Graphene oxide platforms decorated with porphyrinoids and gold nanoparticles for optical detection of H$_2$O$_2$

João Carlos Teixeira Louro
Instituto Superior Técnico – Universidade de Lisboa

The possibility of optical detection of hydrogen peroxide using platforms of graphene oxide (GO) with gold nanoparticles (AuNP) and porphyrinoids was the target of the work. In order to facilitate the dispersion of GO in aqueous solution and to improve its interaction with AuNP and with porphyrinoids, tetrasulfonate porphyrin, TSPP, and tetrasulfonate aluminium phthalocyanine, AlPcS$_4$, a cationic polyelectrolyte (polyethyleneimine, PEI) and a cationic polysaccharide (chitosan, Chi) were employed. The interactions were investigated using UV/vis absorption, steady-state and time-resolved fluorescence. At pH 3.5, well-defined H- and J-aggregates of TSPP were detected, whereas, at pH 6.5 only monomers existed in solution. These aggregates were induced by the presence of the polymers and stabilized in the presence of both GO and AuNP. In opposition, no aggregation tendency was observed in the absence of the polymers, but a strong quenching effect was detected due to increasing concentrations of GO. In the case of AlPcS$_4$ no quenching effect was detected in presence of GO but, rather, an enhancement of the phthalocyanine fluorescence occurred upon addition of AuNP which was further improved when AuNP were deposited on GO sheets, indicating a possible metal-enhanced fluorescence (MEF) effect. The sensing experiments were performed using spectrophotometry with tetramethylbenzidine (TMB), with the assembled system acting as a catalyst in its reaction of oxidation in the presence of hydrogen peroxide. After an optimization using the natural catalyst HRP, the system revealed sensitivity to hydrogen peroxide in the range of 5 to 100 μM.

Keywords: Sensor; Graphene; Nanoparticles; Porphyrinoids; Aggregation; Spectrophotometry.

1 Introduction

Within chemical sensors, optical sensors are those in which the recognition of the analyte molecule is made through a signal transduction caused by an alteration of the optical properties of the sensing molecule leading to a signal that can be followed by photochemical methods like fluorescence or absorption depending on which property is affected. There has been research on optical sensing of a huge variety of analytes: from dissolved gases (e.g. hydrogen, oxygen, ammonia), ions, organic species, humidity, hydrazine or hydrogen peroxide. The last is the analyte of interest for this research. Hydrogen peroxide is a compound that is common in living beings. However, above certain levels it is known to be cytotoxic. Hydrogen peroxide is generated in vivo by the dismutation of the radical superoxide by enzymatic and non-enzymatic ways as well as being directly produced by oxidase enzymes. It is also present in food and beverages, from which is so also absorbed into the human body. H$_2$O$_2$ is also a product of the oxidation of glucose by the enzyme glucose oxidase being remarkably used for sensing of the former, mainly by electrochemical methods. Thus, the assessment of the hydrogen peroxide concentration is very important in areas diverse as food industry or biomedical sciences, both direct or indirect by the detection of compounds in which reaction is involved. The sensing of hydrogen peroxide became a very widespread field of research for the most sensitive, cheap and practical sensors. Recent developments in detection of hydrogen peroxide regard both electrochemical and optical sensors.

The optical detection of H$_2$O$_2$ has been carried out using horseradish peroxidase (HRP) as the catalyst of the reaction that involves the oxidation of the substrate tetramethylbenzidine (TMB). The latter is a colorless substrate which upon oxidation can form a stable blue-colored intermediate with absorption peaks at 370 and 652 nm.

Recently, several studies with the use of composites of carbon materials, metal nanoparticles, quantum dots, lanthanide-based nanoparticles, porphyrinoids as catalysts for the reaction in replacement of the enzyme HRP have been done leading to a promising value of this reaction that was then chosen to be included in this work. The development of the sensor based on the referred reaction was then viewed as possible to be carried out in a composite system with a carbon nanostructure, metal nanoparticles and porphyrinoids.

