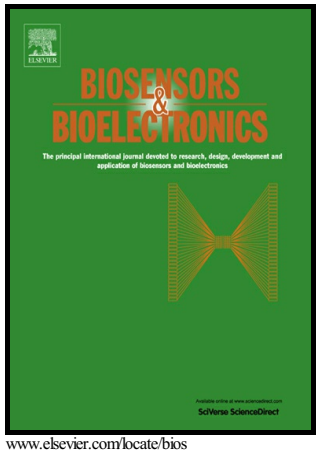

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A novel optical biosensor for direct and selective determination of serotonin in serum by Solid Surface-Room Temperature Phosphorescence

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ABSTRACT

This paper describes a novel biosensor which combines the use of nanotechnology (non-woven nanofibre mat) with Solid Surface-Room Temperature Phosphorescence (SS-RTP) measurement for the determination of serotonin in human serum. The developed biosensor is simple and can be directly applied in serum; only requires a simple clean-up protocol. Therefore it is the first time that serotonin is analysed directly in serum with a non-enzymatic technique. This new approach is based on the covalent immobilisation of serotonin directly from serum on a functional nanofibre material (Tiss®-Link) with a preactivated surface for direct covalent immobilisation of primary and secondary amines, and the subsequent measurement of serotonin phosphorescent emission from the solid surface. The phosphorescent detection allows avoiding the interference from any fluorescence emission or scattering light from any molecule present in the serum sample which can be also immobilised on the nanofibre material. The determination of serotonin with this SS-RTP sensor overcomes some limitations, such as large interference from the matrix and high cost and complexity of many of the methods widely used for serotonin analysis.

The potential applicability of the sensor in the clinical diagnosis was demonstrated by analysing serum samples from seven healthy volunteers. The method was validated with an external reference laboratory, obtaining a correlation coefficient of 0.997 which indicates excellent correlation between the two methods.

Keywords. Nanotechnology; Tiss®-Link; SS-RTP; nanofibre mat; serum; phosphorescence; serotonin; protein clean-up.

1. Introduction

Serotonin or 5-hydroxytryptamine (5-HT) is a biogenic amine that acts as a neurotransmitter in the central and peripheral nervous systems (Berger et al. 2009; Jacobs and Azmitia 1992). It controls a wide variety of physiological and behavioural processes, including depression, mood, sleep, anxiety, addiction, sexual activity, aggression and cognition as well as memory or perception (Berger et al. 2009; Deemyad et al. 2013; Hoyer et al. 2002; Julius 1998; Makris et al. 2016; Meneses and Liy-Salmeron 2012; Piszczek et al. 2015; Ramboz et al. 1998; Udupa and Chen 2015).

Atypical concentrations of 5-HT in physiological fluids (serum and plasma) and body tissues have been related as a critical factor to several mental disorders such as Alzheimer's disease, schizophrenia, depression, infantile autism and obsessive compulsive disorder (Berger et al. 2009; Hoyer et al. 2002; Tecott et al. 1995). Other studies have also shown that high levels of 5-HT can affect the cardiovascular and gastrointestinal systems, and can be related with the carcinoid syndrome (Berger et al. 2009; Brand and Anderson 2011; Kema et al. 2001; Meijer et al. 2000; Pussard et al. 1996).

Due to the multiple roles played by the serotonergic systems, it is important for pathological investigations and clinical diagnostics to establish sensitive, highly selective, rapid and simple methods for determining 5-HT levels in biological samples, mainly in serum.

Many methodologies have been developed for the determination of 5-HT in biological samples, including enzyme immunoassays (Chauveau et al. 1991; Lee et al. 2014; Nichkova et al. 2012), chromatographic (Danaceau et al. 2003; de Jong et al. 2010; Kema et al. 2001; Pussard et al. 1996; Yi et al. 1994; Yoshitake et al. 2004; Yubero-Lahoz et al. 2014), capillary electrophoresis (Peterson et al. 2004; Román et al. 2004), electrochemical (Goyal and Agrawal 2012; Gupta and Goyal 2014; Hasanzadeh et al. 2013; Mazloum-Ardakani and Khoshroo 2014; Wang et al. 2013; Xue et al. 2014) and spectrofluorometric (Bracamonte and Veglia 2011; Peng and Jiang 2007) methods.

