



## Short communication

## Poly(methyl methacrylate) conductive fiber optic transducers as dual biosensor platforms

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## ABSTRACT

Herein the development of an alternative optic-conductive fiber configuration applied for the construction of biosensing platforms. This new approach is based on applying the chemical polymerization of pyrrole onto the surface of polymethyl methacrylate (PMMA) fibers to create a polymer—a conductive surface, onto which an additional photoactive polypyrrole-benzophenone (PpyBz) film is electrochemically generated upon the fiber surface. Irradiation of the benzophenone groups embedded in the Ppy films with UV radiation (350 nm) formed active radicals that allowed the covalent attachment of the desired bioreceptors. Characterization of the amperometric biosensing matrix was accomplished by using a model Urease (Urs) through electrochemical impedance spectroscopy (EIS) and amperometry. Both techniques have shown a low charge transfer resistance (340 kΩ) and a high sensitivity (12.3 μA mM<sup>-1</sup> cm<sup>-2</sup>). Thereafter, the construction of an optical biosensing matrix based on horseradish peroxidase (HRP) production of photons was carried out. The high signal to noise (S/N) ratio (1600) indicated clearly that this approach can serve as a new platform to replace glass optical fibers based on biosensors.

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## 1. Introduction

Fiber optic biosensors are diagnostic tools with specialized features (Marks et al., 1997) that may be utilized in a variety of fields (medical, pharmaceutical, environmental, defense, bioprocessing or food). The fiber optic device serves as the transduction element and the transmitted signal is often proportional to the concentration of a chemical or biochemical to which the biological element reacts (Bosch et al., 2007; Leung et al., 2007; Marazuela and Moreno-Bondi, 2002; Wolfbeis, 2004). Therefore it may be used in a variety of spectroscopic techniques, such as chemiluminescence, absorption, fluorescence, phosphorescence or surface plasmon resonance (SPR) (Bosch et al., 2007). Silica-based fiber optic biosensors have been published as serving a platform for the immobiliza-

tion of various biomaterials (enzymes, antibodies, antigen or whole cells) (Herrmann et al., 2005; Konry et al., 2005; Leshem et al., 2004; Petrosova et al., 2006; Salama et al., 2007; Sobarzo et al., 2007). We have demonstrated that chemiluminescence-based optical fiber immunosensors (OFIS) are more sensitive than their analogous colorimetric and chemiluminescent-based ELISA counterparts (Herrmann et al., 2005; Konry et al., 2005; Leshem et al., 2004; Petrosova et al., 2006; Salama et al., 2007; Sobarzo et al., 2007). However, silica optic fiber chemical modifications are still unreliable fiber-to-fiber, accordingly alternative configuration scenarios would be useful to improve the efficiency and reproducibility for putative mass production of optic fiber probes. Furthermore, optic-conductive biosensors would open the door to a number of wide applications based on a platform that will include both conductivity and light transmission. The alternative fiber optic platform which we tested, was Ppy-coated. PMMA optical fibers display very good optical properties while also proving to be inexpensive and easy to prepare and also easier to cleave in comparison to more commonly used silica-based fibers. The PMMA fiber surface shows a hydrophobic and favorable environment for protein adsorption. However, in order to achieve a more stable bio-conjugate linking,

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chemical modification procedures would need to be implemented to conjugate the surfaces; which in turn would necessitate organic solvents that could damage the fiber surface. PMMA particles were shown to be coated by pyrrole polymerization in an aqueous dispersion medium (Ferenets and Harlin, 2007; Omastova et al., 1998; Omastova and Simon, 2000). This deposition process results in a thin, transparent, conductive and stable Ppy film (Ferenets and Harlin, 2007; Fredj et al., 2008; Wang et al., 2001).