Since 2004, when Geim and Novoselov produced graphene by mechanical exfoliation that the interest in graphene and its properties highly grew due to their unique characteristics that can lead to applications on a wide range of areas of technology and research such as biomedical sciences, materials science and electronics. Some of the most important properties of graphene include high transparency, high mechanical strength and high conduction. Graphene oxide (GO) as a derivative of graphene that contains various oxygen groups is also interesting to study by the fact of maintaining most of the properties of graphene with the addition of being more water...
soluble which is relevant for the biocompatibility of its applications in biological sensing. The option for GO and its possible biological sensing application was made considering the high surface area of the material and the easiness to decorate its surface with more components to achieve a synergetic effect and hence, an improvement of the catalytic activity.

One of those materials are metal nanoparticles. The deposition of metal nanoparticles in GO and graphene was studied mainly for the cases of metals like Ag, Au, Pd and Pt. The main feature of the interaction between metal nanoparticles and the graphene layer is the fact that both have excellent electrons transfer properties. It is important to note that the optical properties of metal nanoparticles are dependent upon their size, interaction with other particles and medium condition\(^{14}\).

For the case of this work the main type of nanoparticles that is going to be used are gold nanoparticles (AuNP). The main feature of AuNP that can be used to follow the integration in sensing platforms is its characteristic band in the absorption spectra that results from its Surface Plasmon Resonance (SPR). Since this changes come from the presence of a molecule or an environmental condition that we want to detect this is very promising for the application. It was also verified that the formation of composites of graphene layers with metal nanoparticles leads to a clearer signal than the case of solely the nanoparticles\(^{15}\).

Nanoparticles have been studied and used in different applications, from biotechnology and targeted drug delivery to magnetic separation and photochemistry. One of the applications that has also gain interest in the last years is the use of metallic nanoparticles as a fluorescence enhancers (metal enhance fluorescence, MEF)\(^{16}\).

The exploration of photochemical features in the sensing targeted study would be achieved using two different types of photoactive molecules: porphyrins and phthalocyanines. Porphyrins are present in living organism in their reduced, oxidized and metalated forms playing an important role in several vital functions like oxygen transport and storage, photosynthesis, electron transport, drug detoxification or hydrogen peroxide biochemistry\(^{17}\). This importance comes from their special properties of absorption, emission, charge transfer and complex formation due to their characteristic aromatic ring. Another relevant aspect is that the cofactor of the natural catalyst HRP enzyme is a heme group, whose structure is a porphyrin.

A relevant feature of porphyrins is the formation of dimers or higher order aggregates, which is dependent on a combination of factors like pH, solvent composition and ionic strength, being a very important process in the study of these molecules. The porphyrin under stud in this work is meso-tetrakis(p-sulfonatophenyl) porphyrin, TSPP. TSPP is an anionic porphyrin due to the presence of sulfonatophenyl groups in the meso positions of the main aromatic ring. This porphyrin presents the special feature of forming pure J and H aggregates. These have a distinct arrangement of the monomeric units, being in a head-to-tail disposition for the J aggregate, whereas in the H aggregate the units are in a face-to-face alignment. The formation of such well aligned J aggregates is less common than that of H aggregates and it is promoted in acidic environments. Characterization of this porphyrin in a system with carbon nanotubes and a supporting cationic polyelectrolyte\(^{18}\) revealed the formation of stable J-type and H-type aggregates under certain conditions, which depended among other on the nature of the polyelectrolyte, its concentration and the solution pH.

Phthalocyanines have an analogous structure to porphyrins compounds since it is also a tetrapyroly macrocycle with the pyrrole subunits being linked by nitrogen atoms instead of methine bridges and having a benzene cycle coupled to the pyroles increasing the conjugation of the macrocycle. Unlike porphyrins they do not exist in nature. They form complexes with metals (e.g. aluminium, zinc, copper) that are used for dying and organic electronics besides being available in free form and with a variety of substituents in its \textit{meso} and \textit{beta} positions. The phthalocyanine used in this work is aluminium phthalocyanine tetrasulfonate (AlPcS4) which is complexed with the metal aluminium and presents as TSPP four sulfonate groups that give the molecule a tetra negative charge which will increase its solubility and will be determinant for the interaction with the charged polymers used.