Both enzyme immunoassay (Darwish 2006; Wu 2006) (EIA) and enzyme-linked immunosorbent assay (Comley 2012; Gan and Patel 2013; Grange et al. 2014) (ELISA) methods are widely used as diagnostic tools in medicine and biomedical researches to analyse 5-HT, among other analytes, in serum samples (Chauveau et al. 1991). These assays require the immobilization of antigens, haptens, or antibodies on a solid surface, involving some disadvantages such as: 1) the presence of natural inhibitors in the samples which can provide wrong results, 2) nonspecific binding of the antibody or antigen to the solid support which leads to false-positive results, and 3) the enzyme reactions are very sensitive to temperature (Berg 2002; Coolbear et al. 1992; Hedstrom 2001; Iyer and Ananthanarayan 2008).

Moreover, these assays need of many steps of reaction (Chauveau et al. 1991; Darwish 2006), incubation processes (Li and Cassone 2015; Nichkova et al. 2012) and washing steps (Huisman et al. 2010; Kim et al. 2009) which increase the analysis time and cost (Kim et al. 2008; Nichkova et al. 2013) as well as require experimented technicians.

Separative methods, in special HPLC with fluorescence detection, are also commonly used for routine clinical analysis of 5-HT in serum. However, chromatographic determination of 5-HT shows significant drawbacks: they usually require previous solid phase, liquid-liquid extractions or protein precipitations (de Jong et al. 2010; Golubchik et al. 2009; Zhen et al. 2011), pre or post-column derivatization reactions (Hirowatari et al. 2004; Mao et al. 2009; Ohkawa et al. 2005), use of relatively large amounts of solvents (Mao et al. 2009; Sa et al. 2012), long preparation times (Hirowatari et al. 2004; Mao et al. 2009; Teradaira et al. 2007) and expensive equipments.

Electrochemical methods are sensitive enough to estimate the concentration of 5-HT in biological samples, but they are not available in clinical laboratories due to problems of maintenance, limited life time of the electrodes and variability on sensitivity (Babaei et al. 2013; Hasanzadeh et al. 2013; Li and Lin 2007; Myers and Lee 2008).

Spectrofluorometric methods have also been developed to determine 5-HT in serum, but optical measurements are not selective enough to develop a good routine method in this field. One of the main problem of the analysis of 5-HT in serum samples by spectrofluorometric methods is the complexity of the matrix, which contains potential interfering compounds such as proteins, antibodies, antigens, hormones, etc. Many of these compounds show weak natural fluorescence and, therefore can affect the reliability of the results. In fact, many proteins show intrinsic fluorescence emission due to the excitation of tryptophan residues (Gorinstein et al. 2000; Kowalska-Baron et al. 2015)

On the other hand, there are only few components of the serum that show intrinsic phosphorescence emission, where 5-HT, tryptamine (TRYP) and tryptophan (TRYPH) are included (Bruxvoort et al. 1993; de Ribamar et al. 1995). They are structurally and metabolically related (D'Andrea et al. 2015). Therefore, phosphorescence could be a good alternative to much more complex methods to determine 5-HT in human serum. Phosphorescence sensors have been successfully used for the direct, quick and easy (in one step) determination of different molecules in complex matrices, solving the main drawbacks of the enzymatic and separative techniques previously discussed (Bi et al. 2014; Ramon-Marquez et al. 2016; Wu et al. 2010).

Since 5-HT has native phosphorescence, in this paper, we have developed a novel biosensor for the direct determination of 5-HT in human serum samples with satisfactory results, by taking advantage of the benefit of the functional material Tiss®-Link (Medina-Castillo et al. 2011a; Medina-Castillo et al. 2011b; Ramon-Marquez et al. 2016), and the high sensitivity and selectivity of Solid Surface-Room Temperature Phosphorescence (SS-RTP). Tiss®-Link is a non-woven nanofibre mat made by electrospinning which has been demonstrated to be useful for covalent immobilization of amines from complex matrices (Medina-Castillo et al. 2011a; Medina-Castillo et al. 2011b; Ramon-Marquez et al. 2016). The use of Tiss®-Link combined with SS-RTP simplifies the procedure to analyse 5-HT directly in serum samples (it only requires minimal clean-up of the samples), reducing considerably the time and cost of the analysis. In addition, the developed biosensor is highly selective and sensitive in the determination and quantification of 5-HT and therefore, it is a good candidate to be used as routine tool in diagnosis and clinical labs.

