Trabajo de Fin de Máster

First steps towards the design of an enzymatic optical (nano)biosensor for the determination of glucose.

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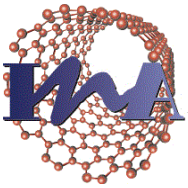

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Abbreviation

AuNCs	Gold nanoclusters
AuNPs	Gold nanoparticles
BSA	Bovine Serum Albumine
UV-Vis-EAM	Ultraviolet visible absorption spectroscopy
FAD	Flavin Adenin Dinucleotide
GOx	Glucose Oxidase
HRP	Horseradish Peroxidase
PBS	Phosphate buffer solution
STEM	Scanning transmission electron microscopy
TEM	Transmission electron microscopy
TRIS	Tris(hydroxymethyl)aminomethane

Summary

This study describes the first steps towards the design of an enzymatic optical (nano)biosensor for the determination of glucose. The aim of this work is to relate the optical properties of gold nanomaterials with glucose concentration. In order to achieve this goal, the redox properties of FAD cofactor of the glucose oxidase enzyme were used. During the enzymatic reaction between glucose oxidase and glucose, the FAD cofactor is reduced to $FADH_2$. A gold precursor in oxidation state 3+ is used to reoxidize the enzyme which at the same time is reduced to gold in oxidation state 0. Gold in oxidation state 0 can be developed into two different types of nanomaterials: gold nanoparticles (AuNPs) or gold nanoclusters (AuNCs). These nanomaterials have different optical properties which could be related to the glucose concentration. On the one hand AuNPs exhibit absorption properties due to surface plasmon resonance (SPR). On the other hand AuNCs are composed by only few atoms, its size is around 2 nm or less and displays fluorescence. The intensity of SPR of gold nanoparticles has been successfully related to the glucose concentration. In addition, kinetics studies have shown a relationship between the velocity at which the fluorescent intensity of AuNCs increases and the glucose concentration.

1. Introduction

1.1. Brief definition of Optical Chemical Sensor and Biosensor.

A chemical sensor is a device that transforms chemical information about a compound (the analyte) into useful analytical information. The International Union of Pure and Applied Chemistry define biosensor as: "*A device that uses biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals*".

If the transformation of the chemical information into useful chemical information is based on the optical properties of the sensor, it is named optical chemical sensor. This transformation is carried out following the next steps. First in the recognition step, the analyte reacts selectively with a chemical compound (the receptor) giving a specific product. Then in the primary transduction step, a primary signal (optical, electrical, mass, magnetic...) is produced. The chemical compound responsible for the primary signal is called the indicator. Later in the secondary transduction step, a secondary transducer converts the primary signal into an electrical signal that is the input for the rest of the sensor (amplifier and signal presenting device). In optical sensors, the secondary transducer is called detector. Finally in the regeneration step, the device is brought to the initial chemical conditions and prepared for another cycle of reaction with the analyte [1]. The IUPAC also consider the single-use sensors describing those devices which can also be used for one measurement.

1.2. Enzymatic reaction between Glucose Oxidase and glucose.

For decades, the determination of glucose is being one of the most important analytical tasks. It has been estimated that 40 % of the blood test are made for glucose quantization. In biotechnology and biochemistry, it is also determined in the production of feed and food. [2]. Despite many reagents have been proposed for this determination, up to date the more reliable results have been obtained with those methods (batch or sensor) based on its enzymatic reaction with Glucose Oxidase (GOx).

GOx is a flavoenzyme which catalyzes the oxidation of glucose to gluconic acid utilizing molecular oxygen as final electron acceptor and by producing hydrogen peroxide as product. [2] For the aim of this work it is very important to briefly review the kinetic of this reaction.

The reaction between GOx and glucose can be divided in two half reactions, a reductive and an oxidative one. In the reductive half reaction, GOx oxides glucose to gluconolactone (which is non-enzymatically hydrolized to gluconic acid) while the

FAD group of the GOx becomes reduced to $FADH_2$. In the oxidative half reaction, the reduced GOx ($FADH_2$) is reoxidized by oxygen to produce hydrogen peroxide (H_2O_2). [3]. In Figure 1. a scheme of GOx reaction is shown.

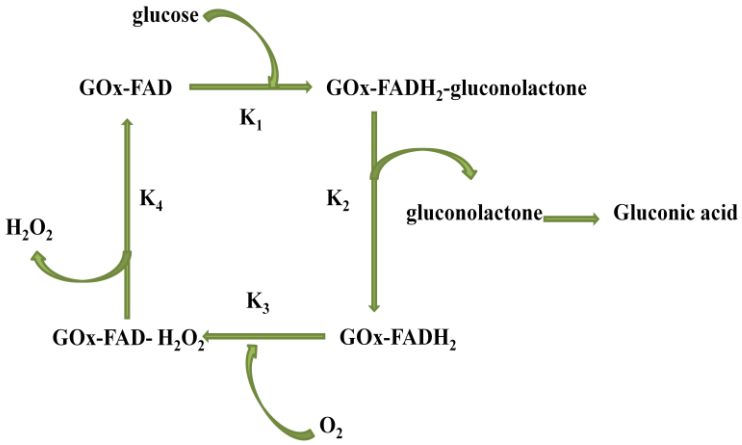


Figure 1. Scheme of GOx reaction.

For the purpose of this work, this mechanism can be simplified to a two step process:

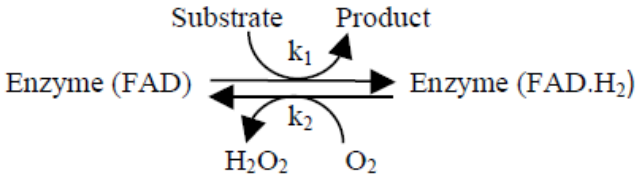


Figure 2. Simplified kinetic mechanism of GOx and glucose reaction. [1]

Taking into account the k1 and k2 values (k2>k1), it can be deduced the oxidation state of GOx during the enzymatic reaction. The change in the concentration of $GOx\text{-}FADH_2$ throughout the reaction is shown in Figure 3.

$GOx\text{-}FADH_2$ concentration depends on the O_2 concentration in solution which is, at the same time, depended on the analyte concentration, and that $[GOx\text{-}FADH_2]max$ does not depend on the glucose concentration but is related to the time at which the change in the oxidation state stars to take place-t_a [4].

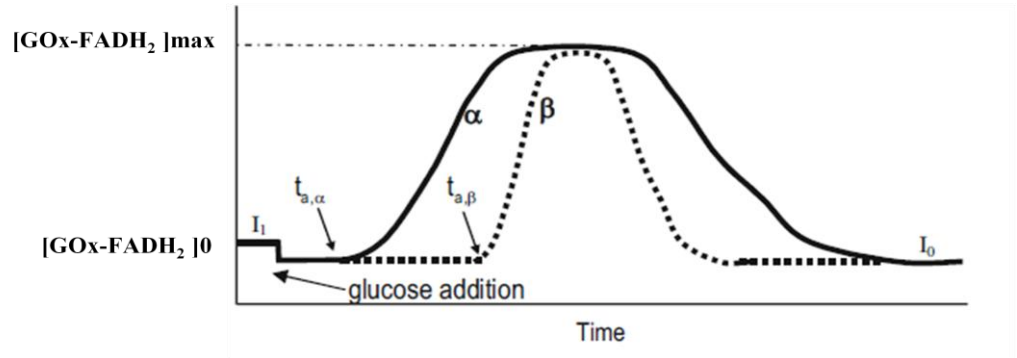


Figure 3. Variation of the reduced form of GOx concentration during the reaction with glucose. (Adapted from ref. 4.)

1.3. Optical methods based on intrinsically fluorescent enzymes, co-enzymes or co-substrates for glucose determination.

Nowadays methods to determine glucose take advantage of the optical properties of intrinsically fluorescent enzymes, their co-enzymes and co-substrates or the measurement of the products of the enzymatic oxidation of glucose by GOx.

Among the most commonly used fluorescence methods there are kinetic methods that measure fluorescence derivatives of the products of the enzymatic oxidation of glucose and GOx [2]; methods based on the consumption of oxygen [5], the production of hydrogen peroxide [6] or the production of protons [7](due to the formation of gluconic acid from gluconolactone) during the enzymatic reaction. Typical probes for oxygen use luminiscent complexes of ruthenium, platinum or palladium which are strongly quenched by oxygen [2].

But there are also a series of fluorescence methods that take the advantage of the intrinsically fluorescent enzymes[8][9]GOx contain aromatic aminoacids as phenylalanine, tyrosine and tryptophan. The presence of these aminoacids gives GOx its intrinsic fluorescence [4]. The coenzyme FAD also displays fluorescence and its spectroscopic properties depends on its oxidation state and the environment [11]. The variation of the fluorescence is due to a differential energy transfer between trytophan and FAD or $FADH_2$. Tryptophan groups transfer more energy to FAD than to $FADH_2$. FAD is the initial form of the enzyme, during the reaction an increase (when $FADH_2$ is formed) and a later decrease (when FAD is regenerated) is observed.

However, the drawback of these methods is that the measurements has to be carried out in spectral areas where a large number of organic compounds have absorption an emission maximums, so real biological samples require pretreatment. In order to overcome this drawback, linking of the GOx to fluorophores, such as fluorescein [c], have been used and more efficient methods for the development of reversible optochemical sensors have been developed[11].

1.4. Methods based on Gold Nanoparticles for glucose determination.

Gold Nanoparticles (AuNPs) are suitable materials for determine biomolecules by a reaction with an enzyme. AuNPs have a strong absorption band due to the surface plasmon resonance (SPR), strong Rayleigh scattering, electric conductance and reversible redox behavior. They exhibit large surface areas and their surface can be modified with molecules through covalents bonds.