This procedure was adapted to coat PMMA fibers with pyrrole polymerized chemically at low temperature, which then display increased hydrophilicity when compared to naked PMMA fibers. The additional step to attach the biomaterial components was accomplished through electropolymerization of an intermediate functionalized pyrrole (biotin, amine or benzophenone) and of these three tested, the finest results were obtained using benzophenone as it was best suited to the required parameters (conductive, stable, thin, controllable and light-transmissible films). The electropolymerization of the PyBz monomer was successfully achieved in an aqueous phase and the observed films showed that the color of the Ppy films varied from light grey to black indicating a stable and typical Ppy film. Irradiation of the benzophenone groups embedded in the Ppy films using UV radiation (350 nm) activated the benzophenone groups and allowed the attachment of proteinaceous enzymes (Urs or HRP) to the fiber surface. The subsequent measurements showed high sensitivity ( $12.3 \mu\text{A mM}^{-1} \text{cm}^{-2}$ ) and high S/N ratio (1600) when compared to unmodified PMMA fiber, respectively.

## 2. Materials and methods

### 2.1. Reagents and materials

Tris-HCl (77-861); pyrrole (13170-9); lithium perchlorate (431567); Urs (9002-13-5); urea (57-13-6); ammonium persulfate 98% (APS) (248614); p-toluene sulphonic acid (PTSA) (402885); 1,1'-ferrocenedicarboxylic acid, 96% (FeCN) (215-068-9); isopropyl alcohol (IPA) (67-63-0); HRP (P8375) were purchased from Sigma-Aldrich (St. Louis, USA) and used as received. PMMA fibers (3 mm diameter unjacketed, NT53-833), mirror and a professional fiber cutter (N54-013) were purchased from Edmund optics (San Jose, USA). Luminescence measurements were carried out using the Immuno-star HRP Chemiluminescent Kit (170-5040) purchased from Bio-Rad Laboratories. PyBz was synthesized as described elsewhere (Cosnier and Senillou, 2003).

### 2.2. Preparation of the PMMA optic-conductive fiber biosensor

#### 2.2.1. Chemical polymerization of pyrrole onto the PMMA fiber-optic endface

PMMA optical fibers (3 mm core diameter) were cleaved manually using a professional fiber cutter with a length of 2.6 cm. The fibers were first soaked in isopropyl alcohol for 5 min, followed by vacuum desiccation for 5 min and finally an additional 5 min of dipping in double distilled water (ddH<sub>2</sub>O). The fibers were dried with airflow through a 45  $\mu\text{m}$  filter, after which they were further dried in an oven at 47 °C for 20 min and underwent vacuum desiccation for 5 min. The clean fibers were dipped into a 1.5 ml tube, containing a mixture 1:1:1 (v:v:v) of the following cold solutions: 25 mmol APS, 25 mmol PTSA and 7.5 mmol pyrrole; to allow the chemical polymerization of pyrrole monomers. The fibers were dipped into the eppendorf immediately after introducing the pyrrole. The 1.5 ml tube was mixed by vortex and transferred to an ice bath (5 °C) (Ferenets and Harlin, 2007) for 4.5 min, after which the fibers were moved into ddH<sub>2</sub>O and gently vortexed, rinsed in ddH<sub>2</sub>O and dried by airflow.

#### 2.2.2. Electrochemical polymerization of PyBz onto the Ppy-coated PMMA fiber-optics

PMMA fibers coated with Ppy films were used as working electrodes to enable the electrogeneration of an additional conductive and functional layer consisting of PyBz monomers so as to electropolymerize PyBz onto fiber-optic tips. This new layer would then enable the subsequent immobilization of the desired receptor protein. The electropolymerization was processed by applying a constant voltage of 0.85 V lasting 10 min. The electropolymerization solution used was prepared by mixing 10 mM PyBz free monomer within a 0.1 M of lithium perchlorate aqueous solution.