2. Experimental

2.1. Reactives and Materials

Tiss®-Link, and PolymP®-Pyridine were kindly supplied by NanoMyP® (http://www.nanomyp.com), Tween 20, potassium iodide (KI), sodium carbonate (Na_2CO_3), potassium phosphate (K_2HPO_4), tryptamine (TRYP), serotonin (5-HT), tryptophan (TRYPH) and human serum albumin (HSA) were all purchased from Sigma Aldrich and used without further purification. Human serum samples were obtained from seven healthy volunteers and stored frozen until assay.

Tiss®-Link is a non-woven nanofibre mat manufactured by electrospinning using PolymBlend® (http://nanomyp.com/es/page.cfm?id=212&title=polymblend%C2%AE) as raw material. PolymBlend® is a polymeric blend formulated with an optimum mixture of two high molecular weight statistic copolymers, containing a high percentage of hydroxyl groups in their structure (40%). Therefore, the nanofibre mats produced with PolymBlend® have high hydrophilicity. During the fabrication process of Tiss®-Link, part of its hydroxyl groups have been functionalized with active vinyl groups, thus the final material has two functionalities: the non-functionalized hydroxyl groups which provide a high hydrophilicity, and the active vinyl groups (330 µmol/g) which can be used for covalent immobilization of primary and secondary amines.

The hydrophylicity of Tiss®-Link was determined by calculating the ω parameter (amount of absorbed water per mass of material) (Stathopoulos et al. 2010). The obtained ω parameter was 2.0, indicating that Tiss®-Link can absorb 2 g of water per gram of material. On the other hand, the pK_a of

the primary hydroxyl groups of the material (which are the only one which can provide ionic charge) is 15, thus, at pH=10 the material is uncharged. Therefore, the properties are kept at pH=10.

Tiss®-Link has excellent mechanical properties (high mechanical strength, high consistency and high flexibility). It is non-luminescent, uncharged, insoluble in aqueous media as well as in apolar solvents (oil, toluene, etc.), showing a high robustness and stability in a wide range of pHs (at least between pH 5 and 10 up to 24 hours), and temperature resistance (up to 100 º C).

PolymP®-Pyridine is a cationic ion exchange material with format of monodisperse and spherical polymeric particles of 2.5 µm of diameter. They have a high surface density of accessible pyridine groups (250 µmol g^{-1}) with a pK_a of 6. These particles are suitable for protein immobilization in a pH range between 3 and 6.

Supporting Material (SM) (see Fig. SM-1) shows images of scanning electron microscopy (SEM) in which the microstructure and morphology of Tiss®-Link and PolymP®-Pyridine are shown.

2.2. Apparatus and measurements

All SS-RTP measurements were performed on a Varian Cary-Eclipse luminescence spectrophotometer equipped with a homemade flow cell. A general description of the equipment for SS-RTP measurements has previously been specified (Ramon-Marquez et al. 2016). Since gaseous oxygen is well known to be a quenching agent, it has to be completely removed from the sample before carrying out the measurements. This was achieved by the application of a gaseous nitrogen stream through the flow cell.

The gas stream was controlled with a mass-flow controller (MFC) (EL-FLOW Select F-201CV, Bronkhorst High-Tech, www.bronkhorst.com, Ruurlo, Netherlands) connected with the flow cell using copper and stainless steel tubing and a PC via a RS-232 serial port by a Flow-Bus Interface (Bronkhorst High-Tech).

All the measurements were carried out at room temperature (21 ºC). The temperature was continuously monitored using a commercial temperature sensor (MicroLite, Fourtec-Fourier Technologies, www.fouriersystems.com) placed at a point close to the flow cell.

The pH of the samples was controlled using a digital pH meter (Crison micropH 2000) calibrated at 20 ± 2 ºC.

2.3. General procedure

For the determination of 5-HT by SS-RTP, three steps are necessary: elimination of serum proteins, covalent immobilization of 5-HT on Tiss®-Link, and SS-RTP measurements.

The elimination of the proteins of serum was carried out following the protein clean-up protocol developed and recommended by the supplier of PolymP®-Pyridine. Briefly, 0.75 mL of serum were incubated with 20 mg of PolymP®-Pyridine at pH=5 (adjusted with 1M HCl) for 30 min at room temperature (RT) in a rotating shaker. After incubation, the samples were centrifuged, the particles with the immobilized proteins were discarded and the pH of the supernatant (free-protein samples) was adjusted to 10 with a solution of 1M NaOH. Finally, it was diluted with carbonate buffer at pH=10 (1:3 v/v, sample:carbonate buffer 20 mM; pH=10).

