Fluorescent optrode for proteins based on a diketopyrrolopyrrole derivative: practical application to total protein determination in urine

Marta Veríssimo[1], Vitor Almovodar[1], Augusto Tomé[1], and Maria Teresa Seabra dos Reis Gomes[1]

[1]University of Aveiro

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Abstract

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The new fluorescent optrode was tested in the determination of total proteins in urine from healthy individuals (low concentrations). Results were not statistically different from the ones obtained with the Ponceau-S/TCA spectrophotometric method.

Keywords: protein, fluorescence sensor, diketopyrrolopyrrole, optical fibre sensor, urine

1. Introduction

All living organisms, large or small, plant or animal, are made of cells, which are partly made of proteins. Proteins, also known as polypeptides, are organic compounds made up of amino acids. Twenty known amino acids link together to form various kinds of protein, which are arranged in a linear chain and folded, to form three-dimensional molecules with complex shapes. Proteins may differ in the type, number, and sequence of amino acids that make up the polypeptide bone. Having different molecular structures, nutritional attributes, and physicochemical properties may also be diverse. Among their many functions, proteins catalyse reactions, transport oxygen and defend organisms from infection. They are crucial building blocks of organisms, they package up the DNA in chromosomes, and they insulate the cells of the nervous system. Proteins participate in virtually every process within cells. In short, protein matters!

Protein quantification is necessary in many different fields, such as food science and technology, biochemistry, physiology, molecular and protein biology, protein chemistry, clinical analysis, and other research applications. The careful selection of the protein quantification method according to the type of sample and the purpose of the analysis is required to ensure accurate data.

A large number of methods have been developed to quantitate complex mixtures of proteins either directly or indirectly (Mæhre, Dalheim, Edvinsen, Elvevoll, & Jensen, 2018). Direct protein determination is based on the analysis of amino acid residues. A protein sample is first hydrolysed (*e.g.* using a strong acid) to release the amino acids, which are then separated by ion exchange, affinity or absorption chromatography. The protein content is calculated as the sum of individual amino acid residues after subtraction of the molecular mass of H_2O. The major drawback of this method is that the hydrolyses of peptide bonds could lead to the destruction of some amino acids, which may result in protein underestimation. High costs could also prevent the use of this method by food science laboratories, despite being the AOAC recommended method for food proteins (FAO, 2003).

Protein determination can be indirectly inferred by the nitrogen content, or after chemical reactions with functional groups within the protein(Mæhre et al., 2018). Two methods are currently used for determining total proteins inferred by the nitrogen content: the Kjeldahl method and the Dumas method. In the Kjeldahl method, the sample is digested with sulfuric acid so that it releases nitrogen in the form of ammonium sulfate, which is converted into ammoniac by treatment with sodium hydroxide, and further quantified by a suitable titration after being trapped in an acid solution. The amount of protein present is calculated from the nitrogen concentration found. Because this method does not measure the protein content directly, a conversion factor (F) is needed to translate the measured nitrogen concentration into a protein concentration. A conversion factor of 6.25 (equivalent to 0.16 g nitrogen per gram of protein) is used for many applications, although the conversion factor depends on the amino acid composition. Although AOAC International considers acceptable the Kjeldahl method for determination of protein based on total N content when data on amino acids analyses are not available (FAO, 2003), it has been proven that the average value factor of 6.25 may lead to errors in the protein content estimation (Mæhre et al., 2018), and several species-specific conversion factors have been suggested in the literature. Besides, the use of concentrated sulfuric acid at high temperatures poses a considerable hazard, requires skilled staff and the technique is tedious and time-consuming to carry out.

In the Dumas method, the sample is combusted in a high temperature (about 900 ^{o}C) chamber in the presence of oxygen. The released gases passed through special columns that absorb CO_2 and H_2O, and the nitrogen content is afterwards measured by passing the remaining gases through a column that has a thermal conductivity detector at the end. Like in the Kjeldahl method, it is necessary to convert the concentration of nitrogen to protein, using a suitable conversion factor. This method is much faster than the Kjeldahl method, does not need toxic chemicals nor catalyst, and many samples can be measured automatically. However, it has a high initial cost, and, just like the previous method, different proteins need different correction factors.

Besides, none of these methods distinguishes between protein-based nitrogen from non-protein nitrogen. Nitrogen from non-protein additives or contaminants in the food, such as the fraudulent addition of melamine to milk, is also measured, and exploitation of this analytical vulnerability has led to serious incidents in the past (Moore, Devries, Lipp, Griffiths, & Abernethy, 2010).

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Indirect protein determination after chemical reactions with functional groups within the proteins comprise spectrophotometric methods such as the direct measurement of UV absorbance at 280 nm, Biuret, Lowry (Folin-Ciocalteau), Smith (Bicinchoninic acid), Bradford (Coomassie Brilliant Blue) and, Pesce and Strande (Ponceau-S/TCA) assays, just to point out the six spectrophotometric methods mostly used (Dilena, Penberthy, & Fraser, 1983; Mæhre et al., 2018; Okutucu, Dinçer, Habib, & Figen, 2007; Zaia, Zaia, & Lichtig, 1998). These six methods will be presented briefly, along with their advantages and disadvantages.

Simple but often unreliable, the direct ultraviolet absorption estimates the amount of protein by measuring the characteristic absorption of aromatic amino acids, mainly tyrosine and tryptophan, at 280 nm. The sensitivity of the method is moderate but has the advantage of not requiring special reagents, being simple, and the fact that the method is non-destructive, allowing to recover valuable proteins. Unless the protein sample is pure, and its extinction coefficient is known, UV determination of protein concentration will invariably be prone to significant errors (Olson & Markwell, 2007). Other disadvantages include the requirement of UV spectrophotometers and quartz cuvettes and the fact that virtually everything, including commonly used buffers, does absorb in the UV region (Zaia et al., 1998). Furthermore, it is incompatible with a wide range of protein extraction methods which frequently employ detergents and denaturing agents.