From the point of view of analytical determination and sensors, the UV-vis surface plasmon band have been used. Different schemes have proposed, the following three being the most interesting:

1. The reaction of enzyme with functionalized metallic NPs results in the assembled and disassembled states of metallic NPs.
2. The reaction of enzymes with free substrates generates thiol-containing products, which leads to the aggregation of metallic NPs.
3. The enzyme-catalyzed oxidation of free substrates generates the product, which induces the growth of metallic nanoparticles. For example, hydrogen peroxide, thiolcholine, nicotamide adenine dinucleotide and dopamine catalyzed growth of AuNPs and increases the intensity of localized SPR [12].

However, as far as we know methods based in the AuNP formation during the enzymatic have not been proposed to date.

1.5. Gold Nanoclusters: properties, synthetic methods and biosensing applications.

Gold Nanoclusters (AuNCs) are gold nanomaterials with a size smaller than 2-3 nm. It is known that Gold Nanoparticles exhibit surface plasmon resonance (SPR) however AuNCs do not have this optical property [13]. Their properties are due to their subnanometric dimensions, which are comparable to the Fermi wavelength of the electron. They bridge the gap between atoms and metallic nanoparticles. The spatial confinement of free electrons in metal NCs provide size-tunable electronic transitions giving them specific properties as fluorescence with long lifetime and large Stokes shift [14]. Optical properties of AuNCs depend on cluster size and aggregation of clusters quenches its fluorescence.

Nowadays different methods, as chemical reduction, photoreduction or chemical etching, are used to synthesized AuNCs.

In chemical reduction AuNCs are prepared through the reduction of Au^{3+} in presence of reducing and capping agents. Thiol compounds are often used as capping agents due to the strong bond formed between gold and sulphur. With thiol as capping agents, $NaBH_4$ is commonly used as reducing agent. Biomolecules as proteins can be used as scaffolds in order to induce the nucleation and growth of AuNCs. A reducing agent as $NaBH_4$ is not necessary as long as some aminoacids can act as reducing agents. One of the most used protein is BSA. The cysteine and histidine residues in BSA can coordinate with Au^{3+} and tyrosine residues reduce Au^{3+} ions to form AuNCs. In order to obtain strong reducing strength of the tyrosine residues, AuNCs synthesis must be do at a pH

value>10, above the pKa of tyrosine [15]. Horseradish Peroxidase (HRP) and Hemoglobin have been also used as capping and reducing agents for AuNCs synthesis. [16] [17]

In chemical etching a large nanoparticle is converted to a nanocluster with few atoms. GOx functionalised with AuNCs can be synthesized by this method [18]. First AuNPs are synthesized and functionalised with phosphonium groups. Then this AuNPs are etched by a bioconjugate composed by GOx and thiotic acid and AuNCs are formed.

Fluorescence AuNCs are gradually emerging as promising materials for sensing biomolecules with enzymatic reaction due to their small size, size-dependant emission wavelength, low toxicity, water solubility and photostability [12].

As has been previously said, cluster aggregation causes quenching in AuNCs fluorescence. In the literature it is possible to find examples of how H_2O_2 induce cluster aggregation and therefore quenching in AuNCs fluorescence. This fact has been used to determine glucose as long as H_2O_2 is a product of the enzymatic reaction of GOx and glucose.

In GOx-functionalized AuNCs the enzyme maintains intact its activity and when glucose is added and H_2O_2 is formed. The fluorescence quenching is proportional to the H_2O_2 concentration and hence to the glucose concentration[18].

The quenching in the fluorescence is due to the evolution of the small clusters to larger ones in the presence of H_2O_2. X-ray photoelectron spectroscopy (XPS) studies of AuNCs says that gold in two different oxidation states are present in AuNCs. Au(0) in the core and Au(I) on the surface. The oxidized state Au(I) on the cluster surface would be reduced to Au(0) in the presence of H_2O_2 because H_2O_2 can reduced oxidized-state gold to atoms [17].

Horseradish Peroxidase (HRP) functionalised AuNCs showed fluorescence with λ_{ex}=365 nm and λ_{em}=650, while accompained with a weak peak λ_{em}=450. When H_2O_2 was added or produced by the reaction of GOx and glucose, notably the fluorescence at 450 nm increase while the peak at 650 nm completely disappeared [16].

Hemoglobin-capped AuNCs showed fluorescence with λ_{ex}=365 nm and λ_{em}=450. On the basis of an aggregation-induced fluorescence quenching mechanism can detect low levels of Cu^{2+}. The fluorescence can be recovered by adding hystidine [17].

2. Objectives

In this work, we began to evaluate the possibility of forming NP or NC during glucose/GOx enzymatic reaction and using the spectroscopic properties of these nanomaterials as analytical signals.

The final goal of this work is to design a nanobiosensor by relating the optical properties of gold nanomaterials with the glucose concentration. For this issue, the growth of gold nanomaterials is carried out by using a gold precursor with gold in 3+ oxidation state and the enzymatic reaction between GOx and glucose as reducing agent. As has been indicated, during the enzymatic reaction O_2 reoxides $FADH_2$ to FAD. The hypothesis set out in this work is to use Au(III) as oxidizing agent with its consequent reduction to Au(0) (see Figure 4.).

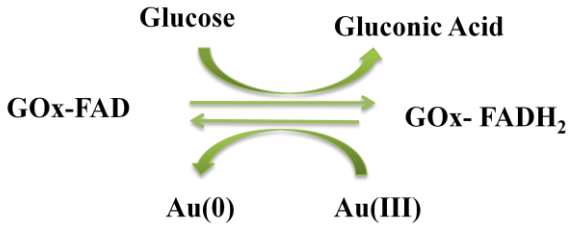


Figure 4. Scheme of gold nanomaterials growth using Au(III) as gold precursor and the reaction of GOx and glucose as reducting agent.

In order to achieve this goal two different but complementary procedures were tested:

- AuNPs free growth using Au(III) as precursor and the enzymatic reaction between GOx and glucose as reductive agent. AuNPs exhibit the optical property of SPR which could be related with the glucose concentration.
- AuNCs growth using Au(III) as precursor, with BSA as the growth restrictingagent, and the enzymatic reaction between GOx and glucose as reductive agent. AuNCs exhibit the optical property of fluorescence which could be related with the glucose concentration.

3. Methods

3.1. Instrumentation

- Fluorescence spectrophotometer Perkin Elmer LS 50 .
- Fluorescence spectrophotometer Time Master Photon Technology International (PTI) TM-2/2003-PTI.
- UV-Vis spectrophotometer Agilent 9453A.
- Transmission Electron Microscope (TEM) FEI Tecnai TF20.
- Analytical scale balance A&D -GR-202.
- pH-Meter micropH from Crison.

3.2. Apparatus

- Incubator: Eppendorf thermomixer compact.

3.3. Reagents

- Bovine serum albumin (BSA), from Sigma-Aldrich (A7906).
- Di-Sodium Hydrogen Phosphate anhydrous (Na_2HPO_4, 141.96 g/mol) from Sigma-Aldrich (S9763).
- Glucose ($C_6H_{12}O_6$, 180.16 g/mol) from Sigma-Aldrich. (G8270).
- Glucose Oxidase from *Aspergillusniger* Type X-Sfrom Sigma-Aldrich (G7141-50KU).
- Sodium carbonate (Na_2CO_3, 105.99 g/mol) from Sigma-Aldrich (222321).
- Tetracholoroauric (III) acid ($HAuCl_4$, 339.79 g/mol) from Sigma-Aldrich (254169).
- Tris(hydroxymethyl)aminomethane (TRIS, 121.44 g/mol) from Sigma-Aldrich (252859).

3.4. Experimental methods

- Incubation method: two different methods were used. On the one hand for standard measurements, the samples were incubated for 24 hours at 37 ˚C; on the other hand for kinetic measurements, a thermostatic sample compartment was used at 37 ˚C. The samples were incubated in buffer solutions (pH 7, 10 or 12) or in milli-q grade water, depending of its later application. When samples were incubated in water the order of mixing of the reagents becomes important because the acidity of the $HAuCl_4$ solutions could denaturalize the proteins. Therefore, first the $HAuCl_4$ solution was added, then the BSA solution, later the GOx solution and finally the glucose solution. BSA was added in high

concentrations and it had a buffer effect in the solution that made that GOx did not denaturalize despite the acidity of the $HAuCl_4$ solution.

- EAM-UV-Vis method: AuNPs exhibit the phenomenon of SPR, therefore its formation can be detected by EAM-UV-Vis. The samples were put inside a 1-cm-path-length absorption cuvette with two clear window and then the cuvettes were put inside the sample compartment.
- Fluorescence method: AuNCs apparition can be detected by measuring its fluorescent emission. The samples were put inside a 1-cm-path-length fluorescence cuvette with four clear window and then the cuvettes were put inside the sample compartment. The excitation wavelength is shown for each measurement in the results section.
- TEM/STEM method: the TEM/STEM samples were prepared by adding a drop of the nanomaterials dispersed in water onto a carbon-coated copper grids.

4. Results and disccusion

Taking into account the proposed objectives, the initial hypothesis was that Au (III) was reduced to Au (0) by the enzymatic reaction between GOx and glucose, thus the glucose concentration could be related with the optical properties of the nanomaterials.

4.1. AuNPs

4.1.1. EAM-UV-Vis characterization

In order to test the capability of the enzymatic reaction between GOx and glucose of acting as reducing agent of Au^{3+} ions for AuNPs formation, three experiments were carried out. $HAuCl_4$ was used as gold precursor. In order to shift the enzymatic reaction to the formation of the product, the enzyme Catalase was used to eliminate H_2O_2 which is a product of the reaction between GOx and glucose. In Table 1 the final concentration of the reagents in each experiment is shown. All the reagents were dissolved in PBS 0,1 M of pH 7 which is the optimum pH for the activity of the enzyme.

Table 1. Final concentration of $HAuCl_4$, GOx, glucose and Catalase.