Regarding the potential use of these components as sensors for \textit{H}_{2}\textit{O}_{2}, a detailed study of the interactions between them was made in aqueous solution and in deposit films using both spectroscopic and microscopic techniques. The role of the polymers in inducing the formation of J and H aggregates was detected for TSPP, but not for AlPcS4. Finally, the most suitable systems were chosen to follow up their potential as sensors for \textit{H}_{2}\textit{O}_{2} using optical detection.

2 Materials and Methods

2.1 Materials

Bidistilled water; Citric Acid (Sigma-Aldrich); Sodium Phosphate dibasic heptahydrate (Sigma-Aldrich); Sodium Acetate anhydrous(M&B Laboratory Chemicals); Potassium Permanganate (Analak); Sodium Tetrachloroaurate(II) (Aldrich); Chitosan, 110 000 g/mol (Sigma-Aldrich); Polyethyleneimine, 25 000 g/mol (Sigma-Aldrich), \textit{meso-tetrakis} (p-sulfonatophenyl) porphyrin – TSPP (Fluka, ≥ 96% purity); Horseradish Peroxidase (BBI Enzymes); Graphite powder, synthetic, conducting grade, -325 mesh, 99,9995% (Alfa Aesar), Aluminum phthalocyanine tetrasulfonate (Porphyrin Products Inc.)
2.2 Synthesis

GO was synthetized according to the modified Hummers method\textsuperscript{19}. For this, 2 g of graphite powder were suspended in 46 ml of concentrated sulfuric acid and left for 12 h under agitation. The suspension was then cooled until 0 °C and 6 mg of KMnO\textsubscript{4} were gradually added, after which it was subjected to sonication for 3 hours. After this last process 92 ml of distilled water were slowly added and it was left boiling for 30 minutes. In order to finish the reaction 10 ml of H\textsubscript{2}O\textsubscript{2} (30%) were added. The purification process consisted in removing the supernatant and adding 50 ml of HOI 5% and then water with the division of the product in several Falcon tubes -with maximum volume of 10 ml in each one – that were centrifuged 20 times (until the final pH was that of distilled water) with supernatant being removed after each centrifugation and distilled water added to fill the 10 ml volume. Only the last centrifugation was performed with the addition of ethanol. Afterwards, the obtained product was displaced in Petri dishes and dried to obtain the desired GO sheets.

AuNP were obtained through the Turkevich method\textsuperscript{20}. A solution of 46 ml of distilled water with 7 mg of gold salt was boiled and a solution of 13 mg of sodium citrate in 4 ml of water, at the same temperature, was added under agitation. The solution after changing the colour from transparent to red was brought to room temperature and stored in the dark.

2.3 Methods

Absorption spectroscopy measurements were performed in a PerkinElmer Lambda 35 UV/Vis spectrophotometer with a fluorescence quartz cell - normal and reduced volume- with an optical path length of 1 cm, at room temperature.

The steady-state fluorescence emission spectra were measured in a Fluorolog Tau-3 spectrophotometer with the same quartz cell as for the absorption.

The equipment used to measure fluorescence decays was a HORIBA Jobin Yvon IBH FluoroLog-3 spectrophotometer adapted time-correlated single photon counting (TCSPC) equipped with a Hamamatsu R928 photomultiplier tube. The samples analysed in this equipment were excited with nanosecond pulses of 445 and 594 nm generated by NanoLED pulsed diodes (Horiba). Emission decays were analysed using the software DAS6 v6.4.

FLIM was performed with the confocal microscope MicroTime 200 (MT200) from PicoQuant which uses the time-correlated single-photon counting (TCSPC) technique. The source of excitation was a pulsed picosecond laser diode with a wavelength of 635 nm.

TEM images were obtained with a Hitachi 8100, 200 kV, LaB\textsubscript{6} filament analytical transmission electron microscope with ThermoNoran model SystemSix energy dispersive X-ray spectrometer (EDS) with light elements detector and digital image acquisition.