The covalent immobilization of 5-HT on Tiss®-Link was carried out by incubating 3 mg (≈ 0.8 cm^2) of nanofibre mat with 3 mL of sample at pH=10 for 15 min at RT in a rotating shaker. Then, the mat was washed (3x5 min) with 4 mL of a solution containing 8.5 g/L of NaCl and 0.5 g L^{-1} of Tween 20 (washing solution) to remove all the no covalently bounded molecules and dried with a nitrogen flow.

The SS-RTP signal was measured at excitation wavelength of 315 nm, emission wavelength of 449 nm, delay time (t_d) 10 ms, gate time (t_g) 5 ms, excitation and emission slits of 10 nm wide and detector voltage 850V. All the measurements were carried out adding 20 µL of 1M KI as heavy atom and under a current flow of 300 mL min^{-1} nitrogen to avoid possible quenching effects from oxygen.

The sensing response was established as the difference between the phosphorescence emission of the 5-HT immobilised on Tiss®-Link and the background (RTP emission of Tiss®-Link in the absence of 5-HT).

All the experiments were replicated six times in order to evaluate the error $\left(st/\sqrt{n}\right)$ where s is the standard deviation, t the student t and n the number of replicas.

2.4. Analysis of 5-HT in serum samples

Seven serum samples were obtained from seven healthy volunteers. The seven samples were analysed with both the proposed biosensor and an external reference laboratory (http://reference-laboratory.es/) located in Barcelona (Spain), which determined the amount of 5-HT by an official enzyme immunoassay method.

The analysis of 5-HT in serum samples by the proposed biosensor requires a standard addition calibration method. It was obtained from the SS-RTP emission of Tiss®-Link (0.8 cm^2) incubated for 15 min at RT directly in the serum samples after the protein clean-up protocol. The calibration was developed diluting the sample with 20 mM carbonate buffer at pH=10 (dilution 1:3 v/v, serum:carbonate buffer) and adding four concentrations of 5-HT (0, 50, 100 and 200 ng mL^{-1}). The SS-RTP emission was measured at the following conditions: $\lambda_{exc/em}$=315/449 nm, t_d=10 ms, t_g=5 ms, slit $widths_{exc/em}$=10/10 nm, and detector voltage 850V.

Three replicas at each concentration level (n=12) were used for obtaining all the standard addition calibrations. The error of the 5-HT concentrations were determined by using the following equations (Bruce and Gill 1999):

$$s_x = \frac{s_y}{|b|}\sqrt{\frac{1}{n} + \frac{\bar{y}^2}{b^2 \sum(x_i - \bar{x})^2}}$$

$$s_y = \sqrt{\frac{\sum(y_i - bx_i - a)^2}{n-2}}$$

where s_y is the standard deviation in the residuals, b is the slope, a is the y-intercept of the line, n is the number of replicas, $\bar{y}$ is the average measurement of the standards, x_i are the concentrations of the standards, and $\bar{x}$ is the average concentration of the standards. The error is expresses as $\pm s_x \cdot t$, where t is the Student-t at 95% probability and n-2 freedom degrees.

3. Results

3.1. Luminescent properties of 5-HT immobilized on Tiss®-Link

To characterize the luminescent spectra of 5-HT immobilized on Tiss®-Link a solution of 1 µg mL^{-1} of 5-HT (pH=10) was prepared and the covalent immobilization of 5-HT on Tiss®-Link was carried out by incubating 3 mg (≈ 0.8 cm^2) of nanofibre mat in 3 mL of solution for 30 min at RT in a rotating shaker. Then the material was washed, and the luminescence emission and excitation spectra of 5-HT covalently attached to Tiss®-Link were characterised (see Fig. 1). It shows that 5-HT immobilised on Tiss®-Link emits fluorescence at 337 nm when it is excited at 280 nm and emits phosphorescence at 440 nm when it is excited at 305 nm. SM (see Fig. SM-2) shows that a heavy atom perturber (KI) and an oxygen scavenger (N_2) have to be used to obtain the phosphorescence emission of 5-HT.