Experiment	**[$HAuCl_4$]**	**[GOx]**	**[Glucose]**	**[Catalase]**
1	1 mM	3 mg/mL	-	-
2	1 mM	3 mg/mL	$6.6.10^{-3}$ M	-
3	1 mM	3 mg/mL	$6.6.10^{-3}$ M	0,5 mg/mL

All the reagents were well mixed and incubated during 24 hours at 37 ˚C. After this time the experiments 2 and 3 changed their colour from yellow to pink and the experiment 1 did not change its colour and it remained yellow. The UV-visible spectra of these solutions is shown in Figure 5.

In the three spectra two peaks at 380 nm and 450 nm appeared. These peaks were due to the FAD group of the enzyme GOx. Another peak in experiments 2 and 3 appeared, at the wavelengths of 575 nm and 560 nm, respectively. These peaks were due to the formation of AuNPs and its property of surface plasmon resonance (SPR). In experiment 1 this peak did not appear so AuNPs growth did not take place. In experiments 2 and 3 GOx and glucose were added, the FAD group of GOx was reduced to $FADH_2$. $FADH_2$ can be reoxidized to FAD and it can reduce Au^{3+} to Au^0. In experiment 1 glucose was not added, the enzyme was in its oxidize state thus it was not able to reduce Au^{3+} and AuNPs were not formed. The difference between the wavelength at which the SPR appeared in experiments 2 and 3 is the AuNPs size. The

higher is the AuNP size the higher wavelength of the SPR. In experiment 2 the SPR appeared at higher wavelengths than in experiment 3. The difference between experiment 2 and 3 was the addition of Catalase and the consequent elimination of H_2O_2 in experiment 3. H_2O_2 increases the size of AuNPs so AuNPs in experiment 2 were bigger than in experiment 3.

This experiment showed that the reaction between GOx and glucose is able to reduce Au(III) to Au(0) and to form AuNPs with a measurable optical property.

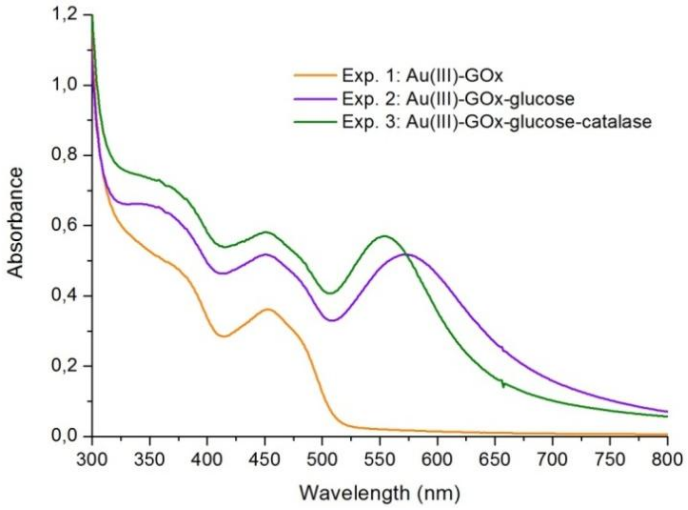


Figure 5. UV-visible spectrum of AuNPs synthesized using the enzymatic reaction between glucose oxidase and glucose (Experiment 1, 2 and 3).

The influence of glucose concentration in AuNPs formation was tested. $HAuCl_4$ and GOx concentration were constant in all the experiments and different glucose concentrations were added. The $HAuCl_4$ and the GOx concentrations were the same than in Table 1., no Catalase was added and the glucose concentrations were 0 M, 10^{-4} M and 10^{-3}M. These experiments were made in PBS 0,1M of pH 7.

All the reagents were well mixed and incubated during 24 hours at 37 ˚C. After this time the experiments 2, 3 and 4 changed their colour from yellow to pink and the experiment 1 did not change its colour and remained yellow. The UV-visible spectra of these solutions is shown in Figure 6.

In the three experiments the peaks at 380 nm and 450 nm appeared due to the presence of GOx. In the experiment without glucose SPR did not appear and AuNPs were not formed. In the other experiments, the SPR peak appeared, thus AuNPs were formed. In the experiment with a glucose concentration of 10^{-4} M the SPR appeared at the wavelength of 561 nm and in the experiment with a glucose concentration of 10^{-3}M the SPR appeared at the wavelength of 569 nm. The wavelengths followed a tendency, the

higher the glucose concentration, the higher wavelengths at which appeared the SPR. Also the intensity values for the absorbance were bigger as the glucose concentration increased. In these experiments the H_2O_2 was not eliminated. The higher was the glucose concentration the higher amounts of H_2O_2 were formed and bigger the size of the formed AuNPs.

These experiments showed that the SPR intensity of AuNPs can be relate with the glucose concentration.

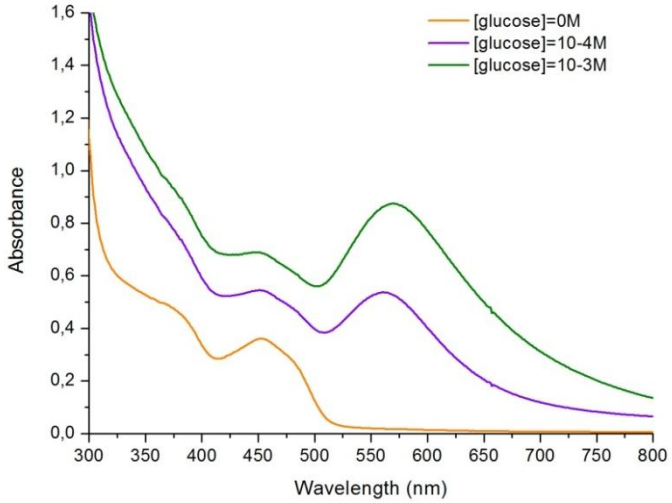


Figure 6. UV-visible spectrum of AuNPs synthesized using the enzymatic reaction between glucose oxidase and glucose.

Following changes in absorbance, the relation between $HAuCl_4$ and GOx concentration and the AuNPs growth, was tested. In Table 2, the final concentration of the reagents in each experiment is shown. All the reagentswere prepared in PBS 0,1 M of pH 7 and they were well-mixed and incubated during 24 hours at 37 ˚C.

Table 2. Final concentration of $HAuCl_4$, GOx and glucose. The $HAuCl_4$ concentration was the same in all the experiments (a). The GOx concentration was the same in all the experiments (b).

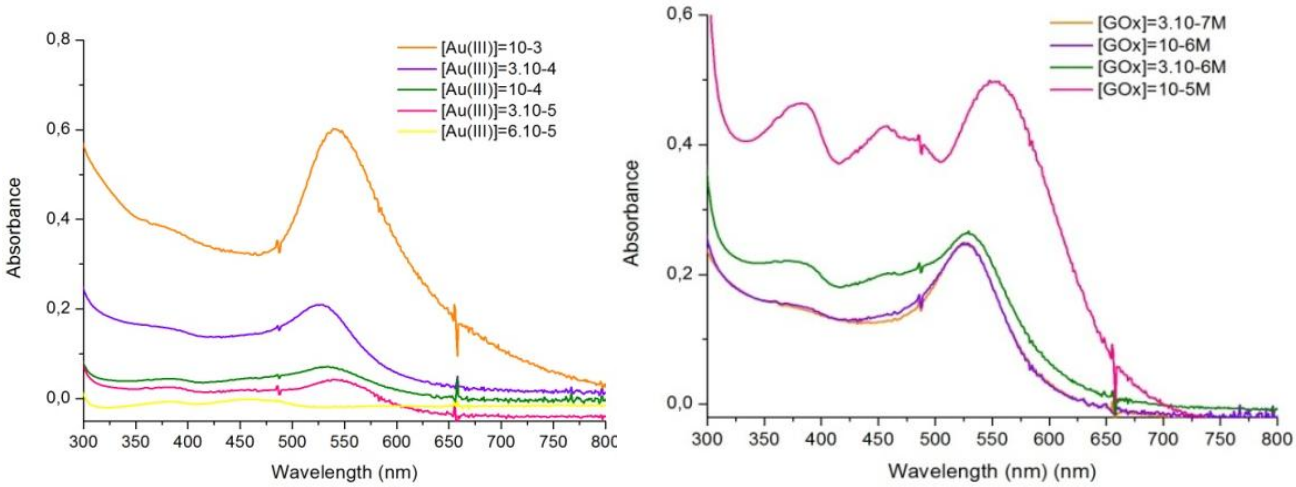
(a)

	[GOx]	[Glucose]	[$HAuCl_4$]
2	10^{-6}M	3,75.10^{-3}M	10^{-3}M
2.b	10^{-6}M	-	10^{-3}M
3	10^{-6}M	3,75.10^{-3}M	3.10^{-4}M
3.b	10^{-6}M	-	3.10^{-4}M
4	10^{-6}M	3,75.10^{-3}M	10^{-4}M
4.b	10^{-6}M	-	10^{-4}M
5	10^{-6}M	3,75.10^{-3}M	3.10^{-5}M
5.b	10^{-6}M	-	3.10^{-5}M
6	10^{-6}M	3,75.10^{-3}M	6.10^{-5}M
6.b	10^{-6}M	-	6.10^{-5}M

(b)

	[$HAuCl_4$]	[Glucose]	[GOx]
a	10^{-3}M	3,75.10^{-3}M	3.10^{-7}M
a.b	10^{-3}M	-	3.10^{-7}M
b	10^{-3}M	3,75.10^{-3}M	10^{-6}M
b.b	10^{-3}M	-	10^{-6}M
c	10^{-3}M	3,75.10^{-3}M	3.10^{-6}M
c.b	10^{-3}M	-	3.10^{-6}M
d	10^{-3}M	3,75.10^{-3}M	10^{-5}M
d.b	10^{-3}M	-	10^{-5}M

After the incubation time the samples without glucose did not change their colour. In Figure 7., the UV-visible spectra of the sample with glucose are shown.