3 Results and Discussion

3.1 Characterization of synthesized material

The AuNP synthetized were subject of observation through Transmission Electron Microscopy to evaluate the shape and size. As seen in the image obtained the nanoparticles reveal the expected spherical shape with a reasonable homogeneity in terms of size. Through software analysis of the TEM images it was determined an average size of 17.5 ± 0.25 nm. An absorption spectrum of the synthetized solution was also performed (Figure 1) revealing its plasmonic band c.a. 525 nm. Taking into account these results, an estimate of the extinction coefficient could be obtained ($e_{525}$=8x10$^8$ M$^{-1}$cm$^{-1}$) and was used to assess the concentration of AuNP in solution\textsuperscript{21,22}.

![Figure 1 – TEM image and absorption spectrum of synthetized AuNP solution](image)

Proceeding in the same way with GO, a TEM image shown below (Fig. 2) was obtained revealing in a half micrometer scale a darker region that corresponds to the presence of the carbon material lattice where the observed wrinkled structure confirms the presence of few-layer GO. The absorption spectrum of GO is depicted in Figure 2-B revealing the main band (c.a. 230 nm) corresponding to electronic transitions in the carbon-carbon double bonds and a shoulder (c.a. 300 nm) from the carbon-oxygen bonds.

![Figure 2 - TEM image of synthetized GO sheet (A) and UV-Vis absorption spectrum of GO dispersed in distilled water (B)](image)

The systems analyzed started with TSPP and involved the presence of the polysaccharide Chitosan and the polyelectrolyte polyethylenimine (PEI) in pH 3.5 and 6.5. A concentration of 2 µM of TSPP was used for all the recorded spectra. Figures 3 and 4 present the absorption and
emission spectra of TSPP in the presence of increasing amounts of chitosan at pH 3.5.

In both absorption and fluorescence spectra, it is clear that the increasing concentration of the polysaccharide leads to the formation of J-aggregates revealed by the increasing intensity of the 494 nm band in absorption and by the splitting of the fluorescence spectra in two bands with a progressive decrease in intensity due to the less fluorescence of the formed aggregates. However, it is verified by the presence of a small band that for the null concentration of chitosan a small amount of aggregate is already present. At the lower concentrations of added chitosan, the Soret band peak shifts to the blue (~400 nm) which concomitantly decreases upon further addition of chitosan followed by the increase of the 494 nm band. The blue-shifted Soret band has been assigned to the formation of an H-aggregate (probably a dimer). This small aggregate is the first arrangement stabilized by the presence of small amounts of chitosan which upon further addition of the polysaccharide, grows and changes to a head-to-tail arrangement. Fluorescence lifetimes were also obtained under these conditions. In the case of the monomer (absence of chitosan) a lifetime of 3.84 ns was obtained, which is in agreement with available data for similar conditions. The following lifetimes reveal the need for a three exponential fit that leads to three time components: a short one in the sub-nanosecond range (varying between 0.1 and 0.6 ns) corresponding to the aggregate, a middle one that varies between 3.7 and 3.9 ns (assigned to the monomer), and a long component of 11 ns which is a value that is shown in literature attributed to a formed complex.

As shown figures 5 and 6 the inclusion of GO and AuNP had no effects in terms of the aggregate promoted by chitosan that maintains stable with the only modifications in the absorption spectra being originated by the increasing scattering.

Alongside with the use of chitosan, experiments involving polyethyleneimine were also carried out.

As shown figures 5 and 6 the inclusion of GO and AuNP had no effects in terms of the aggregate promoted by chitosan that maintains stable with the only modifications in the absorption spectra being originated by the increasing scattering.

Alongside with the use of chitosan, experiments involving polyethyleneimine were also carried out.

As shown figures 5 and 6 the inclusion of GO and AuNP had no effects in terms of the aggregate promoted by chitosan that maintains stable with the only modifications in the absorption spectra being originated by the increasing scattering.

Alongside with the use of chitosan, experiments involving polyethyleneimine were also carried out.

As shown figures 5 and 6 the inclusion of GO and AuNP had no effects in terms of the aggregate promoted by chitosan that maintains stable with the only modifications in the absorption spectra being originated by the increasing scattering.

Alongside with the use of chitosan, experiments involving polyethyleneimine were also carried out.

As shown figures 5 and 6 the inclusion of GO and AuNP had no effects in terms of the aggregate promoted by chitosan that maintains stable with the only modifications in the absorption spectra being originated by the increasing scattering.