The Biuret assay is a chemical test for proteins based on the formation of a coloured chelated complex between cupric ions and peptide bonds, forming a complex with tartrate, which absorbs at 540-545 nm. The intensity of the colour, and hence the absorption at 540-545 nm, is directly proportional to protein concentration, according to the Beer-Lambert law. A significant disadvantage of this assay is its poor sensitivity. Reported linear range is between 0.5-4.0 mg mL^{-1} (Okutucu et al., 2007; Zaia et al., 1998). Another drawback of this assay is that some compounds used in the laboratory, such as Tris buffer and ammonium sulfate, as well as endogenous compounds in crude extracts, can interfere with colour development or generate coloured complexes themselves. Proteins with an abnormally high or low percentage of amino acids with aromatic side groups will give high or low readings, respectively (Saxena, Baunthiyal, & Ravi, 2015).

Lowry's protein assay combines the Biuret reagent with another reagent, the Folin-Ciocalteau phenol reagent. First, copper ions are reduced under alkaline conditions and form a complex with peptide bonds of the protein. Second, the complexed tyrosine and tryptophan react with Folin-Ciocalteau phenol reagent to give an intense blue-green colour, which can be detected somewhere between 500-700 nm. This method has been widely used for protein determination for many decades, due to its simplicity. However, it is susceptible to a wide range of nonprotein substances generally present in most extracts. Besides, the Lowry's phenol reagent is unstable in alkaline solution, and the two-step addition of the colour-developing reagents are cumbersome and tedious (Choi, Chung, Chang, Chun, & Lee, 1993; Mæhre et al., 2018). The absorbance of the reaction mixture is not strictly proportional to protein concentration, and colour development is slow and fades relatively rapidly, therefore, reasonable precise reaction times, and temperatures are required (Zaia et al., 1998). However, Lowry assay is somewhat more sensitive than the Biuret assay, with a reported linear range between 0.02-0.5 mg mL^{-1} (Okutucu et al., 2007).

The Smith assay, also known as the Bicinchoninic acid (BCA) protein assay, has been advertised as an alternative to the Lowry assay. Like the Lowry assay, first the protein complexes with copper ions. Then, in a second step, this protein-bound copper chelates BCA to give an intense purple colour. The chromophore produced is more stable than the one produced in the Lowry assay. Additional advantages include high sensitivity (100 times higher than the Biuret), a simple commercially available two-solution reagent, ease of procedure, and compatibility with a large number of extraneous materials commonly found in protein preparations. However, Smith assay development requires long incubation times (30 minutes to 2 hours), dependence of the incubation temperature of samples and absorbance variation with time. Also, the presence of reducing agents, copper chelating agents, acidifiers, reducing sugars, lipids and phospholipids in the buffer can affect the accuracy of the results (Zaia et al., 1998).

The Bradford assay is the most commonly utilised assay for protein determination as it is relatively easy to perform (Sapan & Lundblad, 2015): negatively-charged Coomassie Brilliant Blue dye binds with positively-charged proteins. The dye in solution is red (absorbs at 465 nm), but when it binds to basic amino acids

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in the protein, it becomes blue (absorbs at 595 nm). The absorption in the sample can then be compared with a standard curve. The Bradford assay has been recognised to be as sensitive as the Lowry method, with a reported linear range 0.02-0.5 mg mL^{-1} (Okutucu et al., 2007) and less prone to interferences (Zaia et al., 1998). However, it exhibited some variations depending on the kind of protein (only detects proteins larger than 3 kDa). Besides, detergents such as sodium dodecyl sulfate (SDS), Triton X-100 and commercial glassware detergents, give excessive interfering colour in the assay (Bradford, 1976), and the method has a narrow range of linearity and poor precision (Dilena et al., 1983). A minor disadvantage of this assay is that the dye adsorbs to glassware, cuvettes, skin, and clothing.

Finally, the Pesce and Strande assay is based on the simultaneous mixing of proteins and Ponceau-S dye with trichloroacetic acid (TCA), and then the resulting precipitate is dissolved in sodium hydroxide giving a violet colour. This assay is considered practical, capable of good precision, and providing adequate accuracy, with a reported linear range of 0.1-0.3 mg mL^{-1} (Okutucu et al., 2007). It is recommended for routine clinical chemistry laboratories (Dilena et al., 1983).

The importance of protein determination is, however, reason enough to justify the investment in the development of a sensor that would allow the determination of the concentrations of interest with minimum sample preparation, and fast.

Recently, fluorescent sensors have caught the attention of many scientists due to their huge advantages such as simple equipment, high sensitivity and selectivity. There is thus a high demand for the development of simple and stable fluorescent sensitive molecules.

Several fluorescent reagents for protein detections and quantifications based on emission enhancement have been reported, such as NanoOrange, fluorescamine, *o* -phthaldialdehyde, cyanine dyes, and SYPRO Ruby (Granzhan & Ihmels, 2005; Tong et al., 2007). All these fluorophores were used in batch analysis in solution, along with lengthy procedures, comprising carefully timed steps, which resulted in small Stokes shifts and nonlinear calibration curves (Tong et al., 2007). Besides, the fluorescamine dye is unstable.

The majority of the fluorophore molecules lose their ability to emit light when they form dimers or aggregate and therefore, can only be used if highly diluted (Jin et al., 2011). The advantage of the fluorophore used in this paper is that it emits when in the solid-state, which is the main reason to use it in fluorescence probes (Jin et al., 2011).

1,4-Diketo-3,6-diarylpyrrolo[3,4-*c*]pyrroles (DPP) and derivatives represent a class of brilliant red and intense fluorescent high-performance pigments, that gained wide attention in recent years due to their outstanding properties, namely large extinction coefficients, high fluorescent quantum yields, environment and heat stability which make them excellent candidates for fluorescent sensors (Kaur & Choi, 2015; Zhang et al., 2018).

The –NH groups of DPP are prone to form strong hydrogen bonds with electronegative ions like F^- or be easily deprotonated. Therefore, an alkylated DPP derivative was synthetized to avoid possible interferences from those ions. The presence of long alkyl chains on both lactam N atoms, besides inhibiting the response to fluoride ion, also favours the solubility of the DPP in organic solvents (Qu, Hua, & Tian, 2010).