3.2. Determination of the optimal conditions for the immobilization of 5-HT on Tiss®-Link

The covalent immobilization of 5-HT on Tiss®-Link occurs through a Michael type-reaction between the primary amino of 5-HT and the activated vinyl groups of Tiss®-Link (Medina-Castillo et al. 2012; Ramon-Marquez et al. 2016). In order to determinate the optimum pH for the covalent immobilization of 5-HT, 3 mg of nanofibre mat were incubated at RT with 4 mL of 1 μg mL^{-1} 5-HT solution at pH=8 (20 mM phosphate buffer) and pH=10 (20 mM carbonate buffer) for different times (1, 2, 3 and 4 h). Then, the 5-HT immobilised on Tiss®-Link were measured by fluorescence. Fig. 2 shows that the immobilization of 5-HT on Tiss®-Link only occurs at pH=10, whereas at pH=8 negligible immobilization is detected. This phenomenon could be attributed to the pK_a of 5-HT. The primary amino of 5-HT has a pK_a=9.97 (Pratuangdejkul et al. 2006), and therefore at pH=8 it is protonated and has not enough nucleophilic character to react with the vinyl groups of the material. However, at pH=10 it is mostly in its neutral form, which has sufficient nucleophilic character to react. This result was similar than others previously published by using the same material (Ramon-Marquez et al. 2016).

Fig. 2 also shows that the amount of 5-HT immobilized on Tiss®-Link increases when the time also increases. Anyway, to decrease the analysis time, 15 min was chosen as immobilization time. At this time the amount of 5-HT immobilized on the solid support is enough to obtain a good sensitivity (as it will be showed in section 3.3) and the amount of potential interfering species (mainly proteins) is not so high (as it will be showed in section 3.4).

Therefore, the incubation of 5-HT with Tiss®-Link was carried out, for the rest of the experiments, in 20 mM carbonate buffer pH 10 for 15 min at RT.

3.3. Effect of coexisting compounds

Since the proposed methodology is based on a kinetic method, the interfering species might be classified in two types: 1) Phosphorescent amines present in serum which can interfere the SS-RTP measurements; 2) Components of serum which can affect the binding kinetic of 5-HT on Tiss®-Link.

3.3.1 Phosphorescent amines present in serum which can interfere the SS-RTP measurements.

Serum is a very complex matrix which contains a lot of molecules and macromolecules such as proteins, vitamins, free amino acid, hormones, etc. Many of these molecules have weak natural fluorescence, but only three of them (5-HT, tryptamine (TRYP) and the amino acid tryptophan (TRYPH)) show phosphorescent emission (Bruxvoort et al. 1993; de Ribamar et al. 1995). Therefore,

most of the luminescent interferences of serum matrix can be avoided by SS-RTP measurements, but TRYP and TRYPH have to be evaluated as potential interference species.

In a previous publication (Ramon-Marquez et al. 2016) we have demonstrated that TRYP is efficiently immobilized on Tiss®-Link at pH=10 (the same condition of the 5-HT immobilization). However, the interference of TRYP in the phosphorescent detection of 5-HT can be eliminated using the appropriate phosphorescence parameters because they have different excitation wavelengths and lifetimes. SM (see Fig. SM-3) shows the effect of the t_d on the excitation spectra of 500 ng mL^{-1} 5-HT and 500 ng mL^{-1} TRYP covalently immobilised on Tiss®-Link at λ_{em}=449 nm. It can be concluded that when the λ_{em}=449 nm, an increase on the t_d provides a decrease on the phosphorescence emission of both 5-HT and TRYP, but it is more pronounced in TRYP. In fact, when λ_{em}=449 nm, [5-HT]= [TRYP]= 500 ng mL^{-1} and t_d is equal or higher than 10 ms, TRYP is not excited between 310 and 330 nm whereas 5-HT can be excited. Therefore, a t_d of 10 ms has to be used in order to eliminate the phosphorescence interference of TRYP.

In addition, to evaluate more deeply the TRYP interference in the detection of 5-HT, several mixtures with a wide range of TRYP:5-HT ratios (1:3, 1:1, 3:1, 5:1, 10:1, 20:1, 50:1) were prepared in 20 mM carbonate buffer at pH=10. Then, they were incubated with Tiss®-Link (0.8 cm^2) for 15 min at RT. Finally, the concentrations of 5-HT in these mixtures were calculated by using standard calibrations carried out at $\lambda_{exc/em}$=315/449 nm and $\lambda_{exc/em}$= 320/449nm (see SM, Fig. SM-4 shows the calibrations). The results of the 5-HT concentrations and the recovery percentages found in these mixtures are shown in SM (see Table SM-1). Table SM-1 shows that exciting at 315 nm, the interference of TRYP does not affect to the 5-HT determination when the concentration of TRYP is equal or lower than 10 times the 5-HT concentration; whereas when 320 nm is used as excitation wavelength, the interference of TRYP occurs for TRYP:5-HT ratios higher than 20:1. The concentration range of TRYP and 5-HT in serum is practically the same (0.1-1 mg L^{-1}), therefore bearing in mind the recovery percentages indicated in Table SM-1, it can be concluded that the TRYP interference can be eliminated by using excitation wavelengths equal or higher than 315 nm and delay times equal o higher than 10 ms.