Alongside with the use of chitosan, experiments involving polyethyleneimine were also carried out.

As shown figures 5 and 6 the inclusion of GO and AuNP had no effects in terms of the aggregate promoted by chitosan that maintains stable with the only modifications in the absorption spectra being originated by the increasing scattering.

Alongside with the use of chitosan, experiments involving polyethyleneimine were also carried out.

As shown figures 5 and 6 the inclusion of GO and AuNP had no effects in terms of the aggregate promoted by chitosan that maintains stable with the only modifications in the absorption spectra being originated by the increasing scattering.
The behavior of the system with the addition of PEI is similar to the previous case with chitosan. Upon the addition of the polycation the formation of H-aggregates followed by that of J-aggregates is promoted. In the fluorescence spectra as previously seen, there is also a clear quenching of the fluorescence with increasing concentrations of the polyelectrolyte. The obtained life-time for the monomer in the absence of the polyelectrolyte is 3.9 ns, which is again in agreement with what was determined before in the chitosan system. Moreover, upon addition, there is an increase of the complexity of the decay, with a long lifetime of 11 ns corresponding to the complex as well as a short lifetime around 0.2 ns that as before is ascribed to the J-aggregate. A lifetime that varies between 8 and 2 ns with the concentration of PEI may be attributed to the formed H-aggregate, considering previous studies. These aggregates are non-fluorescent or have a very low yield of fluorescence.

Upon the addition of GO and AuNP the effects were similar for both polymeric systems, always potentiating the stabilization of J-aggregates. The same happened in the presence of the hybrid. The expected quenching of the TSPP fluorescence was detected with the addition of GO.

3.3 Polymer Interaction Kinetics

Since it was verified an aggregation process of TSPP both in the presence of chitosan and PEI, a kinetic study of the aggregation was performed with both polymeric systems. To evaluate the sensitivity of the system to the order of addition of the components, two measures were performed for each system, with the addition of TSPP to the polymeric system and vice-versa. The spectra for the kinetics of aggregation of TSPP promoted by PEI with both addition methods are shown below. Similar results were obtained with chitosan. (data not shown)

With the obtained spectral data, kinetic curves were built plotting the absorption of TSPP at the wavelength of the formed J-aggregate along time, for both addition methods.

The kinetic curves were adjusted through the use of kinetic models, described in the graph as Method 1 (eq.1) and Method 3 (eq.2), both obtained from the literature.

$$\text{OD} = E_m[M_0]e^{-kt} + E_J[M_0](1 - e^{-kt}) + E_J[J]$$ (1)

where $E_m$ and $E_J$ are the extinction coefficients for the monomer and aggregate respectively, at the aggregate peak, $k$ is the rate constant of the aggregation and $[M_0]$ and $[J]$ are the initial concentration of monomer and the formed concentration of aggregate, respectively. As for the other model, an induction time involving the formation of a critical nucleus (the rate-determining step) has to be accounted for,
OD = OD_{\infty} + (OD_0 - OD_{\infty})((1 + (m - 1)(k_q t + (n + 1)^{-1}(k_c t)^{n+1}))^{1/(n+1)} (2)

where OD_{\infty} is the absorption of the aggregate in the final, and the others are kinetic parameters: k_q is the rate constant for the uncatalyzed growth, k_c is the rate constant for the catalytic pathway; n is a parameter that describes the growth of the chromophore assembly as a power function of time and m, which is a parameter related to the size of the critical nucleus.

The fitted parameters for the models used (Method 1 for PEI-TSPP and Method 3 for TSPP-Chitosan) are displayed next:

**Table 1 - Kinetic parameters obtained for method 1, where TSPP was added to a PEI (Chitosan) solution**

<table>
<thead>
<tr>
<th>Polymer</th>
<th>E_a (M^2 cm^−2)</th>
<th>E_0 (M^2 cm^−2)</th>
<th>k (s^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEI</td>
<td>3E-09</td>
<td>4E+04</td>
<td>3E-04</td>
</tr>
<tr>
<td>Chi</td>
<td>2071</td>
<td>4E+04</td>
<td>5E-04</td>
</tr>
</tbody>
</table>