#### 2.2.3. Immobilization of bioreceptors onto the transducer surface

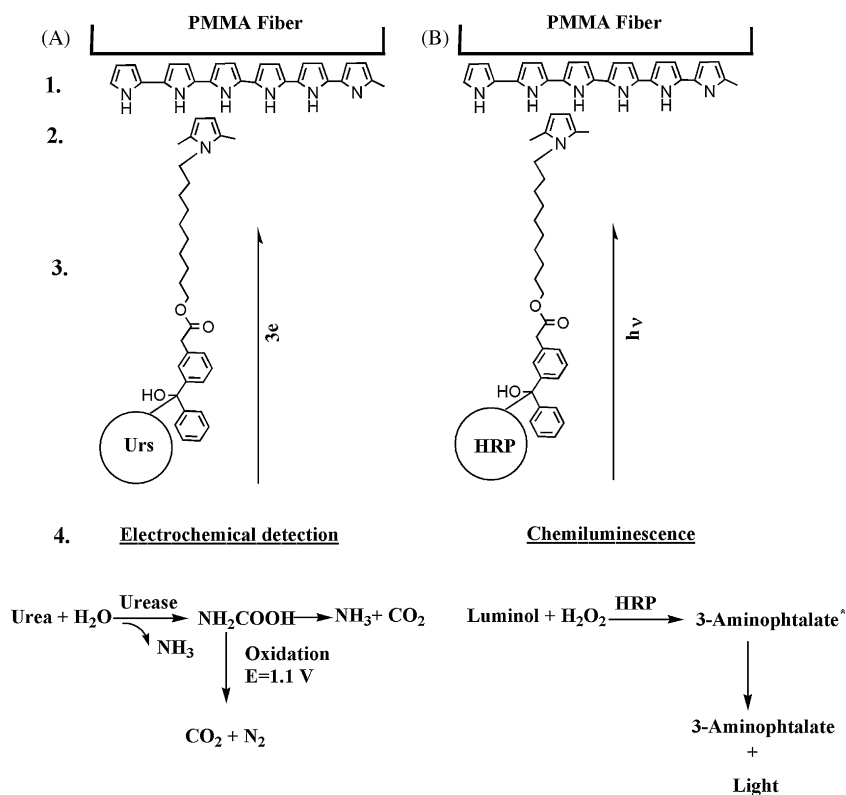
The enzymes Urs or HRP (concentration 0.5 mg/ml) were linked to the PpyBz film through UV –350 nm irradiation (Fig. 1). In order to produce the desired activation radiation we used a 1000 W Xe lamp (Oriol 6271) mount connected to a light condenser (Oriol 66021). The light was reflected through a dichroic mirror (Oriol 66226). The spectrum radiation was then condensed into the monochromator (Oriol 77250) using the appropriate lenses (Oriol, plano convex lenses). After this, a 350 nm wavelength light output, with a light intensity of  $100 \text{ mW cm}^{-2}$  (as measured by an Ophir Optronics power-meter Nova reader, PD300-UV) was projected for 7 min (which was the established optimal time in our lab) into the far end of the optical fiber that was coated at its near end with PpyBz and soaked in 0.5 mg/ml of HRP solution during irradiation (Konry et al., 2008; Leshem et al., 2004; Petrosova et al., 2006). The irradiation of the PyBz film created radicals enabling the attachment of the enzymes covalently. The fibers were then washed with ddH<sub>2</sub>O to remove unattached proteins.

### 2.3. X-ray photoelectron spectroscopy (XPS) analysis and contact angle measurements

An ESCALAB system with parallel X-ray photoelectron imaging (XPI) analysis of small features at a resolution approaching 1 mm was used. The ESCALAB 250 combines the auger electron spectroscopy (AES) and scanning auger electron mapping (SAM). The vacuum in the operation chamber was  $2 \times 10^{-9}$  mbar. XPS analysis was performed using a monochromatic Alk- $\alpha$  X-ray source with a 500  $\mu\text{m}$  surface of analysis and X-ray gun set at 15 kV, 150 W. High resolution spectra of the elements were taken with a pass energy at 15 eV, and a peak position normalized according to C1s (284.6 eV). Static contact angle measurements were taken at 20 °C with a sessile drop method using an OCA 40 system (Dataphysics, German).