(a) (b)

Figure 7. UV-visible spectra of solutions 10^{-6}M of GOx, 3,75.10^{-3}M of glucose and different $HAuCl_4$ concentrations (6.10^{-5}M, 3.10^{-5}M, 10^{-4}M, 3.10^{-4}M and 10^{-3}M) (a). UV-visible spectrum of solutions 10^{-3}M of $HAuCl_4$, 3,75.10^{-3}M of glucose and different GOx concentrations (3.10^{-7}M, 10^{-6}M, 3.10^{-6}M and 10^{-5}M) (b).

In Figure 7.a. we can see that as the $HAuCl_4$concentrationincreased the absorbance of the SPR achieved higher values. For a $HAuCl_4$concentration of 6.10^{-5}M the concentration of Au^{3+} ions was not enough for the growth of AuNPs took place and the

peak due to SPR did not appear. The wavelength at which the maximum absorbance appear, did not follow a tendency.

Figure 7.b. shows how the GOx concentration changed the SPR of the AuNPs. There was not an important change in the SPR with GOx concentrations of 3.10^{-7}M, 10^{-6}M and 3.10^{-6}M. The absorbance values for this GOx concentration stayed almost constant. However there was a high increase in the absorbance for aGOx concentration of 10^{-5}M.

A calibration with glucose was done taking into account the results obtained for the calibrations with $HAuCl_4$ and GOx. The concentrations of $HAuCl_4$ and GOx were maintained constant in all the experiments and different concentrations of glucose were added. The final concentration of $HAuCl_4$ and GOx were 10^{-3} M and 10^{-5}M, respectively, because with these concentrations the highest values of absorbance were achieved (see Figure 7.). The concentrations of glucose were 0 M, 10^{-4} M, 3.10^{-4} M, 10^{-3} M, 3.10^{-3} M and 10^{-2} M. All the reagents were prepared in PBS 0,1 M of pH 7 and they were well-mixed and incubated during 24 hours at 37 ˚C. In Figure 8, the UV-visible spectra for these samples is shown.

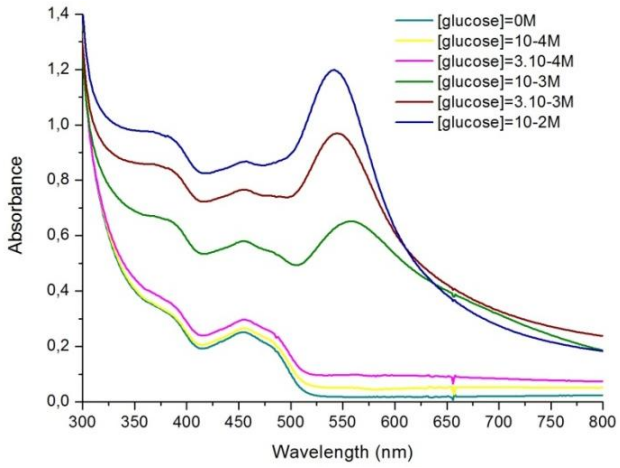


Figure 8. UV-visible spectrum of solutions 10^{-3} M of $HAuCl_4$, 10^{-5} M of GOx and different glucose concentrations (0 M, 10^{-4} M, 3.10^{-4} M, 10^{-3} M, 3.10^{-3} M and 10^{-2} M).

As the glucose concentration increased the absorbance intensity for the SPR achieved higher values. The wavelengths followed a tendency, as higher was the glucose concentration, at smaller wavelengths appeared the SPR. These results contradicted, those of Figure 6. in which as higher was the glucose concentration at higher wavelengths appeared the SPR. Further studies are required to understand the relation between the glucose concentration and the wavelength at which the SPR appeared.

In order to test if the enzyme oxidation state was relevant,two experiments were carried out. On the one hand glucose was added to GOx(in order to reduce the enzyme)and then the $HAuCl_4$ was added. On the other hand, $HAuCl_4$ was added to GOx and then the glucose was added. In both experiments the concentration of $HAuCl_4$, GOx and glucose were respectively 10^{-3}M, 10^{-5}M and 10^{-3}M. All the reagents were well mixed and incubated during 24 hours at 37 ˚C.

In Figure 9, the spectra for both solutions is shown.

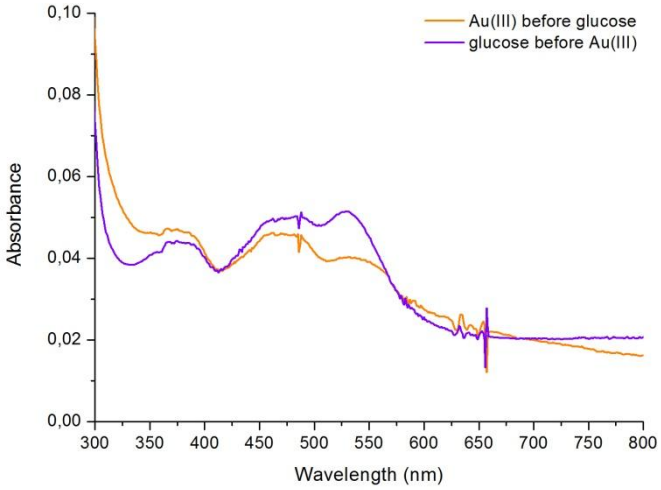


Figure 9.UV-visible spectra of solutions 10^{-3}M of $HAuCl_4$, 10^{-5}M of GOx and 10^{-3}M of glucose adding $HAuCl_4$ before glucose and adding glucose before $HAuCl_4$.

Although the maximum intensity for SPR appeared for both solutions at the same wavelength (531 nm), they had not the same intensity. The intensity was higher when the Au(III) was add to the solution with the reduced enzyme.

As a conclusion, the best procedure for relate the SPR of AuNP with the glucose concentration was 10^{-3}M of $HAuCl_4$, 10^{-5}M of GOx and adding the Au (III) after the enzymatic reaction has taken place.

4.1.2. Fluorescence characterization

AuNPs have not fluorescence properties, but in the literature it is possible to find examples of how AuNCs with fluorescence properties can be synthesized by mixing a gold precursor with enzymes without using an extra scaffold and neither the substrate of the enzyme. Therefore it is possible than AuNCs have been formed at the same time of AuNPs. Fluorescence properties of AuNPs synthesized during the reaction between GOx and glucose were tested. A 3D fluorescence spectrum of the solution of $HAuCl_4$, GOx and glucose with a final concentration of 1 mM, 3 mg/mL and 10^{-3} M, respectively, is shown in Figure 10.a.

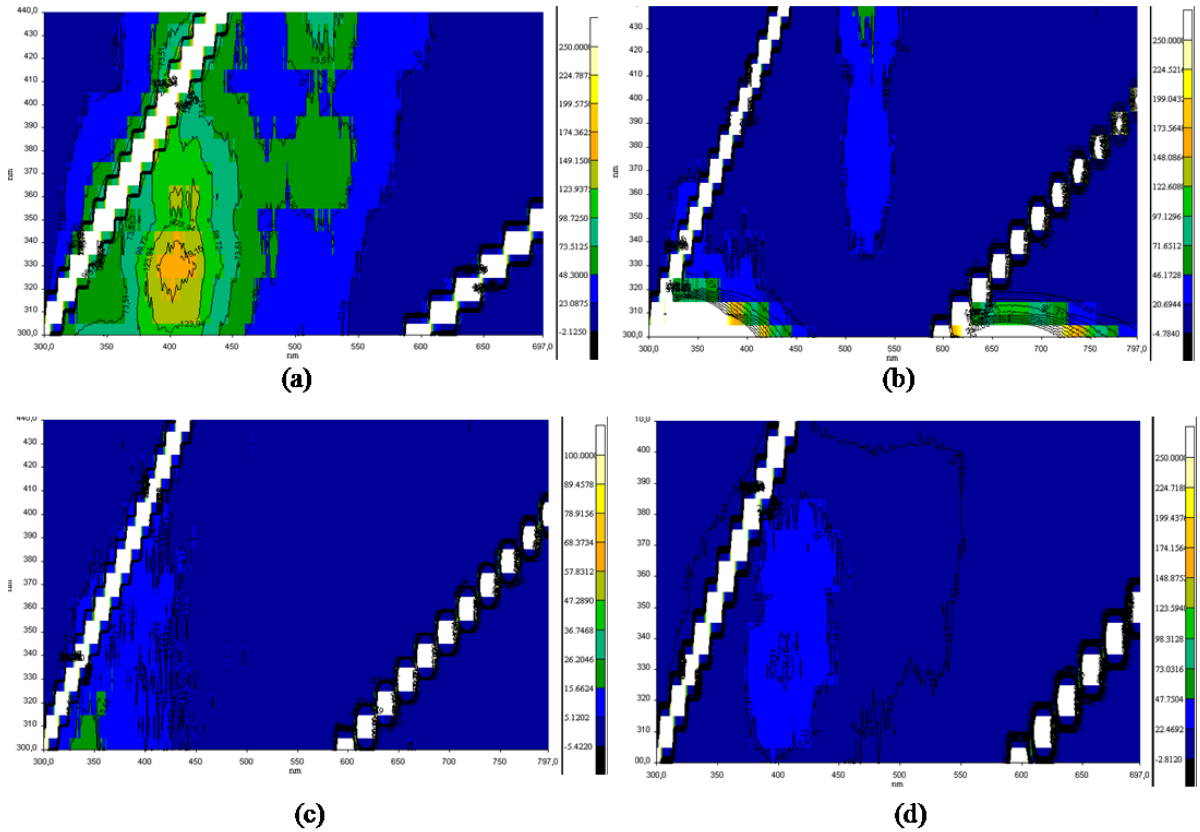
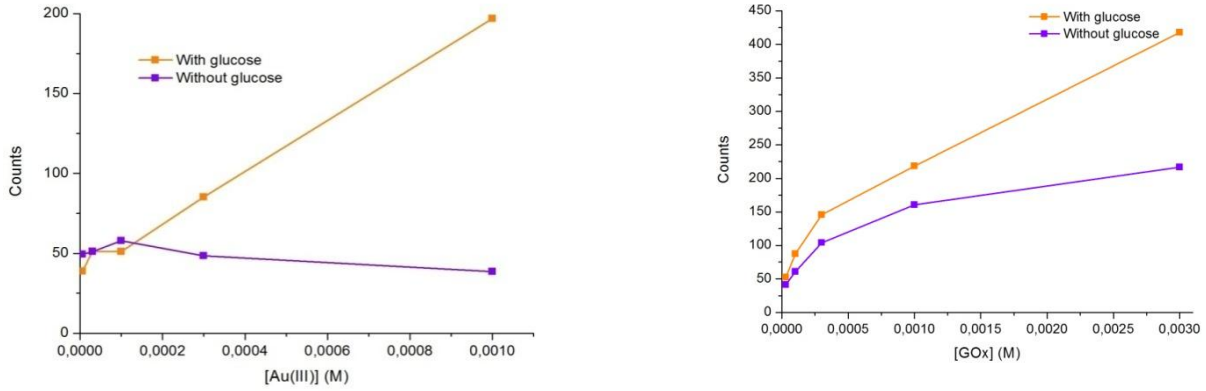