**Table 2 - Kinetic parameters obtained for method 3, where PEI (Chitosan) was added to a TSPP solution**

<table>
<thead>
<tr>
<th>Polymer</th>
<th>m</th>
<th>k_q (s^-1)</th>
<th>n</th>
<th>k_c (s^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEI</td>
<td>27^*</td>
<td>1E-05</td>
<td>0.02</td>
<td>5E-02</td>
</tr>
<tr>
<td>Chi</td>
<td>2</td>
<td>1E-05</td>
<td>2</td>
<td>8E-05</td>
</tr>
</tbody>
</table>

*The value of m is extremely high, which together with the low value obtained for n (<<1) indicates the existence of covariance between the two values. Therefore, we cannot take any information from such values and the remain kinetic parameters have to be viewed as indicative of its order of magnitude.

The same kinetic models described well the system with chitosan, which demonstrates a resemblance in the kinetic behavior of the aggregation process in the presence of both polymeric systems. When the polymer is added to the TSPP solution there is a lag time until the absorption of the aggregate starts ascending quickly, in opposition to the inverse addition in which this is immediate. This is in accordance with available kinetic studies with TSPP and it originates from the fact that when TSPP is pipetted to the bulk solution it is highly concentrated and immediately enters in contact with aggregating medium, whereas for the inverse addition the porphyrin is already diluted in solution. Nonetheless, the values obtained for PEI added first display a certain covariance and its physical meaning is not easily explainable. For the system in which the template is added second, chitosan leads to a faster aggregation kinetics.

### 3.4 Porphyrin without Polymer Systems

Since it was verified that the polymers used promoted the aggregation of TSPP, an analysis without their presence was made to verify how the systems would behave. This experiment was made at acidic pH since it is the medium in which the aggregates occurred.
regions upon the addition of the polymer (C) and the existence of blue spots distributed around the shown graphene sheets (D) that correspond to short lifetimes. The inset D' shows a formed J-aggregate. This is in accordance with previous fluorescence analysis in which it was verified that the addition of chitosan to the porphyrin at pH 3.5 induced the formation of J-aggregate, with a short lifetime as well as the fact that the presence of GO in the system quenched the fluorescence leading to a shorter average lifetime.

3.5 Phthalocyanine Systems

For the systems with phthalocyanine the interaction with the polymeric systems was weaker than with TSPP and the inclusion of the AuNP and GO didn’t lead the system to any particular feature. In turn, an important Metal Enhanced Fluorescence (MEF) effect was observed with the addition of AuNP. This effect was more prominent in the system with all the components in the presence of chitosan.

![Figure 14 - Stem-Volmer plot for the quenching of fluorescence](image)

From the fit the following data was retrieved: a value of 7 ml mg⁻¹ for \( K_a \) for \( k_q \) of 10⁹ ml mg⁻¹ s⁻¹ and finally a value for \( K_{SV} \) of 4.7 ml mg⁻¹.

In the same system with addition of nanoparticles, it is possible to distinguish the plasmonic band of the nanoparticles in terms of absorption. There was a small quenching (compared to GO) observed in the fluorescence spectra which wasn’t a novelty in comparison to the studied in the presence of the polymers.

3.6 FLIM Characterization

The first system here analyzed is the one of the porphyrin with chitosan and GO. As seen in Fig. 13-A in the lifetime distribution upon the addition of each component to the system the average lifetime decreases. The FLIM images on the right show this by the appearance of darker

![Figure 15 - Fluorescence spectra of AlPCS4 in aqueous solution in the presence of Chitosan, GO and increasing concentrations of AuNP (λex = 600 nm)](image)

Comparatively to the system with only AuNP present, the hybrid system shows a more pronounced effect which highlights the contribution of GO to the process. The MEF effect depends on the distance between the NP and the fluorophore and this distance may be better controlled when the AuNP are deposited in the GO sheet.