### 2.4. Electrochemical instrumentation

The amperometric and impedance measurements, as well as the electropolymerization, were carried out using a PGSTAT30 potentiostat and a conventional electrochemical cell (Metrohm). The modified fibers were used as the working electrodes; a saturated Ag-AgCl-KCl electrode (Ag/AgCl) was used as a reference electrode. A platinum (Pt) wire, placed in a separate compartment containing an aqueous solution of 0.1 M Tris-HCl buffer electrolyte (pH 7), was used as a counter electrode for the amperometric measurements; while in the electropolymerization procedure, a Pt wire was used as a counter electrode and immersed directly in a 0.1 M LiClO<sub>4</sub> aqueous solution. EIS faradic measurements were conducted in a solution containing 2 mM FeCN, in which the electrode set up was the same as that used in the electrochemical deposition. An alternating current sinusoidal signal of (10 mV) amplitude was used and the direct potential was set to 0.7 V. The value of the impedance was determined over a range 10–10<sup>5</sup> Hz, and the impedance measurements were performed with a PGSTAT30 electrochemical workstation. The



**Fig. 1.** Amperometric and optical biosensor constructions, (1) Modification of PMMA fiber's surface by chemical polymerization of pyrrole, (2) electrochemical polymerization of PyBz, (3) irradiation of benzophenone moieties by UV and attachment of the enzyme Urs or HRP to the benzophenone groups, (4) monitoring of the current as a function of urea concentration in the amperometric biosensor case (A) and light recording in the chemiluminescence's biosensor (B) when adding oxidizing reagent and luminol into the wells in a 1:1 ratio.

capacitance values and resulting data modelled were calculated and matched with the fit and stimulation software integrated in the FRA software (Eco Chemie B.V. Utrecht Netherlands).

### 2.5. HRP chemiluminescence measurement

Chemiluminescence measurements were performed by applying hydrogen peroxide and luminol into fiber holding wells in a 1:1 ratio. Measurements were carried out using a single photon avalanche diode (SPAD) photodetector based on a SPAD module purchased from SensL (PCMplus module, 10 μm sensor). Data was collected using SensL Integrated Environment software and recorded in RLU (relative light units).

## 3. Results and discussion

Steric hindrance forces formed by benzophenone functional groups limited the chemical polymerization of PyBz onto the naked PMMA fiber surfaces. To overcome this obstacle a chemical polymerization of pyrrole was carried out to form a conductive surface, which enabled the subsequent electrochemical polymerization of PyBz with the ultimate target of an efficient immobilization of receptor proteins. Polymerization time was set to 4.5 min, while longer polymerization periods resulted in rather unstable quality with non-uniform films. These samples required additional cleaning in order to remove the loosely fixed film layers.

### 3.1. X-ray photoelectron spectroscopy (XPS)

The shift on binding energy for samples in the O1s region from PMMA (532 eV) to PMMA-Ppy (531 eV) was attributed to the intensity increase of the O1s featuring the polymerization of pyrrole

molecules on the PMMA surface. This was further supported by the observation that the O1s intensity increased substantially as a result of PyBz electropolymerization on the PMMA-Ppy which correlated directly to the binding energy shift to higher values as the electropolymerization tightened the thin polymeric film structure (C/O increases relative to PMMA-Ppy).

### 3.2. Surface hydrophilicity

To gain a better understanding of the modification, the surface hydrophilicity was characterized by measuring its contact angle which for PMMA was 78.4°, very similar to the values recorded in the literature (83°) (Bull et al., 2006). The PMMA-Ppy fiber has displayed a better contact angle (48°) due to a very thin film of Ppy coating and the existence of amine groups on the fiber surface. The contact angle was further decreased in the PMMA-Ppy-PpyBz case showing a better hydrophilicity (18°), when compared to naked PMMA, which directly correlates to the large amount of hydroxyl groups on the PpyBz film. The resulting hydrophilic surface provided an excellent microenvironment for enzyme immobilization.

The morphology of the surface was investigated by scanning electron microscopy (SEM). The SEM study of Ppy films demonstrated that the surface was covered with grains (diameter of approximately 10–100 nm). The body of the film was approximately hundred nanometers thick. The PpyBz films created a smooth layer upon the Ppy films.