Initially, it was envisaged that aldehyde groups in the fluorescent molecule would be needed to assure the necessary chemical affinity with the target proteins through their amino functional groups. Nevertheless, a DPP derivative with ester groups was also prepared and tested, for comparison. Figure 1 shows the molecular structure of the DPP-dialdehyde, and DPP-diester used in this work. The interaction of the formyl groups with proteins are expected to increase the rigidity of the recognition molecule, leading to an increase in the fluorescence intensity.

The measurement of proteins in human urine provides an essential tool in the diagnosis of renal diseases. Healthy kidneys should only filter tiny (trace) amounts of proteins into the urine as most protein molecules are too large for the filters (glomeruli). The reference range in healthy adult males and females is 10-150 mg L^{-1} or 20-140 mg/24 h (Yalamati, Karra, & Bhongir, 2016). Higher levels indicate a condition known as "Proteinuria", requiring medical follow-up.

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The new sensor was tested analysing healthy people's urine samples (detection of low protein levels), and results were compared with the ones obtained with the highly sensitive spectrophotometric Pesce and Strande (Ponceau-S/TCA) method.

2. Experimental

2.1 Reagents

Ponceau-S dye from Sigma-Aldrich (Madrid, Spain), trichloroacetic acid (TCA) and sodium hydroxide from Panreac (Barcelona, Spain), and sodium chloride from Pronolab (Lisbon, Portugal) were used. Bovine serum albumin (BSA) was from Sigma-Aldrich and has been kept in a cold dark place. Ultrapure water was produced by a Milli-Q system from Millipore (Mississauga, Canada). Sodium fluoride from Fluka (Buchs, Switzerland), sodium dodecyl sulfate from Riedel-de Haën (Seelze, Germany), and acetone and glucose from Merck (Darmstadt, Germany) were used to test for possible interferences.

The DPP derivatives were synthetized by a new procedure that will be reported elsewhere(Agazzi et al., 2019). The DPP derivatives applied on the optical fibre tip were dissolved in chloroform (Sigma-Aldrich).

2.2 Solutions and standards preparation

Ponceau-S stock solution was prepared by dissolving 1 g of Ponceau-S dye in 25 mL of Milli-Q water. A trichloroacetic acid (TCA) solution at 300 g L^{-1} was prepared by diluting the appropriate volume of TCA in Milli-Q water. The Ponceau-S/TCA concentrated reagent was prepared by diluting 10 mL of the Ponceau-S stock solution to 500 mL with the TCA solution.

Sodium hydroxide solution (0.8 g L^{-1}), sodium chloride solution (0.9%) and a stock solution of 1 g L^{-1} BSA were prepared by dissolving the appropriate amounts in Milli-Q water.

For the Ponceau-S/TCA method, BSA standards were prepared daily in a saline solution (0.9% sodium chloride) from the BSA stock solution. An equivalent sodium chloride amount was added to the urine samples (Ballantyne, Gibbons, & O'Reilly, 1993). For the optrode method, BSA standards were prepared daily by diluting the BSA stock solution with Milli-Q water.

2.3 Samples

Seven urine samples were collected from the same family (adults and children) and analysed a few hours after collection. All samples were previously filtered through a Whatman filter paper grade 4, pore size 20-25 μm (Maidstone, England) to remove coarse particles.

2.4 FTIR-ATR measurements

In order to chemically characterise and follow the interaction of the protein with the Di-CHODPP sensing layer, infrared spectra of Di-CHODPP before and after being in contact with BSA were acquired. An infrared spectrometer (Bruker Alpha Platinum, Germany) equipped with a single reflection diamond ATR module, with a resolution of 4 cm^{-1} and 32 scans, in the mid-infrared region (4000-600 cm^{-1}) was employed. Analyses were performed in a room with controlled temperature (23 ºC) and humidity (35%).

Background was obtained recording a spectrum at the ambient air. A 1 cm x 1 cm of a clean metal plate was used as deposition surface for FTIR-ATR measurements. A spectrum of the clean metal plate was acquired. The metal plate was then coated with the Di-CHODPP compound dissolved in chloroform, the solvent was left to evaporate, and the metal plate with the Di-CHODPP was analysed. Afterwards, a drop of a 500 mg L^{-1} BSA solution was placed above the Di-CHODPP coating, left to evaporate and a new spectrum was acquired.

2.5 Coating of the optical fibre tip

The optical fibre used was a pure fused silica core of 1000 μm, from Sarspec (Vila Nova de Gaia, Portugal). Before coating the fibre, the fibre tip needed to be prepared: around 1 cm of the external buffer was cut with a blade and removed, exposing the cladding. Then, using a Micro-strip from Micro Electronics Inc.

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(Massachusetts, USA), the cladding of the fibre was removed, exposing around to 2 to 3 mm of the fibre core. Finally, the core was gently cleaned with ethanol before coating. The exposed core of the fibre was then dipped into the Di-CHODPP chloroform solution, removed, and left to dry for at least 3 hours, but preferably overnight.

2.6 Optical sensor set-up

Figure 2 shows a scheme of the experimental set-up. A light source DH-2000 from Ocean Optics (Florida, USA) equipped with a deuterium lamp was connected to the sensing fibre by an SMA-905 connector. The UV excitation energy reaches the other end (the coated tip) of the fibre, which was introduced into a PEEK home-made cell, possessing approximately 1 mL reservoir with a filling entrance at the top, and an exhaust aperture at the bottom (drain). The coated tip of the optical fibre was positioned at a 90^{o} angle from a collimating lens, a convex flat lens 1", with a focal distance of 30 mm from Sarspec. The visible light emitted from the coated tip and collected by the collimator lens was guided to another pure fused silica fibre of 600 μm (core) connected to a fluorescence spectrophotometer Scanspec from Scansci (Vila Nova de Gaia, Portugal). The fluorescence spectrophotometer was connected to a PC by a USB cable. The acquisition software used was Spectrascan from Scansci.

2.7 Procedure for analysis

2.7.1 The Ponceau-S/TCA method

Total protein was quantitated following the already described procedure for Ponceau-S/TCA method (Marshall & Williams, 2000; Pesce & Strande, 1973): 500.0 μL of Ponceau-S/TCA concentrated reagent was mixed with 5.0 mL of urine sample or BSA standard, and the mixture was centrifuged with a centrifuge Mixtasel Mod. 540 from Selecta (Barcelona, Spain) at 3500 rpm for 20 minutes (Pesce & Strande, 1973). After centrifugation, the supernatant was discarded, and the precipitate was dissolved in 5.0 mL of sodium hydroxide (0.8 g L^{-1}).