Although we have also demonstrated that TRYPH is not immobilized on Tiss®-Link at low concentrations (1 µg mL^{-1}) (Ramon-Marquez et al. 2016), the concentration of TRYPH in serum is highly variable (depending on the amount of proteins consumed in the diet), and much higher than 1 µg mL^{-1}. The normal concentration of free TRYPH in serum is usually between 40 and 50 µg mL^{-1}. In addition, TRYPH can be found as an amino acid of the serum proteins, principally human serum albumin (HSA) and human serum globulin (HSG) (Gorinstein et al. 2000; Kowalska-Baron et al. 2015).

The concentration of proteins in serum is very high, and proteins can be covalently attached to Tiss®-Link (by their amine or thiol groups). Therefore, the interference of TRYPH (free and as a component of the proteins) has to be also carefully evaluated.

In order to study the interference produced by high concentrations of TRYPH (free and as a component of the proteins), 4 mL of 48 µg mL^{-1} TRYPH and 4 mL of 1500 µg mL^{-1} HSA solutions were prepared in 20 mM carbonate buffer at pH=10. Tiss®-Link (3 mg) was incubated in both solutions for 15 min at RT. After incubation the materials were washed 3 times with the washing solution, and the SS-RTP spectra of immobilized TRYPH and HSA were characterized (see Fig. 3).

Fig 3 shows that free TRYPH is poorly immobilised on Tiss®-Link at the tested conditions even when the concentration of the free TRYPH is 480 times higher than the concentration of 5-HT. In addition, free TRYPH is excited at a shorter wavelength than 5-HT. Therefore, it can be concluded that the interference of free TRYPH can be eliminated by using the same conditions than for eliminating the interference of TRYP ($\lambda_{exc} \geq 315$ nm and $t_d \geq 10$ ms). But the interference produced by the TRYPH of the proteins cannot be spectroscopically eliminated and, therefore, a protocol for protein clean-up is required. In this case, we have used a cationic ion exchange material (PolymP®-Pyridine) for protein clean-up.

We evaluated the binding capacity of PolymP®-Pyridine using HSA as protein model. For this, 40 mg of PolymP®-Pyridine were added to 2 mL of solutions containing different concentrations of HSA (100, 200, 400, 800, 1500 y 2000 mg L^{-1}) at pH=5 (adjusted with HCl). Then, the suspensions were sonicated for 30 s and incubated for 30 min under shaking at RT. After incubation, the samples were centrifuged and the HSA concentrations in the supernatants were calculated by fluorescence. The amount of adsorbed HSA onto PolymP®-Pyridine was calculated as the difference between the initial concentration of HSA and those found in the supernatants. Adsorption isotherm (see SM, Fig. SM-5) was fitted to the Langmuir-Freundlich model by using a non-linear regression method (Yoon et al. 1999), and the obtained setting parameters: adsorption constant (K=0.56), maximum amount of adsorption (C_m=83.92 mg g^{-1}), and exponential factor (n=0.78), showed that PolymP®-Pyridine exhibits a high affinity for serum proteins by electrostatic interactions (Yoon et al. 1999).

3.3.2. Components of serum which can affect the binding kinetic of 5-HT on Tiss®-Link.

There are lot of molecules and macromolecules in the serum which can originate different rheological properties in serum, regarding to buffer media. Besides, these molecules can be adsorbed on Tiss®-Link surface during covalent immobilization of 5-HT. These facts can affect the diffusion rate of

5-HT from serum to the active sites of Tiss®-Link. Therefore, the reaction rate of covalent immobilization of 5-HT on Tiss®-Link in serum could be different than the covalent immobilization in a buffer solution.

In order to study the reaction rates in serum, serum sample C was selected, and the binding kinetic of 5-HT on Tiss®-Link in 20 mM carbonate buffer at pH=10 was compared with the binding kinetics of 5-HT performed directly in serum (dilution 1:3 v/v, serum:carbonate buffer) before and after the protein clean-up protocol described in section 2.3. The initial concentration of 5-HT was the same in the three cases ($[5\text{-HT}]_0$=75 ng mL^{-1}; concentration of 5-HT after serum dilution).