Figure 10. 3D fluorescence spectra for a solution of $HAuCl_4$, GOx and glucose with a final concentration of 1 mM, 3 mg/mL and 10^{-3} M (a), a solution 3mg/mL of GOx (b), a solution 1 mM of $HAuCl_4$ (c) and a solution of $HAuCl_4$ and GOx with a final concentration of 1 mM and 3 mg/mL (d). In the y-axis the excitation wavelength is plot and in the x-axis the emission wavelength is plot.

In this spectrum appear the signals due to the enzyme fluorescence. The FAD group of GOx emits light at 510 nm when it is excited at 380 nm or 450 nm, both signals are shown in the spectra. The fluorescence aminoacids emit light at 340 nm when they are excited at 280 nm. The peak due to the aminoacids fluorescence in this sample was weaker in comparison with a sample composed only of GOx of the same concentration (Figure 10.b.). However the FAD fluorescence was increased in the sample with AuNPs. In the AuNPs fluorescence spectrum, an emission peak appeared at 420 nm when the sample is excited at 335 nm or 355 nm. A 3D fluorescence spectra of a $HAuCl_4$solution was made (Figure 10.c.), the peak at 420 nm did not appear. A 3D fluorescence spectra of aHAuCl$_4$and GOx solution was made (Figure 10.d.). Although the peak at 420 nm appeared, it had a lower intensity than in the AuNPs sample. The peak at 420 nm appeared when Au^{3+} was put in contact with GOx and its intensity increased when the enzymatic reaction between GOx and glucose took place.

The relation between $HAuCl_4$ and GOx concentration and the AuNPs growth was also tested by following the changes in the fluorescence signal founded in the 3D spectra (λ_{ex}=335 nm and λ_{em}=420). The same solutions of Table 2 were used for these experiments.Changes in the fluorescence of experiments of Table 2 had been also measured (Figure 11.).



(a) (b)

Figure 11. Fluorescence intensity versus $HAuCl_4$ concentration (a) and GOx concentration (b) with and without glucose at λ_{ex}=335 nm and λ_{em}=420.

In Figure 11.a. it is shown how by maintaining the GOx concentration constant and increasing the $HAuCl_4$ concentration, the fluorescence intensity increases with the concentration of glucose. Nevertheless when glucose was not added the fluorescence intensity was nearly the same for all the $HAuCl_4$concentrations. The difference of fluorescence intensities between the samples with and without glucose was significant for $HAuCl_4$concentrations of 3.10^{-4}M and 10^{-3}M. For smaller concentrations the difference in fluorescence intensities were no significant. In Figure 7.11it is shown how by maintaining the $HAuCl_4$ concentration constant and increasing the GOx concentration, the fluorescence intensity changed. Although when glucose was added the fluorescence intensity grew more than when glucose was not added, in the latter case the fluorescence intensity grew also as the GOx concentration increased.These results agreed with the results obtained in the 3D spectra. The fluorescence with λ_{ex}=335 nm and λ_{em}=420 appeared when $HAuCl_4$and GOx were put in contact and its intensity is higher when glucose was added.

A set of experiments was done for studying the relationship between the glucose concentration and the fluorescent signal. The concentration of $HAuCl_4$and GOx was

maintained constant and different concentrations of glucose were added. The $HAuCl_4$and GOx concentrations were 10^{-3} M and 10^{-5} M, respectively. These concentrations were chosen because the highest values for the fluorescent signal were achieved (see Figure 11.) The concentrations of glucose were 0 M, 10^{-4} M, 3.10^{-4} M, 10^{-3} M, 3.10^{-3} M and 10^{-2} M. All the reagentswere prepared in PBS 0,1 M of pH 7 and they were well-mixed and incubated during 24 hours at 37 ˚C. In Figure 12, is shown the fluorescence intensity versus the glucose concentration.

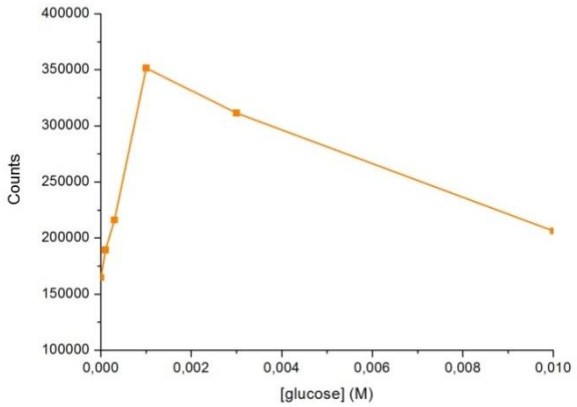


Figure 12. Fluorescence intensity versus glucose concentration at λ_{ex}=335 nm and λ_{em}=420.

A linear increase was observed for the fluorescence intensity as the glucose concentration increased from 0 M to 10^{-3} M. For higher glucose concentrations, the fluorescence intensity decreased linearly. This reduction in the fluorescent signal could be due to internal filter or to autoabsorption phenomena. Further studies are required to understand this fact and it will be object of study.

4.1.3. Kinetic measurements

Measurements in function of time were made in order to establish the velocity of appearance of the absorbance and fluorescence signals.

A glucose solution was mixed with a GOx solution and when the enzyme was in its reduced state, $HAuCl_4$ was added. The final concentration of GOx, glucose and $HAuCl_4$ were respectively 10^{-6} M, $3{,}75.10^{-3}$ M and 10^{-3} M. Two identical solutions were prepared. All the reagents were dissolved in PBS 0,1 M of pH 7.

As soon as the $HAuCl_4$was added, one of these reactions was followed by measuring the absorbance changes and the other one the fluorescence intensity.

Fluorescence spectra with and excitation wavelength of 335 nm were recorded for 30000 s. In Figure 13.all the fluorescence spectra are plotted together.

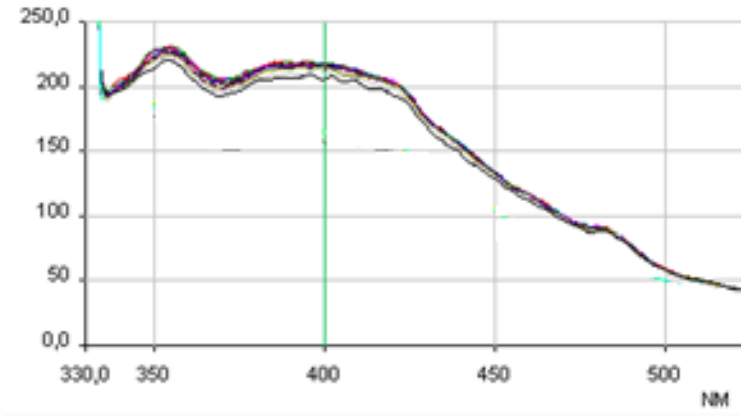


Figure 13. Fluorescence spectra of a solution of GOx, glucose and $HAuCl_4$ (λ_{ex}=335 nm).

Two peaks appeared in these spectra. On the one hand a peak with λ_{em}=355 nm which was attributed to GOx fluorescence. On the other hand a peak with λ_{em}= 400 nm which was the same peak observed in the 3D fluorescence spectra. This peak achieved its maximum intensity at the beginning of the reaction and did not increase its intensity throughout the time.

Absorbance spectra were recorded for 30000 s. In Figure 14. all the absorbance spectra are plotted together.

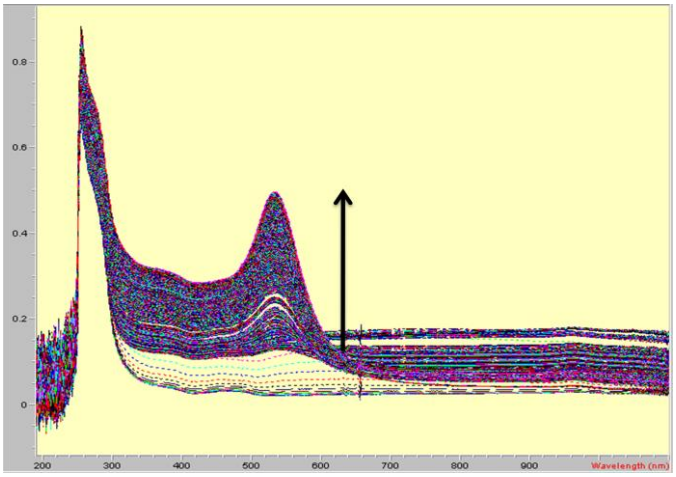


Figure 14. Absorbance spectra of a solution of GOx, glucose and $HAuCl_4$. The arrow shows how the peak at 539 nm increased along the time.