The absorbance of the basic solutions was measured at 560 nm with a double beam UV-Vis spectrophotometer Shimadzu UV-2101 PC (Kyoto, Japan), and measurements were made against Milli-Q water. Calibration line was drawn plotting the absorbance of the BSA treated sample *vs* . BSA concentrations, and quantification of proteins in the urine sample was obtained by entering with the absorbance corresponding to urine treated solution in the calibration line equation.

2.7.2 The optical fibre sensor method

BSA standards were prepared from 10.0 to 60.0 mg L^{-1}.

Urine samples were prepared according to the following procedure: 500.0 μL of TCA (300 g L^{-1}) was added to 5.0 mL of the urine sample. The mixture was filtered using a disposable syringe to force the mixture through a membrane filter of 0.45 μm from Millipore, mounted in an appropriated filter holder Swinnex, also from Millipore. After filtration, the filter was removed with a plastic tweezer and carefully washed with 4.0 mL of sodium hydroxide solution (0.8 g L^{-1}) to dissolve all the proteins retained in the filter membrane. The solution was transferred to a 5.0 ml volumetric flask, and volume was completed with a sodium hydroxide solution using a Pasteur pipet.

Before initiating measurements, the coated optical fibre was introduced into the home-made liquid chamber, as represented in Figure 1, and the light source (deuterium lamp) was left to stabilise for 30 minutes.

After light source stabilisation, measurements could be initiated. Each BSA standard or the treated urine sample solution was poured through the top hole of the cell with a Pasteur pipet, and the chamber top was closed with a screw thread. The Vis spectrum (in intensity units) was saved after stabilisation. Once the measurement was saved, the chamber was opened, unscrewing the two screws, one at the top and another at the bottom, and the sample was evacuated by gravity. The chamber was washed with Milli-Q water between analysis. A calibration line was derived with the fluorescent intensity obtained with the BSA solutions. The

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maximum fluorescent intensity for each urine treated sample was entered in the equation of the calibration line.

3. Results and discussion

3.1 Interaction between Di-CHODPP and the proteins

Experiments with the diester Di-CO_2MeDPP did not show any changes in the emission spectrum when the compound was put in contact with BSA. This result seems to indicate that the interaction between the protein and the Di-CHODPP does not involve the formation of a hydrogen bond but, probably, a covalent bond involving the formyl groups (that are much more reactive than ester groups). Because the recognition process is very fast and reversible, that covalent bond must be weak. Presumably, a hemiaminal bond (and not an imine bond) is formed from the interaction of the amino groups of the protein and the formyl groups of the recognition molecule, as illustrated in Figure 3. This interaction is reversible, and the di-aldehyde functions are quickly recovered after immersion of the coated fibre in water.

Figure 4 shows the IR spectrum of the Di-CHODPP before and after contacting with BSA solution. There are two bands in the spectra of Di-CHODPP related with C=O vibration: at 1662 cm^{-1} and at 1696 cm^{-1}. The first one can be attributed to the C=O stretching mode of the lactam, and, as expected, its intensity remained unchanged after the interaction with the protein. The second band can be attributed to the carbonyl group of the aldehyde, and its intensity, as expected, did decrease after the interaction with the protein.

A small shoulder at 1111cm^{-1} is consistent with the formation of a C–N bond and is highly characteristic of a C–N stretching band of a secondary amine. This band was not found in the ATR spectra of pure BSA, as can be seen in Figure 4.

The increasing intensity of the band at ˜3300 cm^{-1}, typical of O–H and N–H bonds(Bellamy, 1975), also corroborates the formation of the hemiaminal function.

All the listed observations are consistent with the hemiaminal formation hypothesis, and with the reversibility of the protein interaction, very important to assure sensor calibration and reuse.

3.2 Sensor analytical signal and calibration

After solvent evaporation, the coating (Di-CHODPP) on the tip of the optical fibre was easily confirmed by visual inspection, due to the presence of the orange colour of the compound. Figure 5 shows the fluorescent emission spectra of the coated fibre in contact with Milli-Q water and in contact with a 100 mg L^{-1} BSA solution.

Figure 5 shows the fluorescent emission spectrum of the coated fibre in contact with Milli-Q water. When the sensor is put in contact with a 100.0 mg L^{-1} BSA solution, the intensity of the peak at 581 nm showed a small increase, compared to the peak intensity in water. Despite of looking like a modest increase in the fluorescent intensity, due to the yy scale of Figure 5, the changes on the emission intensity at 581nm were significant enough to be selected as the analytical signal for protein quantification. The interaction between Di-CHODPP and BSA was reversible, and intensity emission found in water was rapidly restored after flushing the cell with Milli-Q water and refilling it.

Figure 6 shows the emission intensity at 581 nm obtained for the BSA standards analysed. The graph shows a linear working range between 10 mg L^{-1} (LOQ) and 100 mg L^{-1} of BSA.

3.3 Validation of the direct BSA calibration

To evaluate the possibility of using BSA standards directly, without TCA pre-treatment similar to the one used in the Ponceau-S/TCA method, a 60.0 mg L^{-1} BSA standard was analysed by the optrode, both directly, and after pre-treatment. The pre-treatment consisted in the TCA addition, followed by filtration and protein dissolution with sodium hydroxide solution, as described in 2.7.2 procedure. It was found that fluorescence emission spectra were equal, and the intensity of the peak at 581 nm was exactly the same. These results

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allowed to conclude that BSA standards could be analysed directly, without any pre-treatment, making calibration simpler and fast than in the Ponceau-S/TCA method.

The removal of interfering substances from urine, by protein precipitation with TCA, and redissolution of the proteins in NaOH has been widely used in the vast majority of methods for protein determination (Choi et al., 1993; Noble & Bailey, 2009; Yalamati, Bhongir, Karra, & Beedu, 2015). The elimination of this step would allow saving a considerable amount of time, and although it seems to be an unrealistic simplification, it deserves to be tested with the urine samples. Therefore, an aliquot of urine was analysed directly and after TCA addition, filtration and proteins dissolution with sodium hydroxide solution.