Fig. 4 shows that the binding kinetics of 5-HT on Tiss®-Link in buffer, serum before clean-up, and serum after clean-up are different. The binding kinetic in free-protein serum is closer to the binding kinetic in buffer, but they are not equal. Therefore the diffusion rate of 5-HT to Tiss®-Link is affected by the media, and this matrix effect cannot be eliminated. Thus, a standard calibration method cannot be used and the proposed biosensor requires a standard addition method for the calibration.

3.4. Application and sensor validation

Firstly, in order to evaluate the necessity of carrying out both the protein clean-up protocol and the standard addition method, two of the serum samples (samples A and B) were analysed by the proposed method before and after protein clean-up protocol and the results were compared with those provided by the reference laboratory. The protein clean-up protocol is described in section 2.3 and the analysis of these samples were carried out following the procedure described in section 2.4 (Analysis of 5-HT in Serum Samples). The results obtained for samples A and B before the protein clean-up protocol were 315 ± 20 ng mL^{-1} and 128 ± 15 ng mL^{-1}, after the protein clean-up protocol were 137 ± 16 ng mL^{-1} and 75 ± 15 ng mL^{-1}, and by an external reference laboratory were 122 ng mL^{-1} and 62 ng mL^{-1}, respectively. It is possible to conclude that both the protein clean-up protocol and the standard addition method have to be used in order to obtain reliable results.

In order to evaluate the practical applications, the 7 samples of serum were analysed by both the proposed assay protocol and an external reference laboratory following the procedure described in section 2.4 (Analysis of 5-HT in Serum Samples). Table 1 summarises the results. A correlation coefficient of 0.997 was calculated by comparing the concentrations of 5-HT obtained with the proposed biosensor (by using SS-RTP), with those obtained by the external reference laboratory (by using enzyme immunoassay), which indicates excellent correlation between the two methods; SM (see Fig. SM-6) shows the calculation of this correlation coefficient.

In comparison with other methods previously published in the literature for determining 5-HT in serum samples, the use of Tiss®-Link combined with a protein clean-up protocol and a SS-RTP measurement offers several advantages such as: 1) it is one of the most simple methods, requiring only a protein clean-up and the incubation with the material, while the rest of the methods require more complicated sample treatment, e.g. solid phase extractions, preconcentration techniques, derivatization reactions, the use of immunogenic conjugates and antibodies specific for serotonin, etc.; 2) it is the most environmentally friendly method using only aqueous solutions, whilst the other methods require organic solvents and hazardous reagents.

4. Conclusions

In this paper, a novel optical biosensor for analysing 5-HT by SS-RTP has been developed. The developed biosensor combines the nanotechnology (non-woven nanofibre mat made by electrospinning) with the detection by solid surface-RTP, which has led to a significant improvement in the selectivity and sensitivity of the detection and quantification of 5-HT. It is the first time to our knowledge that 5-HT has been quantified directly in a complex matrix such as human serum by using a non-enzymatic technique. This novel method is very simple and solves the two major problems of the methods proposed to date for the analysis of 5-HT in serum: 1) the large interference in the measurements of 5-HT produced by coexisting species, such as proteins, hormones, amino acid, vitamins, etc.; 2) the low sensitivity that hinders the determination of 5-HT due to it can be found at very low concentrations in serum (between 40 and 450 ng mL^{-1}). In addition, the proposed biosensor simplifies the methodology to determine 5-HT in complex matrices, avoiding pre-concentration and derivatization steps, and allowing the direct analysis in serum samples.

In conclusion, the proposed sensor has proved to be valid for the detection of 5-HT in serum with good accuracy and short analysis time (55 min), which make it a good candidate to be applied in routine clinical analysis.

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Caption Figures

Fig. 1. Fluorescence (a) and Phosphorescence (b) excitation (black lines) and emission (grey lines) spectra of 5-HT immobilised on Tiss®-Link (solid lines) and the nanofibre mat free of 5-HT (dotted lines). [5-HT]=1 µg mL^{-1}.

Fig. 2. Binding kinetics of 5-HT on Tiss®-Link at pH=8 (red dashed line) and pH=10 (solid black line). $[5\text{-HT}]_0$= 1 µg mL^{-1}, $\lambda_{exc/em}$=280/337 nm, monochromator slits $width_{exc/em}$=5/5 nm, detector voltage 500V.