The peak at 280 nm was due to the aminoacids. The peaks at 380 nm and 450 nm are due to the FAD group. The peak at 539 nm is due to the SPR of the nanoparticles. All these peaks at the beginning did not appear, the peaks increased its intensity along the time.

In the literature there are examples of AuNCs with fluorescence signals at same wavelengths at which we found fluorescence (λ_{ex}=335 nm and λ_{em}=420) [10]. These AuNCs were formed immediately after all the reagents were put together. The fluorescence intensity of these AuNCs was maintained constant over the time, thus the AuNCs concentration did not change along the time. However the AuNPs growth did not follow the same process. At the beginning a peak of SPR did not appear, SPR increased along the time, so the AuNPs did not appear immediately.

With these results the following hypothesis was made, the AuNCs and the AuNPs concentration were in equilibrium. AuNCs were formed immediately and when the AuNCs started to increase their concentration, some AuNCs started to aggregate and to form bigger AuNPs in order to maintain the AuNCs concentration always constant.

4.2. AuNCs

4.2.1. Preliminary experiments

As has been reported in the introduction, AuNCs growth can take place by chemical reduction. By using a reducing agent as $NaBH_4$ and capping agents or by using biomolecules as proteins (BSA) which acts as reducing agent and as scaffolds.

The capability of GOx for acting as reducing agent and scaffold for AuNCs growth was tested by following the same method used for BSA [15]. Two solutions were prepared, on the one hand a solution of $HAuCl_4$ and GOx with a final concentration of 5 mM and 25 mg/mL, respectively and on the other hand a solution with $HAuCl_4$, GOx and glucose with a final concentration of 5 mM, 25 mg/mL and 10^{-3} M. These solutions were incubated during 24 hours at 37 ˚C.

After the incubation time the color of the solutions did not change from yellow to brown as expected. Fluorescence spectra for these solutions were recorded but no fluorescence was found. GOx could not be used as reducing agent and scaffold for AuNCs growth.

In light of these results, in the following experiments BSA was used as scaffold and reducing agent. The reaction between GOx and glucose was added in order to enhance the reducting activity of the protein and to relate the fluorescence signals of AuNCs with the glucose concentration.

4.2.2. Study of the pH of the reaction

For the growth of AuNCs to took place by using BSA as scaffold the pH must be higher than 10. However, the working pH of the enzyme GOx range from pH 6 to pH 8. In this range of pHs the enzyme has the highest activity. The optimal pH for AuNCs growth was not consistent with the working pH of the enzyme. To overcome this drawback there were two possibilities. On the one hand, to study if the growth of AuNCs could take place in a pH closer to the optimal one of the enzymatic reaction. On the other hand, to perform the enzymatic reaction at the optimal pH for AuNCs growth.

In order to study the influence of pH in the AuNCs growth different experiments were carried out. In a set of experiments 750 μL of a $HAuCl_4$ solution and 750 μL of a BSA solution with a final concentration of 5 mM and 25 mg/mL, respectively, were mixed. This experiment was carried out at two different pHs, 10 and 12. Each pH was achieved by adjusting with NaOH or with a buffer solution. For pH buffer of 10, TRIS with a concentration 0,1 M was used. For pH buffer of 12, PBS of the same concentration was used. These solutions were excited at 365 nm, which is the excitation wavelength of the AuNCs synthesized with BSA [15] (Figure 15.).

The spectra shows that in the case of the TRIS buffer solution, the AuNCs were not formed. In the other cases AuNCs were formed because a peak in the range from 610 nm to 640 nm appeared. The maximum intensity was achieved with the AuNCs synthesized at pH 12 by adjusting the pH with NaOH. The AuNCs synthesized at pH 10 adjust with NaOH gives also fluorescence. AuNCs were also formed at pH 12 with PBS 0.1 M.

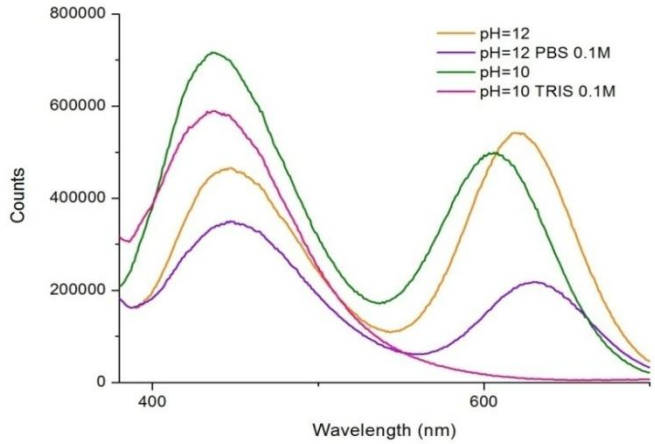
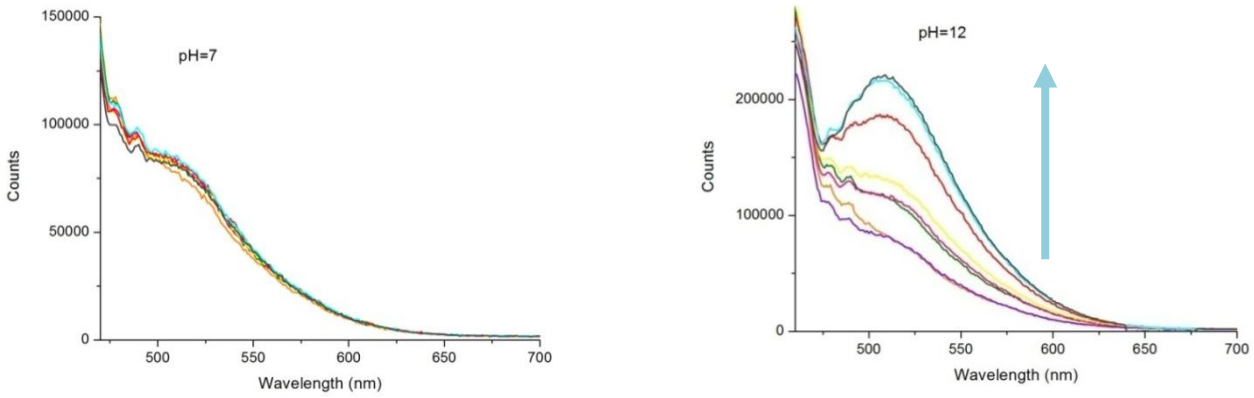


Figure 15. Fluorescence spectra of AuNCs synthesized with $HAuCl_4$ and BSA at pH 12 adjusted with NaOH and PBS 0.1M. pH 10 adjusted with NaOH and TRIS buffer solution 0.1M (λ_{ex}= 365 nm).

However, there are not many studies about GOx behavior in basic media because at pH 12 the enzyme could denaturalize. In order to test this, a study of the effect of high pH values on the GOx stability was carried out. As has been indicated before, GOx has a FAD groups which fluorescence properties are characterized by excitation at 380 nm and 450 nm, and emission at 510 nm. This fluorescence is highly quenched by the environment surrounding FAD in the enzyme, so at low GOx concentrations the fluorescence is very poor and in some cases it cannot be detected, however when the enzyme denaturalizes the FAD group is released to the media and the fluorescence at 510 nm increases; this effect will be used for testing the GOx stability with pH.

Two solutions of GOx 0,75 mg/mL were made at two different pHs. The first at pH 7 in a buffer solution of Na_2CO_3 and the second one at pH 12 in PBS. The fluorescence intensity at 510 nm was measured over the time under excitation at 450 nm (Figure 16).

In Figure 12 and figure 13 it is shown that at pH 7 there was not an increase in the fluorescence intensity and the enzyme stays stable. However at pH 12 there is an increase in the fluorescence intensity throughout the time. At pH 7 the enzyme did not change its conformation over the time and the fluorescence intensity did not increase. At the time of 12120 seconds glucose has been added to these solution. The GOx solution at pH 7 remains active however the GOx solution at pH 12 has become inactive and there was no evidence of the formation of peroxides thus the enzyme is inactive. At pH 12 at the time of 3300 seconds GOx starts its denaturalization and at the time of 12120 seconds the enzyme is completely denaturalize and inactive. At pH 12 the enzyme is active only for 30 minutes and then it starts to denaturalize.



(a) (b)

Figure 16. Fluorescence spectrum of a GOx solution 0.75 mg/mL at pH 7 (a) and pH 12 (b) at different time (λ_{ex}=450 nm). The arrow indicate the increase in the fluorescence intensity as the time increases.

In Figure 17. the change in the fluorescence intensity at λ_{ex}=450 nm and λ_{em}=510 nm is plotted versus the time.

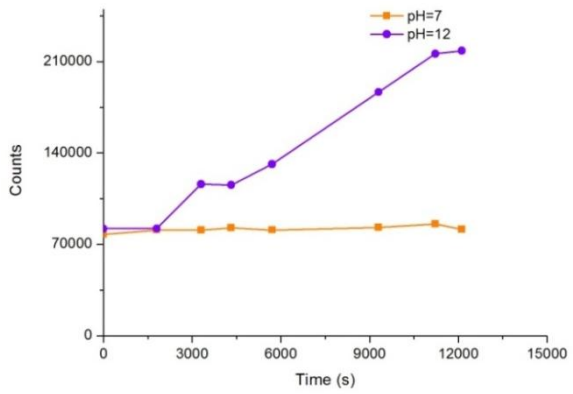
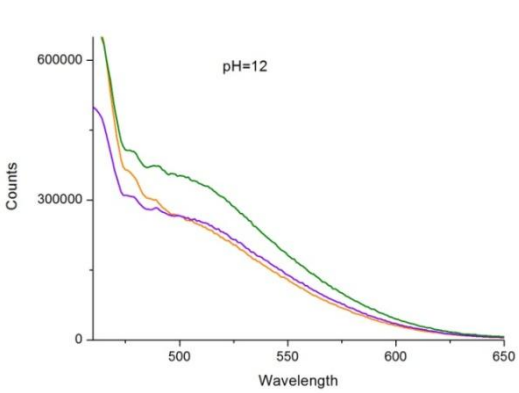


Figure17. Fluorescence intensity versus time at λ_{ex}=450 nm and λ_{em}=510 of a GOx solution 0,75 mg/mL at pH 7 and pH 12.

