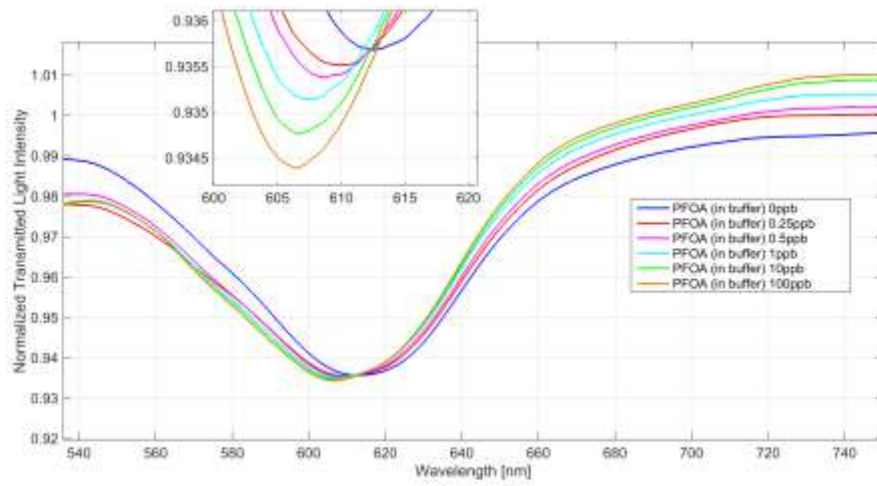


Fig. 5 A

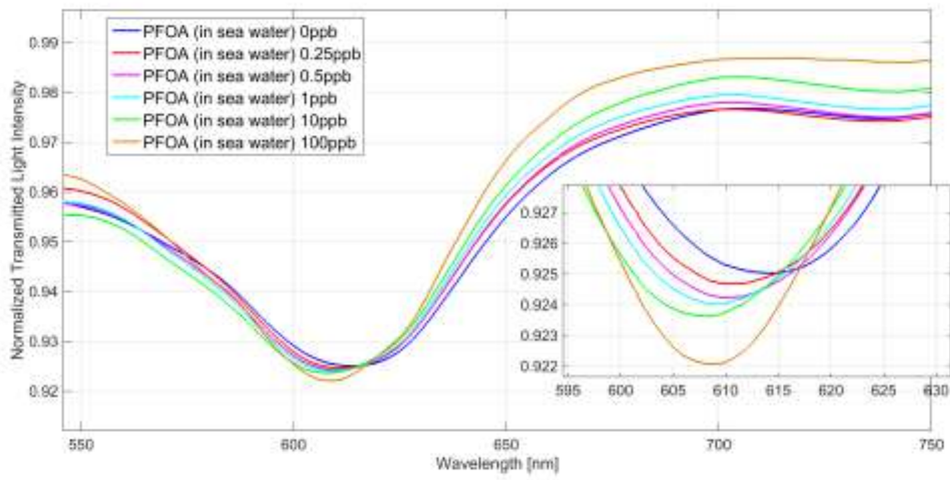


Fig. 5 B

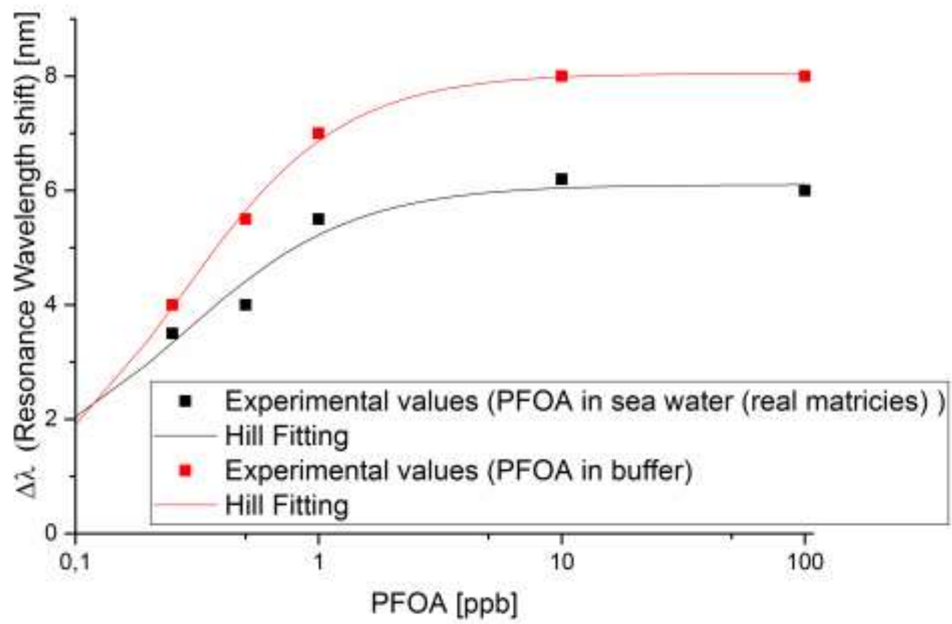