The analysis of both aliquots, with and without TCA addition, with the new optic fibre sensor, revealed that sample without treatment showed a less intensity signal at 581 nm when compared with the one obtained with the treated sample. This study allowed to conclude that urine samples needed to be pre-treated (TCA/filtration/dissolution), like the vast majority of methods for protein determination, to remove interfering substances from urine that could inhibit the interaction between proteins and the coated sensor, giving low and erroneous results.

3.4 Interference studies

Although each method for protein determination counts with particular advantages and disadvantages, the great majority of them is prone to interferences from numerous substances that could be found in biological samples, such as Tris buffer, acetone, sodium dodecyl sulfate (SDS), Triton-X, sugars, EDTA, and ethanol, among others (Bradford, 1976).

A 50.0 mg L^{-1} BSA standard solution was analysed using the optical sensor, using the procedure reported in section 2.7.2 for BSA standards, and the respective emission spectrum was recorded. To test some of the most often reported interference compounds, solutions containing the same amount of BSA (50.0 mg L^{-1}) and the substances presented in Table 1A were prepared and analysed with the fluorescent optical sensor.

DPP compounds were reported to be highly reactive and selective for fluoride anions (Qu et al., 2010), due to the intermolecular proton transfer between a hydrogen atom on the lactam N positions of the DPP and the fluoride anion. In spite of having blocked those positions by choosing a compound where the referred protons have been replaced by alkyl groups, the sensor was also tested with aqueous fluoride solutions (see Table 1B).

Fluorescence emission spectra obtained with BSA solutions with and without the substances listed in Table 1A were compared, and no interference was noticed with SDS, glucose and acetone, as no changes in the BSA fluorescence intensity were observed.

The intensity of fluorescence observed with the fluoride ion solution (Table 1B) was compared against the fluorescence spectrum in Milli-Q water. As expected, no differences were observed between spectra, meaning that fluoride ion did not interact with the Di-CHODPP. This result validates the choice of N,N-dialkylated Di-CHODPP.

3.5 Analysis of urine samples by the Ponceau-S/TCA method and by the new optrode sensor

Protein was quantified in seven urine samples using the new optrode. BSA solutions of known concentration were used to prepare a standard calibration line covering the range from 10.0 to 60.0 mg L^{-1}. The concentration of the unknown urinary protein samples was then determined by entering with the intensity of emission of the TCA treated sample in the BSA calibration line. For comparison, the protein amount in the urine samples was also measured by the Ponceau-S/TCA spectrophotometric method. Table 2 shows the obtained protein content found in urine samples using both methods.

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated from the calibration curve and from the standards errors estimate S_{yx}, that measures the variability or scatter of the observed values around the regression line. The LOD (concentration corresponding to a signal = intercept + $3S_{yx}$) for the optrode was 3.1 mg L^{-1}while the LOQ (concentration corresponding to signal = intercept + $10S_{yx}$)

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was 10.4 mg L^{-1}. LOD and LOQ for the optrode method were not significantly different from the values found for the Ponceau-S/TCA method, 3.4 mg L^{-1} and 11.4 mg L^{-1} for LOD and LOQ, respectively.

The values in Table 2, obtained with the two methods, were statistically compared using a paired *t*- test, and it was found that the difference in the mean values between the results of the two methods was not great enough to exclude the possibility that the difference was due to random variability (P = 0.05). An *F* -test was running for comparison of standard deviations, and the Ponceau-S/TCA method was more precise than the optical sensor method ($\alpha = 0.05$), in all cases, except for sample 4, where standard deviations did not differ significantly ($\alpha = 0.05$).

Healthy adults excrete less than 150 mg of total protein per 24 h (Waller, Ward, Mahan, & Wismatt, 1989). Considering an average volume of urine excretion/day of 1.5 L (Dube, Girouard, Leclerc, & Douville, 2005), the values from Table 1 correspond to total protein values ranging from 23 to 52 mg. These are low values classified as "negative result" (less than 100 mg L^{-1}) typically found in healthy individuals. Results show the power of the method to quantify low concentrations, and higher values, like the ones found in proteinuria conditions, above 300 mg L^{-1}, could be easily detected and quantified, by simply diluting the sample. The sensor was used daily for two weeks with no loss in sensitivity.

4. Conclusions

A fluorescent optical sensor for the detection of total proteins based on Di-CHODPP is present. The Di-CHODPP showed interesting and unusual properties of retaining its fluorescent capability when in solid-state, which is particularly important when used as a recognition molecule in an optical sensor. Besides, it is insoluble in water and experienced a fluorescence enhancement in the presence of proteins. The designed analytical setup consisted of a UV light injected in the optical fibre coated with the Di-CHODPP at stripped the end, which entered a home-made cell with a 1 mL cavity with the sample. The interaction between proteins and the coated tip of the fibre is very fast and reversible with a simple water flush. Experiments with BSA allowed to compare the sensor detection limit (3.1 mg L^{-1}) with the LOD of other conventional methods, such as Ponceau-S/TCA spectrophotometric method (3.4 mg L^{-1}).

The new fluorescent sensor was applied to the detection of the total proteins in human urine and results were not statistically different from the ones obtained with the Ponceau-S/TCA spectrophotometric method. The new optical sensor, showed, however, some advantage over the Ponceau-S/TCA traditional method, namely in the economy of reagents and analysis time, as standards do not need to be treated with trichloroacetic acid, nor centrifuged. The new sensor does not suffer from interference from sodium dodecyl sulfate, acetone or glucose, the most common interfering compounds in conventional spectrophotometric methods. Besides, the presence of fluoride ions in solution did not change the fluorescence intensity of the sensor. Summing up, sensor reliability in the determination of total proteins in urine samples has been demonstrated.

Conflicts of interest

There are no conflicts to declare.