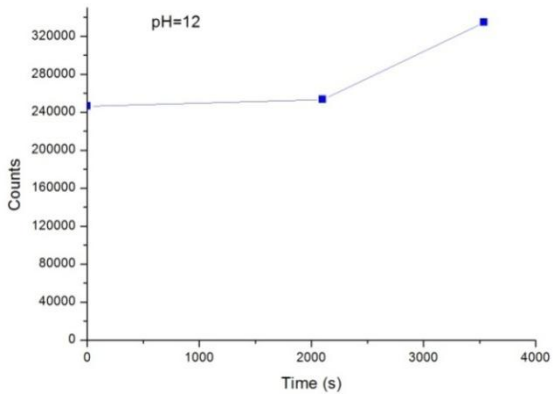
In Figure 16 and figure 17 it is shown that at pH 7 there was not an increase in the fluorescence intensity and the enzyme stays stable. However at pH 12 there is an increase in the fluorescence intensity throughout the time. At pH 7 the enzyme did not change its conformation over the time and the fluorescence intensity did not increase. At the time of 12120 seconds glucose has been added to these solution. The GOx solution at pH 7 remains active. However the GOx solution at pH 12 has become inactive and because no evidence of the formation of peroxides was observed.

At pH 12 at the time of 3300 seconds GOx starts its denaturalization and at the time of 12120 seconds the enzyme is completely denaturalize and inactive. At pH 12 the enzyme is active only for 30 minutes and then it starts to denaturalize.

It has also to be taken into account that the enzyme is not alone, there is a high concentration of BSA in the media. The same experiment has been made but adding BSA (50 mg/mL) in PBS at pH=12 (Figure 18.).



(a)



(b)

Figure 18. (a) Fluorescence spectrum of a GOx solution 0.375 mg/mL and BSA 25 mg/mL at pH 12 at different time (λ_{ex}=450 nm). The arrow indicate the increase in the fluorescence intensity as the time increases. (b) Fluorescence intensity versus time at λ_{ex}=450 nm and λ_{em}=510 of a GOx solution 0.375 mg/mL and BSA 25 mg/mL at pH 12. The presence of BSA made that the enzyme stay stable more time.

As a conclusion, it can be said that the presence of BSA made that the enzyme stay stable more time and it could be possible to work in these conditions.

4.2.3. AuNCs transformation and characterization

The growth of AuNCs was performed in a set of experiments. All the reactions were made at PBS pH 12 0.1 M. Although higher intensities were achieved with pH 10 or pH 12 adjusted with NaOH, PBS was chosen because very low volumes were used and adjust the pH with NaOH gives inaccuracies of the real pH of the solution.

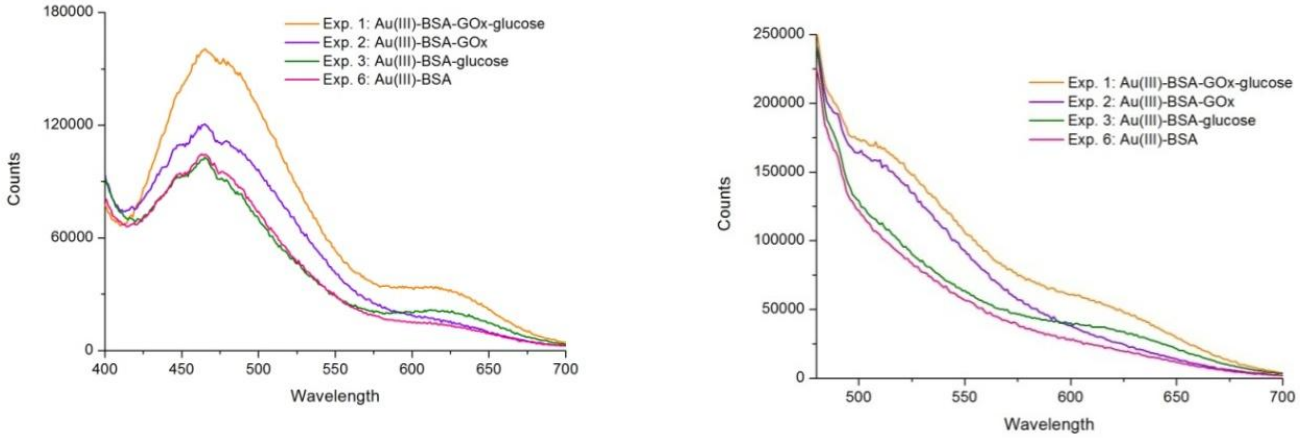
750 µL of a solution of $HAuCl_4$ were mixed with 750 µL of a BSA or GOx or BSA and GOx solution. Then in some experiments 50 µL of a glucose solution were added and the solutions were incubated 24 hours at 37 ˚C.

In Table 4.the final concentration of $HAuCl_4$, BSA, GOx and glucose of the different experiments is shown.

Table 4. Final concentration of $HAuCl_4$, BSA, GOx and glucose.

Experiment	**[$HAuCl_4$]**	**[BSA]**	**[GOx]**	**[Glucose]**
1	5 mM	25 mg/mL	0,375 mg/mL	10^{-3} M
2	5 mM	25 mg/mL	0,375 mg/mL	-
3	5 mM	25 mg/mL	-	10^{-3} M
4	5 mM	-	0,375 mg/mL	-
5	5 mM	-	0,375 mg/mL	10^{-3} M
6	5 mM	25 mg/mL	-	-
7	5 mM	-	-	10^{-3} M
8	0 mM	25 mg/mL	0,375 mg/mL	-

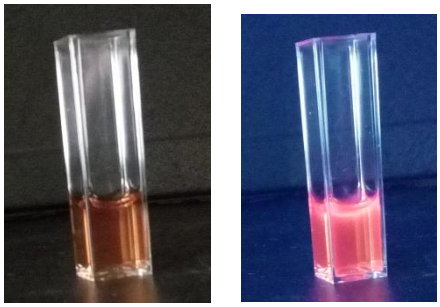
Only for the experiments which had $HAuCl_4$ and BSA (Conditions corresponding to tests 1, 2, 3 and 6) the colour of the solution changed from yellow to brown These solutions had an emission peak at 610 nm when excited at 390 nm or 470 nm (Figure 19.)



(a) λ_{exc}=390 nm (b) λ_{exc}= 470 nm

Figure 19. Fluorescence spectra of conditions given fortests 1, 2, 3 and 6 of Table 4 at two excitation wavelengths 390 nm (a) and 470 nm (b).

In Figure 20.we can see two pictures of conditions of test 1 with and without excitation. Without excitation AuNCs had a brown colour, when they were excited with UV-light they emitted light and they become orange.



(a) (b)

Figure 20.v AuNCs without excitation (a) and under UV-light (b).

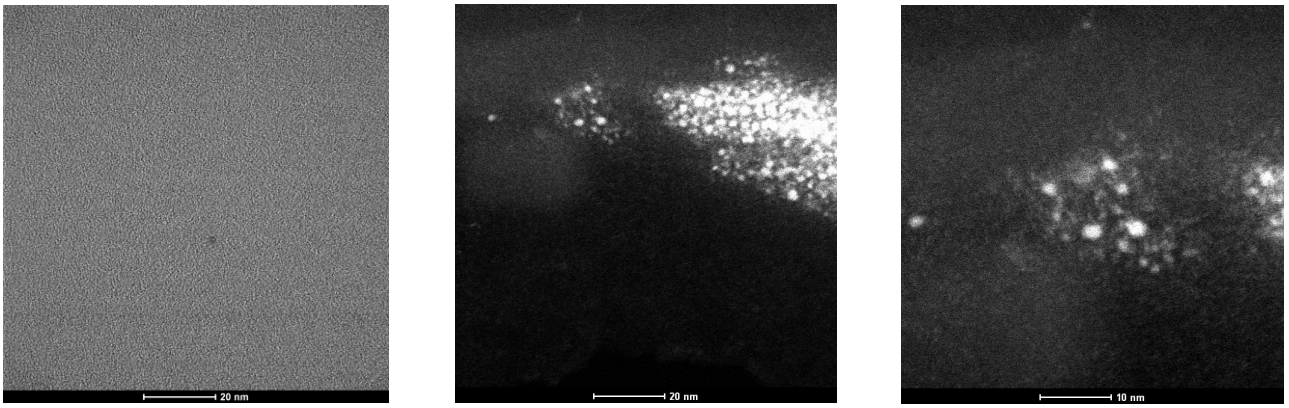
In order to determine the size and size distribution of AuNCs some image were recover with TEM and STEM. The experiment 1 which has $HAuCl_4$, BSA and glucose with a final concentration of 5 mM, 25 mg/mL and 10^{-3} M was repeat but without PBS 0.1M, in this case the pH was adjust to 12 with NaOH. Buffer solution cannot be used because crystals of the salt of which the buffer solution was made of are formed. These crystals are too big in comparison with the size of the AuNCs and their presence would not let see the AuNCs.

Some droplets of this solution were put on top of a glass in order to evaporate the water. Buffer solution cannot be used because crystals of the salt of which the buffer solution

was made of are formed. These crystals are too big in comparison with the size of the AuNCs and their presence would not let see the AuNCs.