### 3.3. Electrochemical impedance analysis

Fig. 2 presents the imaginary part versus the real part of the modified fiber impedance which shows typical characteristics of conducting polymers. The high frequency semicircular regions in

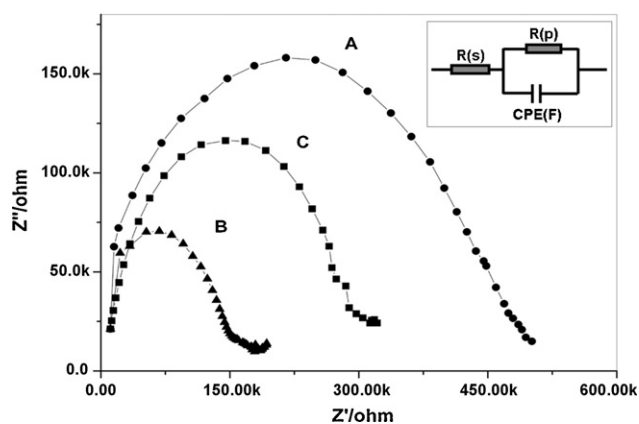


Fig. 2. Equivalent electrical circuit of EIS data and Nyquist plot for (A) PMMA-Ppy (B) PMMA-Ppy-PpyBz (C) PMMA-Ppy-PpyBz-Urs in 0.1 M Tris-HCl (pH 7).

the PMMA-Ppy case showed good conductivity (when compared to the non conductive PMMA), with a radius of curvature equivalent to 500 k $\Omega$ , indicating a moderate electrical resistance of the charge transfer, while the value of the radius in the PMMA-Ppy-PpyBz was approximately 3 times lower (160 k $\Omega$ ), due to the additional layer of PpyBz generated on the PMMA-Ppy fiber surface which further decreased the electrical charge transfer resistance. In the PMMA-Ppy-PpyBz-Urs case the charge transfer resistance increased to higher values as the protein on the surface contributed to the decreasing values of the analyte diffusion, hence increasing the charge resistance accordingly to 400 k $\Omega$ . In order to characterize the response of the monolayer Urs-modified sensor, the apparent Michaelis–Menten constant  $K_m^{app}$  can be calculated for the immobilized enzyme by the amperometric method (Shu and Wilson, 1976), where  $J$  is the steady state current density,  $J_{max}$  is the maximum current density under conditions of enzyme saturation, and  $C$  is the urea concentration.  $K_m^{app}$  calculated from the Lineweaver–Burk  $1/J$  vs.  $1/C$  plot is as the relation with a slope of  $K_m^{app}/J_{max}$  and an intercept of  $1/J_{max}$ .

$$1/J = K_m^{app}/(J_{max}C^{-1}) + 1/J_{max} \quad (1)$$

Typical  $K_m^{app}$  and  $J_{max}$  values were calculated as 1.1 mM and 50  $\mu\text{A cm}^{-2}$ , respectively. These values were comparable with those previously reported for the immobilization of Urs in electrochemically prepared Ppy films (Gambhir et al., 2001) and indicate a steric hindrance toward the diffusion of the products of the enzyme reaction toward the fiber surface as shown in Fig. 3 (due to the existence of the bulky enzyme on the surface). The capacitance values of PMMA-Ppy-PpyBz and PMMA-Ppy fibers modelled and fitted by Randles circuit were  $2 \times 10^{-7}$  F and  $1 \times 10^{-6}$  F, respectively. These values indicate that the PMMA-Ppy-PpyBz set up provides a better conductivity than PMMA-Ppy alone.

#### 3.4. Amperometric analysis

In the optical PMMA fiber surface modification process presented in Fig. 1, pyrrole was polymerized chemically on the fiber surface to create a conductive surface, and then PpyBz films were electropolymerized by applying a voltage at 0.85 V. Benzophenone and most of its derivatives absorb photons at approximately 350 nm, resulting in the promotion of one electron from a non-bonding  $sp^2$ -like  $n$ -orbital of oxygen to an antibonding  $\Pi^*$ -orbital of the carbonyl group. The actual electron deficient oxygen  $n$ -orbital becomes electrophilic and therefore interacts with weak C–H  $\sigma$ -bonds, resulting in hydrogen abstraction to complete the half-filled  $n$ -orbital. When amines or similar heteroatoms are in the vicinity of the excited carbonyl, an electron transfer step may occur, followed