Fig. 6

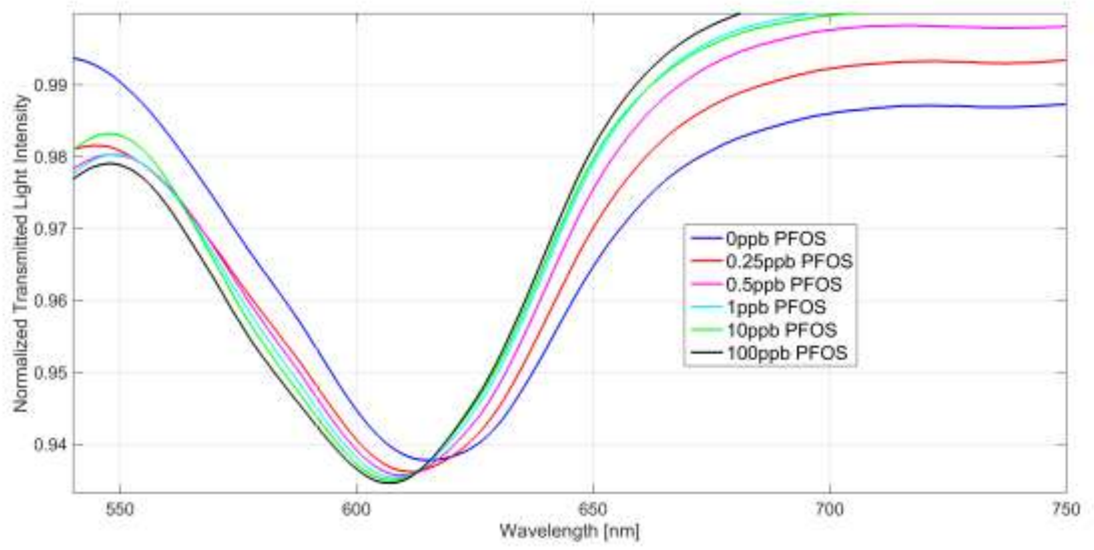


Fig. 7

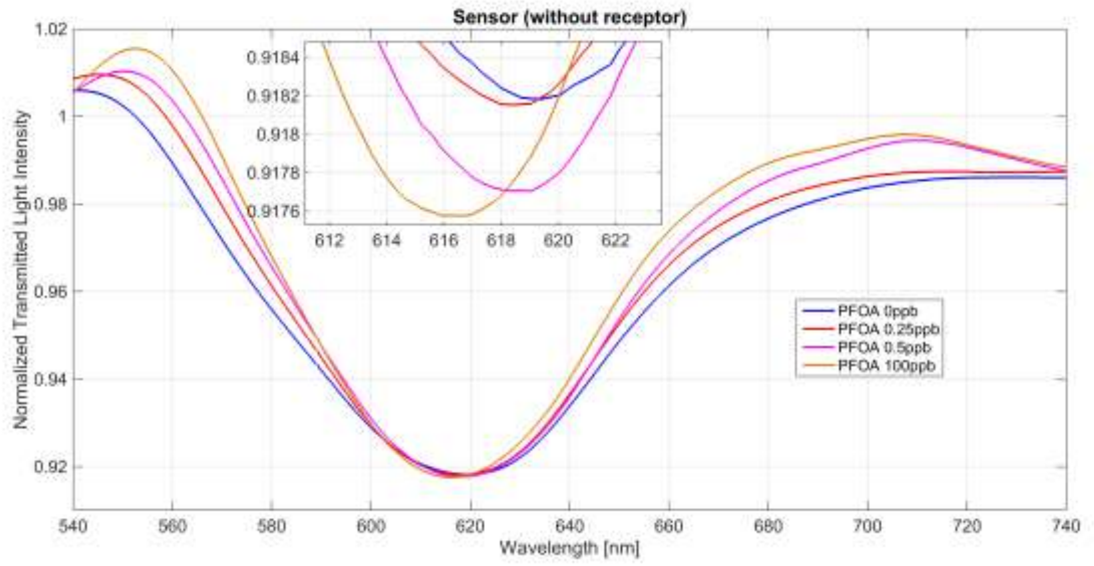


Fig. 8