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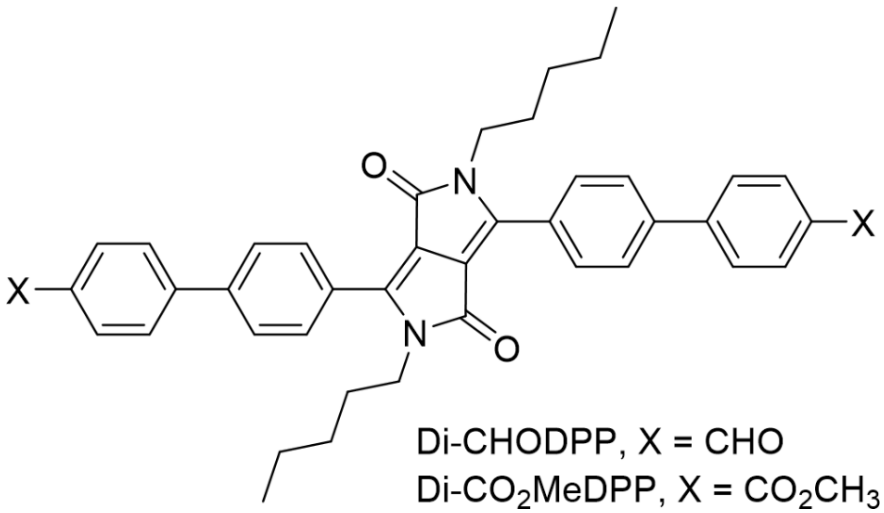

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Figure 1 Molecular structures of the two DPP derivatives tested as selective membranes of the optical sensor.

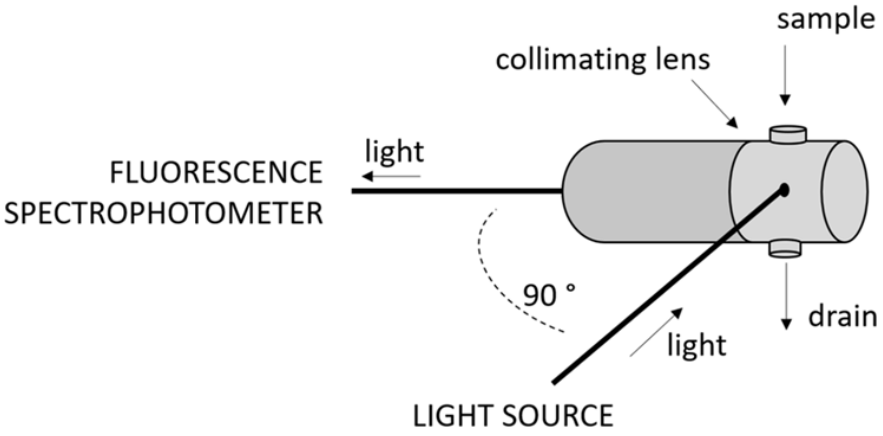

Figure 2 Experimental setup used with the fluorescence sensor.

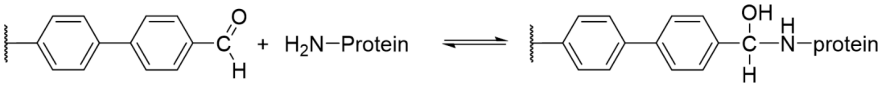

Figure 3 Envisaged interaction between the Di-CHODPP and the protein amino group.

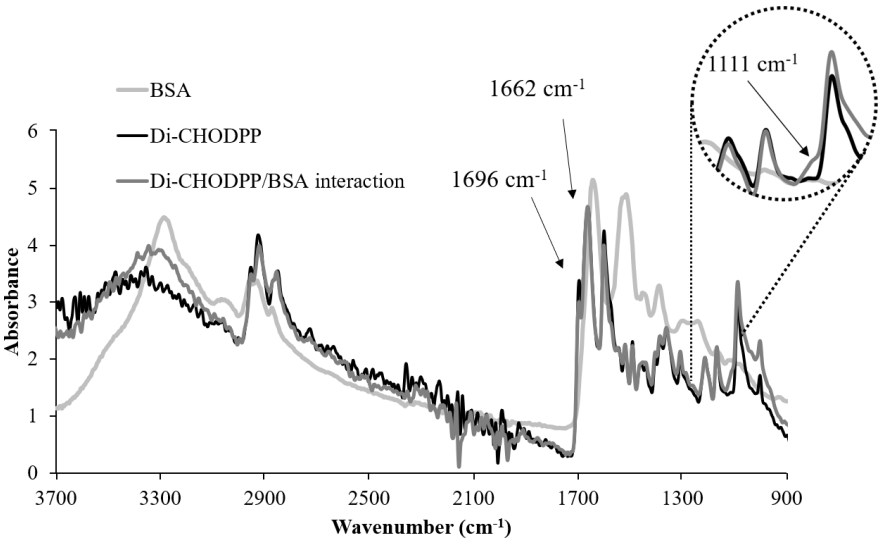

Figure 4 IR Spectrum of the Di-CHODPP with (grey line) and without (black line) BSA. The light grey line represents pure BSA spectrum.

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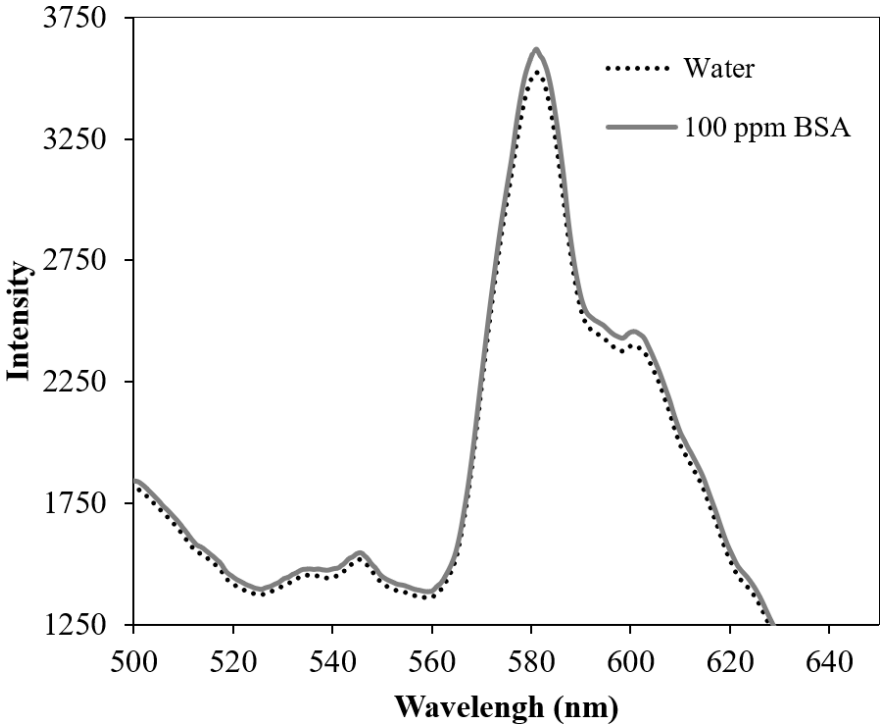


Figure 5 Fluorescence emission spectra of the Di-CHODPP in contact with Milli-Q water (black dots), and in contact with a 100 mg L^{-1} BSA solution (gray line).