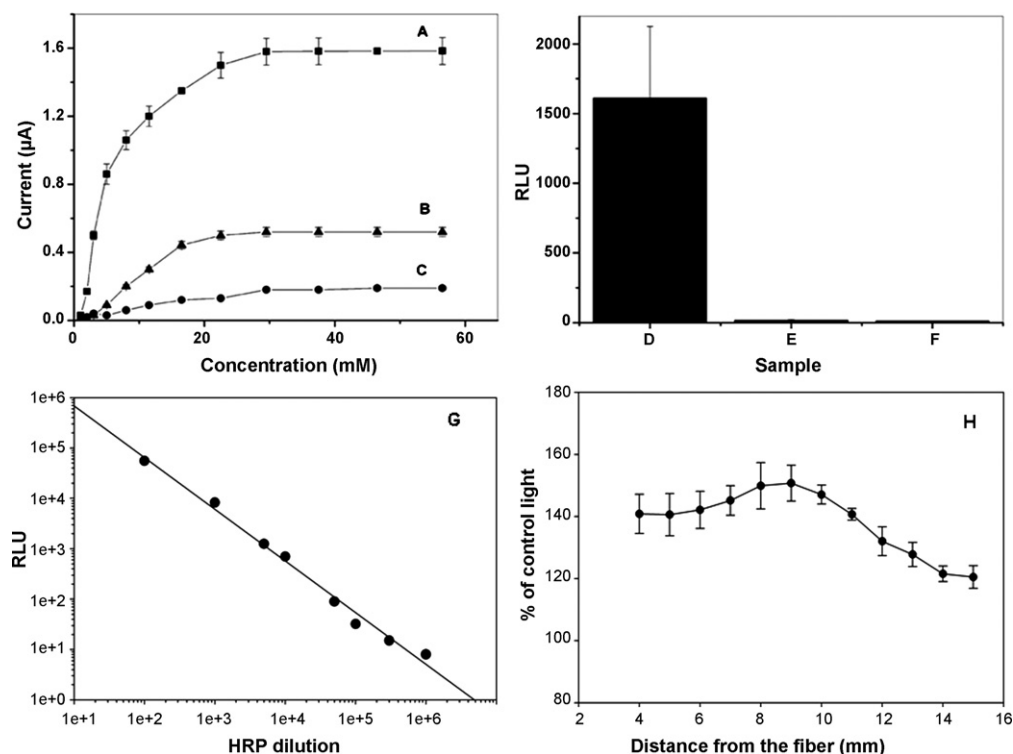
by proton abstraction from an adjacent group. In biological systems the most effective H-donors include backbone C–H bonds in amino acids; thus, methylene groups of amino acid side chains are good candidates providing abstractable hydrogens (Leshem et al., 2004). This enables the enzyme attachment and the catalytic reaction of the analyte that could be detected by either electrochemical or optical means. The analytical performance of such a platform was demonstrated by constructing an amperometric biosensor for urea monitoring. The detection principle of urea is based on the carbamic acid oxidation. As shown in Fig. 1, Urs catalyzed the hydrolysis of urea to form intermediate products (carbamic acid and ammonia). The electrolysis was conducted by applying a 1.1 V to oxidize the carbamic acid (three electron oxidation) (Wang et al., 2007) which created a current that correlated to the urea concentration.

The PMMA-Ppy-PpyBz-Urs fiber in Fig. 3A exhibits a better performance than those of the controls PMMA-Ppy-PpyBz-Urs without UV irradiation (Fig. 3B), and PMMA-Ppy-Urs without PyBz linker (Fig. 3C). The biosensor PMMA-Ppy-PpyBz-Urs performance indicates that the irradiation of the PyBz did activate the benzophenone and did attach the enzyme Urs specifically. Furthermore, the sensitivity of the PMMA-Ppy-PpyBz-Urs (12.3  $\mu\text{A mM}^{-1} \text{cm}^{-2}$ ) probe was 4 times higher than the set up without having UV radiation treatment (2.3  $\mu\text{A mM}^{-1} \text{cm}^{-2}$ ), indicating that the enzyme was attached covalently to the benzophenone group although some may bind non-specifically. The biosensor limit of detection, dynamic range, response time and sensitivity were 0.5 mM, 0–10 mM, 2 s and 12.3  $\mu\text{A mM}^{-1} \text{cm}^{-2}$ , respectively. These values indicate significant improvement compared to the values reported in the literature (limit of detection 0.2 mM, dynamic range 0–5 mM, response time 40 s and 0.47  $\mu\text{A mM}^{-1} \text{cm}^{-2}$  sensitivity) (Rajesh et al., 2005). Hence this biosensor presents rapid, sensitive and efficient analytical performance.