The images were recorded with TEM (Figure 21.). The presence of organic matter (BSA) and the fact that the contrast in TEM is due to the thickness, limit the visibility of AuNCs. The difference between the thickness of the substrate and the thickness of the substrate and the AuNCs on top of it is not different enough to give a good contrast.

Some images were recorded with STEM (Figure 21.). In this technique the contrast is related with the atomic number. In this case the atomic number of gold is different enough to the atomic number of the substrate to give a good contrast.



(a) (b) (c)

Figure 21. TEM image of AuNCs synthesized with $HAuCl_4$, BSA, GOx and glucose at pH 12 (a). STEM images of AuNCs synthesized with $HAuCl_4$, BSA, GOx and glucose at pH 12 (b) (c).

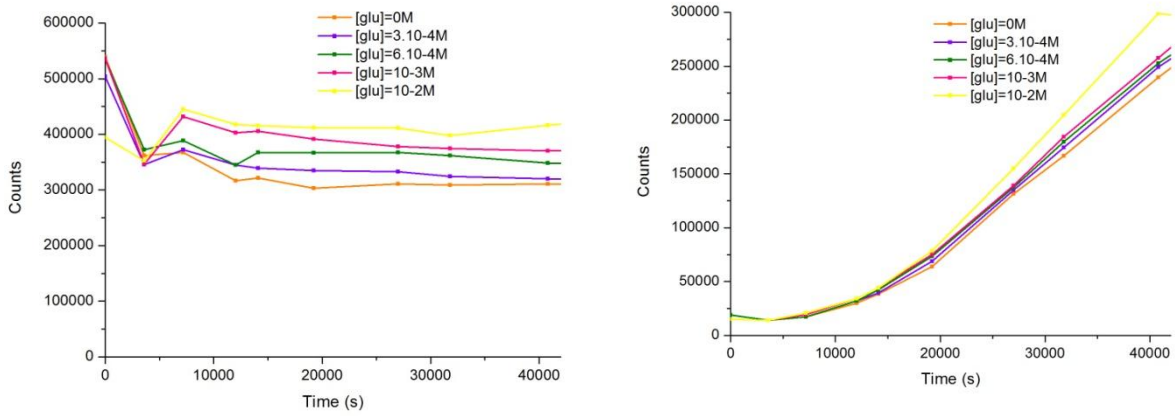
The AuNCs size was in the range from 0,5 nm to 2,5 nm.

4.2.4. Glucose concentration: calibration line

Some kinetics studies have been made in order to study how could we related the glucose concentration with the velocity at which the fluorescent intensity of AuNCs increased.

Five solutions with $HAuCl_4$, BSA, GOx, with a final concentration of 5 mM, 25 mg/mL and 0,375 mg/mL, and glucose with different concentrations (0, 3.10^{-4}, 6.10^{-4}, 10^{-3} and 10^{-2}M) have been prepared in PBS 0.1M at pH 12. These samples showed emission fluorescence at 450 nm and 610 nm when excited at 390 nm (Figure 22). The signal at 450 nm in the beginning decreased and then it stayed almost constant. As higher was the glucose concentration, higher was the final fluorescence signal. The

signal at 610 nm increased over the time. The higher the glucose concentration, the faster the velocity at which the fluorescent intensity of AuNCs increased.



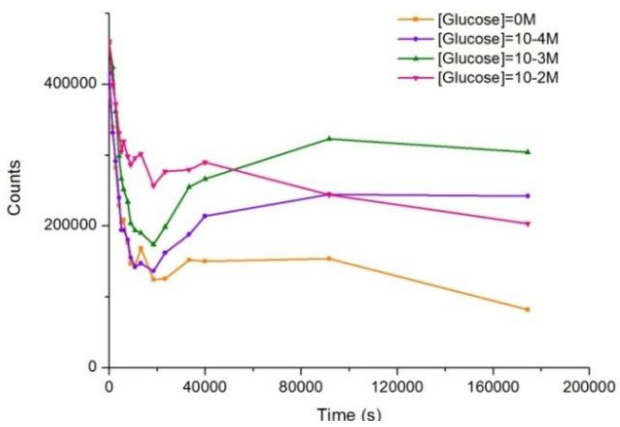
(a) λ_{ex}= 390 nm λ_{em}=450 nm (b) λ_{ex}= 390 nm λ_{em}=610 nm

Figure 22. Fluorescence intensity versus time at λ_{ex}=390 nm, λ_{em}=450 (a) and λ_{em}=610 nm (b) of a $HAuCl_4$ 5mM, GOx 0.375 mg/mL, BSA 25 mg/mL and different glucose concentrations (0M-10^{-2} M) solutions at pH 12.

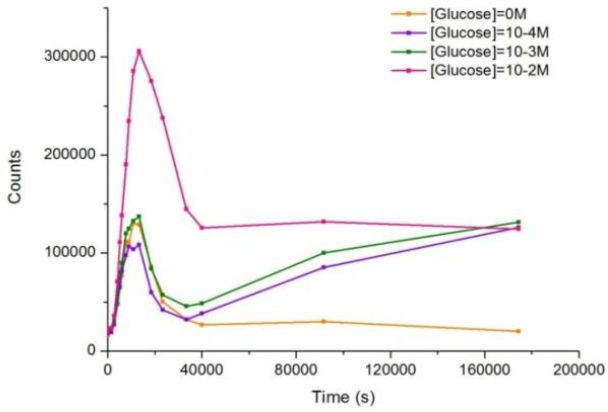
In order to study how the temperature affects the kinetics of the AuNCs growth some experiments at 37 ˚C have been made. Four solutions with $HAuCl_4$, BSA, GOx, with a final concentration of 5 mM, 25 mg/mL and 0,375 mg/mL, and glucose with different concentrations (0, 10^{-4}, 10^{-3} and 10^{-2}M) have been prepared in PBS 0.1M at pH 12. The fluorescence intensity have been measured at 450 nm and 610 nm with an exciting wavelength of 390 nm throughout the time (Figure 12.). The solutions have been incubated at 37 ˚C for all the experiment.

In Figure 23.a. the signal at 450 nm at the beginning decreased its intensity, then it increased although it did not reach the initial value. As higher was the glucose concentration, higher was the final fluorescence signal, except for highest glucose concentration. In Figure 23. c. the fluorescence intensity signal at 610 nm versus the time until 12000 seconds in shown. The increase in the fluorescence intensity was faster for the highest glucose concentration. For the other glucose concentration there was not a significant difference in the velocity at which the fluorescent intensity of AuNCs increases. After 12000 seconds the fluorescence intensity decreased for all the samples (Figure 23.b.). Then, the fluorescence intensity for the sample without glucose kept

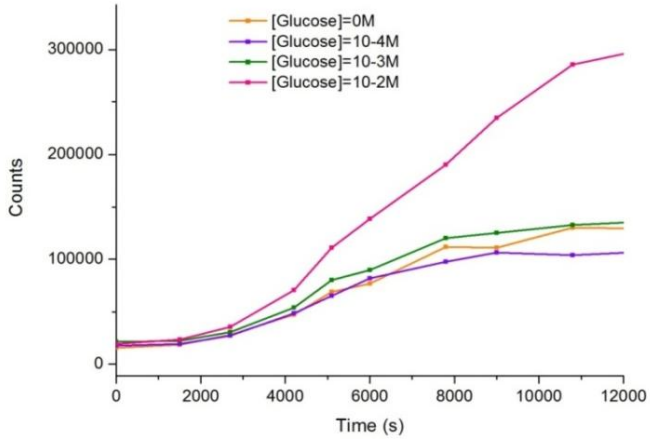
constant throughout the time. The samples with 10^{-3}M and 10^{-4}M of glucose increased it signal over the time. The sample with 10^{-2}M of glucose kept constant it signal along the time. At the end the fluorescence intensities for all the samples with glucose achieved the same value. The sample without glucose did not reach a signal as high as the samples with glucose.



(a) λ_{ex}= 390 nm λ_{em}=450 nm



(b) λ_{ex}= 390 nm λ_{em}=610 nm



(c) λ_{ex}= 390 nm λ_{em}=610 nm

Figure 23. Fluorescence intensity versus time at λ_{ex}=390 nm, λ_{em}=450 (a) and λ_{em}=610 nm (b) and (c) of a $HAuCl_4$ 5mM, BSA 25 mg/mL and different glucose concentrations (0M-10^{-2} M) solutions at pH 12.

The velocity at which the fluorescent intensity of AuNCs increased was higher when the reaction is carried out at 37 ˚C than at room temperature.

As a conclusion, the best procedure for relate the fluorescence of the AuNCs with the glucose concentration was adding BSA, GOx, glucose and to work at pH 12 and at 37 ˚C.

5. CONCLUSIONS

The conclusions obtained of this in work in which the first steps towards the development of a optical nanobiosensor for glucose sensing were studied are:

- The enzymatic reaction between GOx and glucose is able of acting as a reducing agent and to induce the free growth of gold nanomaterials from a gold precursor in oxidation state 3^{+}. During the enzymatic reaction Au(III) reoxides $FADH_2$ to FAD giving to the formation of Au(0) in the form of AuNP. Optical signals (SPR) due to the presence of AuNPs appear when the substrate (glucose) is added. Nevertheless without glucose the signal of SPR does not appear and the growth of AuNP does not take place. The concentration of glucose can be related to the SPR signals because the higher the glucose concentration the higher the absorbance intensity. Fluorescence signals also appear during the free growth of these gold nanomaterials.

- The growth of AuNCs using the protein BSA as scaffold and reducing agent can be enhanced with the enzymatic reaction between GOx and glucose. The velocity at which the fluorescent intensity of AuNCs increases and the glucose concentration can be related.

The obtained results are encouraging, and provide new opportunities and challenges for future work and the completely development of an optical nanobiosensor for glucose sensing based on the growth of gold nanomaterials during the enzymatic reaction.

6. References

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