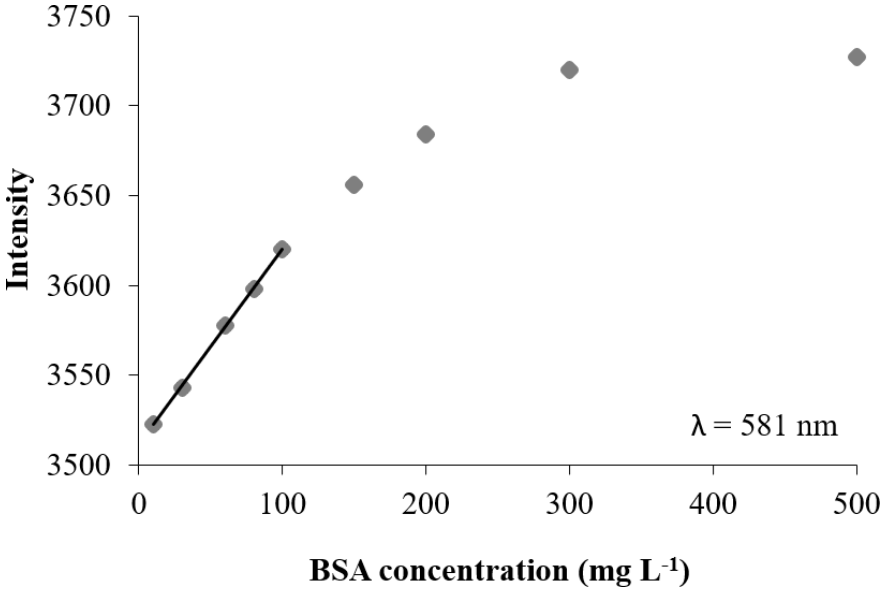


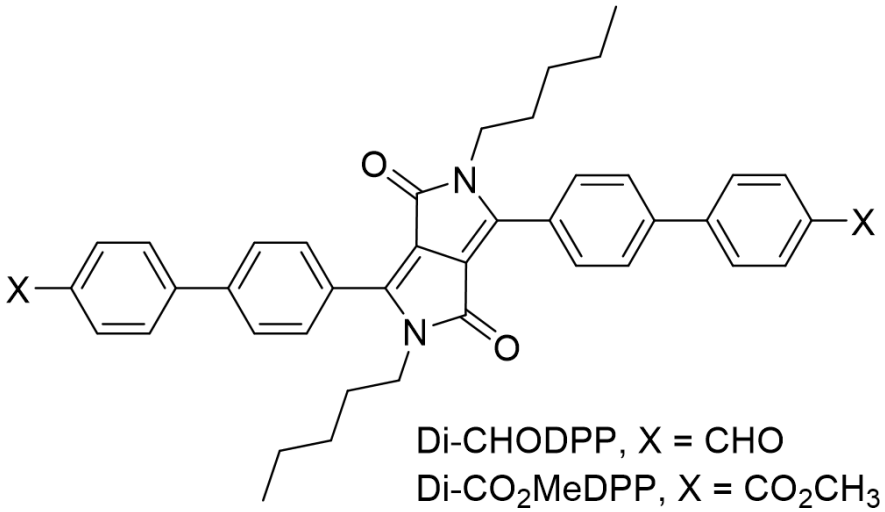
Figure 6 Intensity of fluorescence emission at 581 nm*vs.* BSA concentration (mg L^{-1}).

Table 1 Substances tested for interference.

Substances in 50.0 mg L^{-1} BSA	**Concentration**
Sodium dodecyl sulfate (SDS)	1% (w/v)
D-Glucose	1 M
Acetone	5% (v/v)
Substances in Milli-Q water	**Concentration**
Fluoride ion (NaF)	500 mg L^{-1}

Table 2 Protein content in urine samples obtained by the optical sensor and by the Ponceau-S/TCA spectrophotometric method.

Samples	**Optical sensor method (mg L^{-1})**	**Ponceau-S/TCA method (mg L^{-1})**
1	23.3 ± 3.7	23.6 ± 2.4
2	14.7 ± 4.3	15.1 ± 2.2
3	11.7 ± 3.4	12.0 ± 2.2
4	26.0 ± 2.1	26.3 ± 2.6
5	15.1 ± 2.5	14.6 ± 2.3
6	23.5 ± 2.8	23.3 ± 2.6
7	14.3 ± 3.2	14.6 ± 2.5



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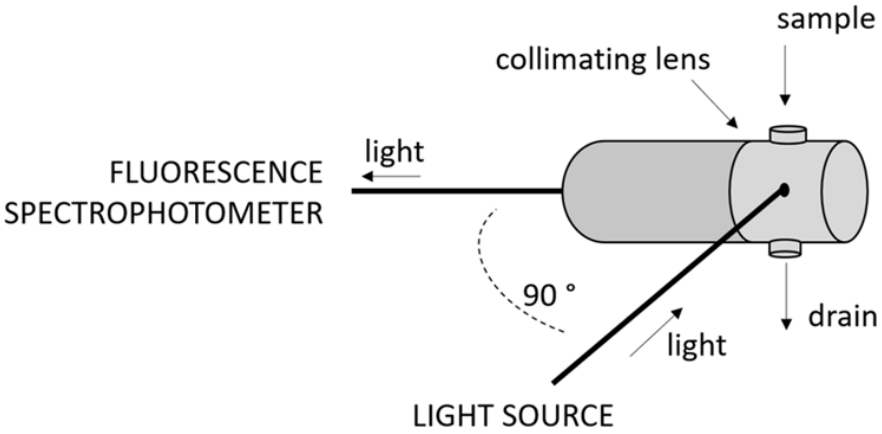