![Figure 16 - Average fluorescence lifetime distribution (A) FLIM images (B, C, D) obtained from decay analysis of about 20 point measurements of cast drop solutions at pH=3 of TSPP (B), TSPP-CHI (C) and TSPP-CHI-GO (D) (with inset D' of image D)](image)

For the equivalent system with PEI, the results are similar with the novelty being the fact that upon the addition of the polyelectrolyte there is no great reduction of the average lifetime, only the enlargement of the distribution. This fact is coherent with the fact that in the lifetime analysis it was identified the molecular complex caused by the strong interactions between the porphyrin and PEI that produces a long lifetime at the same time as the referred J-aggregate produces a short lifetime, with the distribution being enlarged. For the presence of GO the effect is similar to the last analysis. The imaging shows the loss of uniformity with the presence of PEI (Fig. 14-C) and pictures, in the presence of GO, two of its sheets: one with a lighter blue color revealing high presence of quenched TSPP in it, and a greener sheet where the effect is not so clear.

![Figure 17 - Average fluorescence lifetime distribution (A) FLIM images (B, C, D) obtained from decay analysis of about 20 point measurements of cast drop solutions at pH=3 of TSPP (B), TSPP-PEI (C) and TSPP-PEI-GO (D)](image)

The choice for catalyst was the system of TSPP without polymer at acidic pH. The porphyrin instead of the phthalocyanine comes from the stronger interaction with the other components of the system (i.e. GO and AuNP) in comparison to the phthalocyanine. The discard of the polymeric systems is based on the observed aggregation that reduced the fluorescence of the system while
the option for an acid pH is determined by the optimum medium for the occurrence of the sensing reaction.

3.7 Sensing Analysis

After an optimization study with the natural catalyst HRP that established the usage of TMB and \( \text{H}_2\text{O}_2 \) concentrations of 100 and 50 µM, one of the first sensing tests consisted in the evaluation of a simple system with TSPP at a concentration of 1 µM and a fixed concentration of 100 µM of TMB. The concentration of hydrogen peroxide was varied between 5 and 100 µM and, as seen below, it is noticeable a tendency of increasing of the absorption at the main peak of the oxidized TMB with the concentration of \( \text{H}_2\text{O}_2 \) which shows the peroxidase-like catalytic ability of TSPP. Since this analysis was made in the micromolar range this reveals a good sensitivity that could lead to a promising sensing application.

![Absorption peak at 652 nm value for oxidation of TMB in the presence of increasing concentrations of \( \text{H}_2\text{O}_2 \)](image)

Using the procedure of letting the reaction to happen overnight, the system was tested with all the components studied before. We started by exploring the pH effect upon the system, pertinent considering the results of our studies above which involved two charged species (TSPP and GO) with pKa in the range of 4 to 6. Besides, the catalytic activity of HRP was also reported to be dependent on the solution pH.

![Spectra of oxidized TMB in the presence of \( \text{H}_2\text{O}_2 \) using (A) GO and (B) TSPP as catalysts after 21 hours at different pHs (blue – pH 4.4; orange – pH 5; grey – pH 5.6).](image)

The solution pH has a significant effect on the catalytic ability of both GO and TSPP. Another curious pH effect was detected when testing the catalytic ability of TSPP working at pH 3. Under such conditions the formation of J-aggregates was induced which was completed in the first 3 minutes of the reaction. Afterwards, an increase of the band corresponding to the oxidized TMB occurred. This can be understood by the role of the amino groups of TMB that stabilize the in-line aggregate through a hydrogen bond network which acts between the anionic sulfonate groups and the charged protonated nitrogen atoms of the porphyrin. This must be similar to the effect reported above for PEI and chitosan.

Considering these results, pH 4.4 was selected for the posterior tests. A preliminary study using just one of the components, TMB or \( \text{H}_2\text{O}_2 \), showed that in all cases the reactions only took place when both were present. Temperature is known to also influence the reaction, therefore it was kept constant for all experiments at 25 °C.

It is shown in Fig. 17 the system without the porphyrin, in which the oxidation is achieved in all cases. Nonetheless, the system composite of GO and the AuNP was the most efficient, which highlights the synergistic effect of the interaction between the two components.