Fig. 3. Excitation spectra of 5-HT (green dotted line), TRYPH (red dashed line) and HSA (black solid line) immobilized on Tiss®-Link. [5-HT]= 0.1 µg mL^{-1}, [TRYPH]= 48 µg mL^{-1}, [HSA]= 1500 µg mL^{-1}, λ_{em}= 449 nm, slits $width_{exc/em}$=10/10 nm, t_d=10 ms, t_g=5 ms, detector voltage 800V, 20 µL of 1 M KI, in a current flow of 300 mL min^{-1} nitrogen.

Fig. 4. Binding kinetics of 5-HT on Tiss®-Link in 20 mM carbonate buffer at pH=10 (green solid line), serum C after protein clean-up (dilution 1:3 v/v, serum:carbonate buffer) at pH=10 (red dotted line), and serum C before protein clean-up (dilution 1:3 v/v, serum:carbonate buffer) at pH=10 (black dashed line). $[5\text{-HT}]_0$=75 ng mL^{-1}, [KI]=1 M, $\lambda_{exc/em}$=315/449 nm, t_d=10 ms, t_g=5 ms, slit $widths_{exc/em}$=10/10 nm, and detector voltage 850V in a current flow of 300 mL min^{-1} nitrogen.

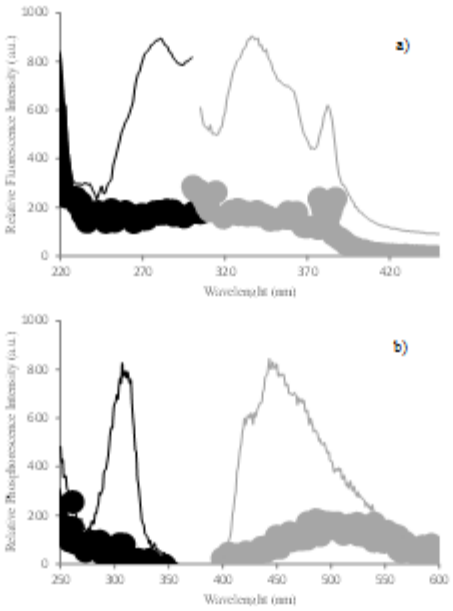

Figure 1

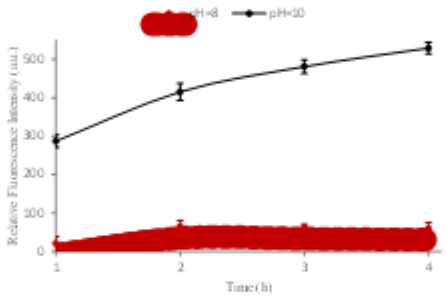

Figure 2

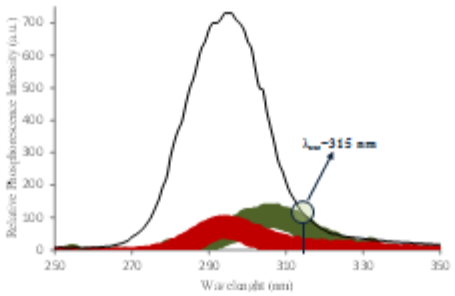

Figure 3

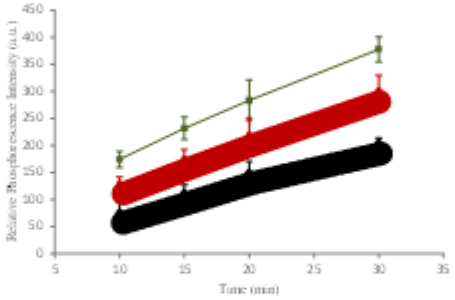

Figure 4

Table 1. Results of the 5-HT determination by the proposed biosensor and an external reference laboratory.

Sample	[5-HT] (ng mL^{-1}) proposed biosensor	[5-HT] (ng mL^{-1}) External reference laboratory
A	122 ± 16	122
B	75 ± 12	62

C	304 ± 6	303
D	114 ± 8	104
E	226 ± 12	209
F	449 ± 13	463
G	197 ± 9	202

Highlights

- We have developed a novel optical sensor for analysing serotonin in complex matrices
- It is free of pre-concentration and derivatization protocols
- It is fast, simple, chip and the most environmental friendly method published so far.
- It is the first time that serotonin is analysed directly in serum with a non-enzymatic technique.