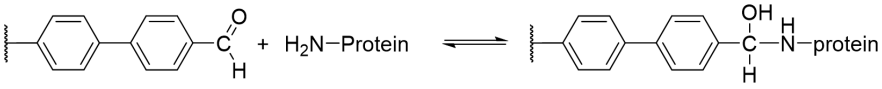

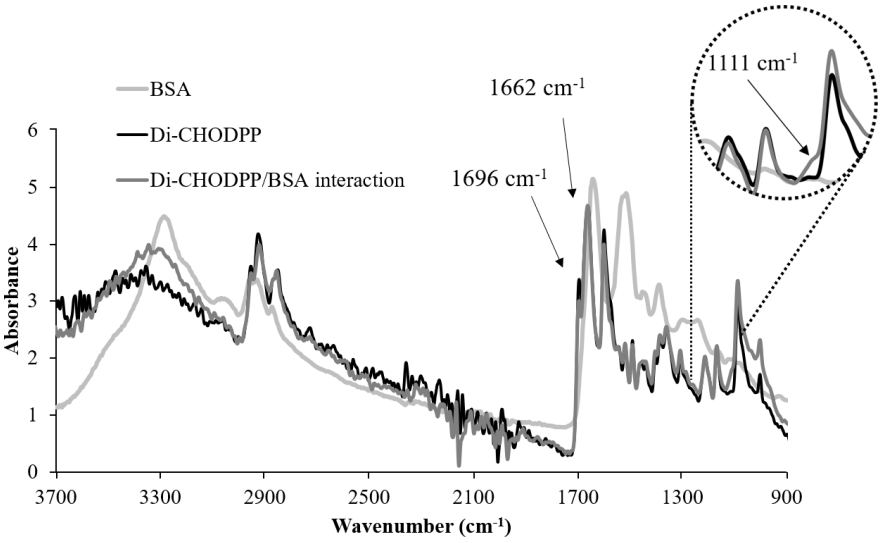

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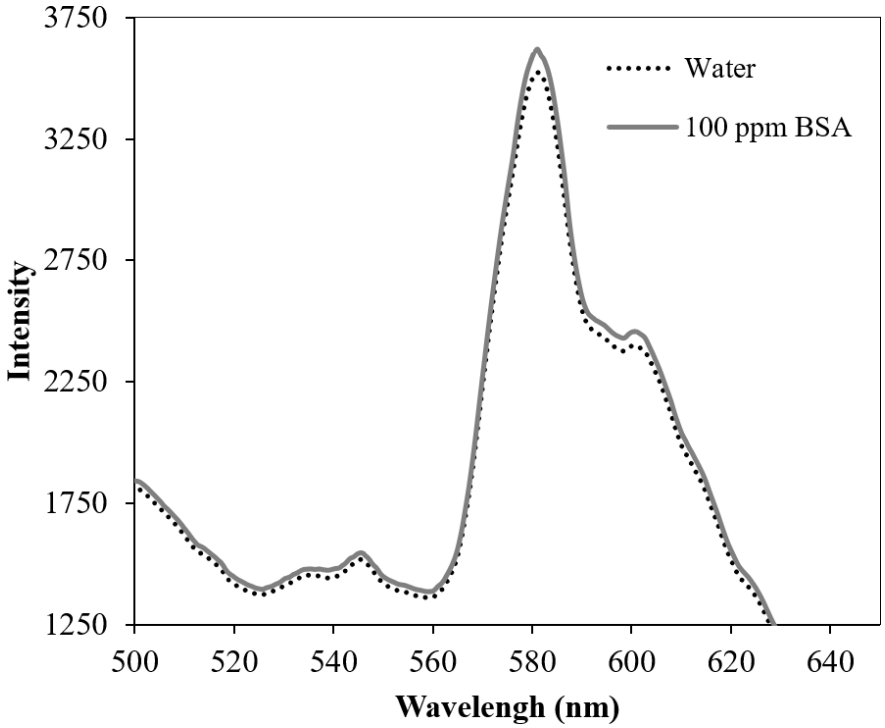

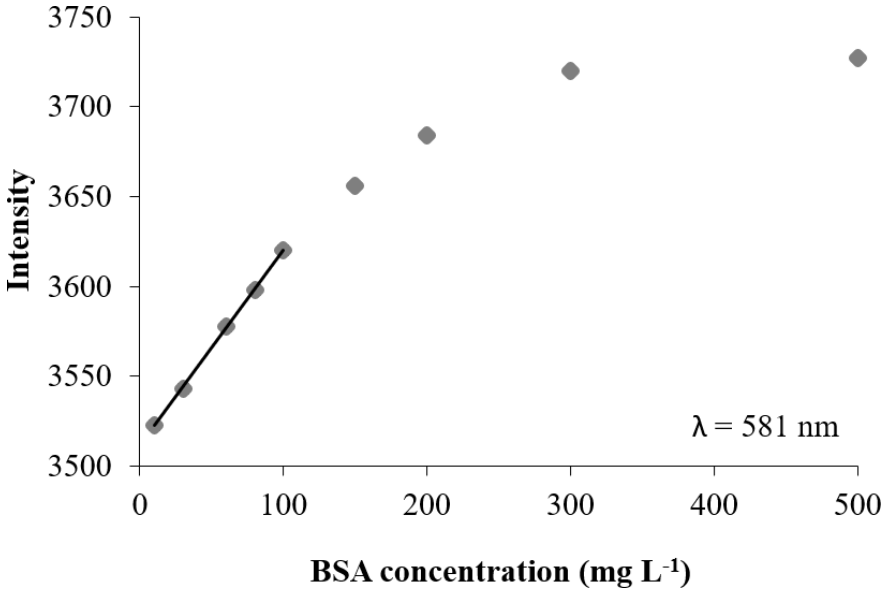

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