![Absorption spectra for the oxidation of TMB in the presence of \( \text{H}_2\text{O}_2 \) and GO and gold nanoparticles alone or in composite](image)

Proceeding to the analysis with the porphyrin, Fig. 18 shows that the reaction occurred in the presence of the porphyrin, as shown already, and that the addition of AuNP led to an enhancement of the signal of the reaction, again pointing out the synergistic effect of the interaction between the two components. A strange result was the non-occurrence of significant reaction in the presence of GO that is not in accordance with all the remaining data. The interaction TSPP – GO seems to prevail and therefore, none of the two components is available for the oxidation of TMB.

![Absorption spectra for the oxidation of TMB in the presence of \( \text{H}_2\text{O}_2 \) and TSPP without and with AuNP or GO](image)

4 Conclusions

The spectroscopic analysis revealed that the presence of the chitosan and PEI promoted the aggregation of TSPP in its acid form, leading to the
formation of mainly J-aggregates, with H-aggregates being also noticed in the presence of PEI. A strong interaction between the porphyrin and the polymers was translated in the appearance of a molecular complex that was noticed by the red-shift in the spectra and the presence of a long lifetime in the fluorescence decay analysis. With the addition of GO there is a quenching of the fluorescence. The addition of only AuNP produced little effect on the spectra with exception of the acid form of TSPP in the presence of chitosan. For the composite with the nanoparticles and the carbon material it was also shown nothing more than a small quenching of the fluorescence of the porphyrin with the increasing volume of nanoparticles, for all cases. The kinetics of TSPP aggregation process induced by polymers was analyzed using distinct aggregation kinetic models. Curiously, the aggregation kinetics of TSPP was not particularly affected by the nature of the template used (PEI vs. chitosan) but showed a remarkable dependence on the order of mixing components.

In the phthalocyanine spectrophotometric analysis, only PEI seems to have a clear effect in the spectral properties of AlPcS4, where the blue-shift of the absorption spectra together with the appearance of a shorter lifetime, indicated the formation of non-specific aggregates. GO alone is known to have almost no effect on the spectral features of the phthalocyanine due to very effective electrostatic repulsions. However, in the presence of chitosan an efficient quenching of the dye’s fluorescence took place. In the presence of AuNP at their highest volumes an increasing in fluorescence that was attributed to a possible Metal Enhanced Fluorescence effect. This effect was even more pronounced for the systems with all the components (GO-AuNP-polymer). These results highlight the importance of GO in the support and distance control of the AuNPs and the dye. This was a clear case of a synergistic effect gained from the combination of both nanomaterials.

Evaluating the system with H2O2 and TMB in the presence of the natural catalyst HRP, the optimal conditions in terms of reaction signal and velocity were achieved with concentrations of 100 μM of TMB and 50 μM of H2O2. An experiment with increasing concentrations of hydrogen peroxide in between 5 and 100 μM in the presence of TSPP and TMB lead to a response of the system with an increase of the signal of the TMB oxidation that showing a sensitivity of the system to the analyte. This is a good indicator to the possibility of the building of a sensor in that range of concentrations. However, the reaction of oxidation of TMB was very slow with the synthetized composites and the experiments involving the reaction occurred overnight. It was tested the easing of the reaction upon irradiation of the reaction solution making it occur quicker but with lower intensity. Experiments with the other components were performed, revealing the occurrence of the reaction except for the TSPP/GO system. TSPP/AuNP and GO/AuNP revealed to be the most efficient systems.

It was possible to achieve great information about the behaviour of the interaction of the system with the porphyrin and the phthalocyanine in the presence of GO and nanoparticles. The MEF effect is of paramount importance in the field of bioimaging and cancer therapy either by exploring its potential in photodynamic therapy (due to the presence of the porphyrinoid sensitizer) or the photothermal effect provided by the presence of AuNP. Another important indication from this work was the reinforcement of the potential peroxidase-like activity of the hybrid, which indicate the possibility of the usage of the studied system to build a molecular sensor for hydrogen peroxide. This field is of great importance considering the possibility to be extended to measures of glucose levels with improved sensitivity. The combination of GO and AuNP provide several advantages as compared to others, which include: their extremely large surface area and the extended possibilities of further functionalization (e.g. with porphyrinoids: biomarkers, etc.); they are robust and stable system; easy to prepare and to purify; and their cost is lower than those with the natural peroxidases.

REFERENCES