#### 3.5. Light measurements and calibration curves

During the chemiluminescent reaction, the light is emitted in all directions (360°) from the solution just above the fiber interface. Thus a certain amount of photons are not directed into the fiber and are lost into the measuring chamber. In order to decrease the light loss and improve the sensitivity of our system, an elliptic mirror was placed above the well containing the chemiluminescent reaction and was calibrated. For this purpose, 10  $\mu\text{l}$  of a 1 mg/ml HRP solution diluted by 1:100 was mixed with 30  $\mu\text{l}$  of the chemiluminescent substrate into the reaction well. The light level was first measured without the reflecting system (control) and then the mirror was placed above the fiber at a distance ranging from 4 to 10 cm. The mirror was finally situated 9 cm above the fiber based on the measurements displayed in Fig. 3H. Consequently, a serial dilution of HRP was used to examine the PMMA modified fiber surface as a platform for light transmission. A chemiluminescence mix was added to the well and the signal recorded as relative light units (RLU). Eight HRP dilutions were tested in triplicate in the biosensor ( $10^2$ – $10^6$ ). The results are displayed in Fig. 3G. A linear distribution can be observed in the optical system down to a dilution of 1:1,000,000 of the HRP stock solution (1 mg/ml) with a strong correlation factor ( $r^2 = 0.99$ ).

The ultimate target of this research was to demonstrate the applicability of a platform that would enable the attachment of biomolecules to a fiber surface so as to transmit light efficiently. This was demonstrated by the attachment of the enzyme HRP (Fig. 1) to the fiber surface, introducing hydrogen peroxide and luminol reagents into the measurement cell in a 1:1 ratio and collecting the light by a single photon avalanche diode photo-detector (Ashkenazi et al., 2009). The observed values for the sample (Fig. 3D) PMMA-Ppy-PpyBz-HRP have displayed very sensitive results (1600 S/N) obtained on a small integrated optical system, when compared with



**Fig. 3.** Calibration curves amperometric measurements corresponding to (A) PMMA-Ppy-PyBz-Urs/UV, (B) PMMA-Ppy-PpyBz-Urs, (C) PMMA-Ppy-Urs/UV modified PMMA fiber as a function of urea concentration ranging from 0 to 60 mM.  $E = 1.1$  V vs. Ag/AgCl. Light measurements corresponding to (D) PMMA-Ppy-PyBz-HRP/UV, (E) PMMA-Ppy-PyBz-HRP, (F) PMMA-Ppy-HRP/UV modified PMMA fiber. Chemiluminescence measurements were performed by adding oxidizing reagent and luminol into the wells in a 1:1 ratio, (G) HRP calibration in the PMMA/SPAD reader configuration. Eight HRP dilutions were tested in triplicate in the biosensor ( $10^2$ – $10^6$ ). A linear distribution can be observed in the optical system until a dilution of 1:1,000,000 of the stock HRP with a good correlation factor  $r^2 = 0.99$ . (H) An elliptic mirror was placed above the fiber in a distance ranging from 4 to 14 cm in order to improve the light collection efficiency. The results show an improvement in the light signal from 20% up to 50% if the fiber is placed in the mirror point (9 cm).

the same set up without UV radiation treatment (11 S/N), thereby indicating that the enzyme was linked specifically to the transducer surface which was further supported by the value of the same set up but without PyBz (16 S/N).

#### 4. Conclusions

In this study we have demonstrated the development of an alternative fiber-optic configuration designated for the construction of biosensing platforms. This new approach was developed and based on the chemical polymerization of pyrrole applied onto the surface of PMMA fibers to create a conductive surface, which enabled the subsequent electrogeneration of photoactive PpyBz upon the fiber surface. Irradiation of the benzophenone groups embedded on the Ppy films using UV radiation (350 nm) enabled the attachment of enzymes based sensing matrices.

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