

## Article

# Highly Sensitive Voltammetric Glucose Biosensor Based on Glucose Oxidase Encapsulated in a Chitosan/Kappa-Carrageenan/Gold Nanoparticle Bionanocomposite

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**Abstract:** In this work, an enzymatic sensor, based on a bionanocomposite film consisting of a polyelectrolyte complex (PEC) [Chitosan/kappa-carrageenan] doped with gold nanoparticles (AuNPs) encapsulating glucose oxidase (GOD) deposited on a gold electrode (Au) for glucose sensing, is described. Using the electrocatalytic synergy of AuNPs and GOD as a model of enzyme, the variation of the current ( $\mu\text{A}$ ) as a function of the log of the glucose concentration ( $\log [\text{glucose}]$ ), shows 3 times higher sensitivity for the modified electrode (283.9) compared to that of the PEC/GOD modified electrode (93.7), with a detection limit of about  $5 \mu\text{M}$  and a linearity range between  $10 \mu\text{M}$  and  $7 \text{mM}$ . The response of the PEC/AuNPs/GOD based biosensor also presents good reproducibility, stability and negligible interfering effects from ascorbic acid, uric acid, urea and creatinine. The applicability of the PEC/AuNPs/GOD based biosensor was tested in glucose-spiked saliva samples and acceptable recovery rates were obtained.

**Keywords:** chitosan; kappa-carrageenan; polyelectrolyte complex; gold nanoparticles encapsulation; glucose oxidase; bionanocomposite.

## 1. Introduction

Many polysaccharide-based PECs have been used 'to make capsules to encapsulate drugs, in order to enhance their controlled and prolonged release. Chitosan/kappa-carrageenan PEC capsules have been used for theophylline encapsulation. It has been shown that its release rate decreases when the concentration of chitosan increases, the capsule diameter increasing accordingly [1]. Better results have been obtained with chitosan/alginate PEC capsules, in the case of diltiazem encapsulation, because carrageenan promotes the entry of water into the matrix, leading to capsule desintegration instead of swelling [2]. Upon cross-linking with glutaraldehyde, prolonged drug release was obtained: over 24 hours for sodium diclofenac [3]. Glucose oxidase enzyme (isoelectric point 4.2) was negatively charged when the  $\text{pH} > 4.2$  and could form a complex with chitosan through electrostatic interactions. The resulting cationic GOD-loaded chitosan was then combined with carrageenan to form a complex via electrostatic interaction. Higher encapsulation efficiency of GOD in chitosan/carrageenan complexes was observed as compared to that of a single polymer. The efficiency was doubled in polymer blends, which exhibited greater ability to encapsulate protein compared to that of a single polymer. At a charge ratio of 3, chitosan/kappa-carrageenan complexes showed the highest encapsulation efficiency (79%) [4]. The ability of chitosan- $\kappa$ -carrageenan PEC

capsules to protect protein integrity under acidic conditions makes them a promising drug delivery system for the oral administration of peptides and proteins.

Chitosan-based electrochemical biosensors use chitosan as an electrode coating in order to limit interference, glucose oxidase being grafted on top of the chitosan layer [5, 6]. Our recent paper reported the use of a chitosan-carrageenan polyelectrolyte in a voltammetric glucose biosensor. This easy-to-prepare biosensor exhibits a broad linear range, a low detection limit, excellent selectivity and is shown to be applicable to saliva samples [7].

In recent years, considerable attention has been paid to improving the construction of biosensors using metal nanoparticles such as silver (Ag) [8] platinum (Pt) [9] and gold (Au) [10, 11] because of their unique physical and chemical properties. In particular, gold nanoparticles have been used in the development of biosensor performance due to their ability to play an important role in immobilizing biomolecules while maintaining biocatalytic activity, their large specific surface area, their excellent biocompatibility and electrocatalytic properties.

Many types of electrochemical biosensor, such as immunological [12], the DNA [13] enzyme [14–16] sensors, with improved analytical performance, have been prepared on the basis of gold nanoparticles (AuNPs). The interaction of chitosan with gold nanoparticles has been studied; the stabilization of gold nanoparticles and the self-organization effect has been demonstrated [17, 18]. In this work, the effect of AuNP doping of chitosan-carrageenan polyelectrolyte on the analytical performance of a voltammetric glucose biosensor is studied.

## 2. Materials and Methods

### 2.1. Reagents:

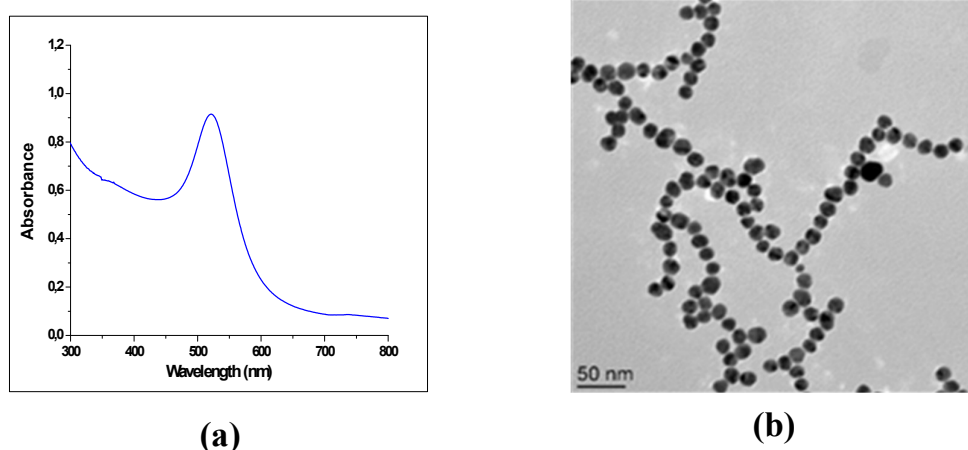
Kappa-carrageenan a natural polysaccharide, extracted from red algae of the genus *Halurus.SP* (MW=750 kDa with a polydispersity index of 1.7. Chitosan (average MW= 45 kDa with a degree of acetylation >75%) was obtained from Sigma Aldrich. Citrate gold nanoparticles (AuNPs, 23 nm) were synthesized using the classic citrate reduction method [19]. Glutaraldehyde (GA, 25 wt% aqueous solution), 4-aminothiophenol (4-ATP, 97%), the enzyme glucose oxidase extracted from *Aspergillus niger* (GOD, 100–250 kU/g solid, type X-S) was purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), acetic acid (99.8 %), D-(+)-Glucose were also purchased from Sigma-Aldrich. The ethanol, acetone, sulfuric acid (95%), and hydrogen peroxide (30% in water) were purchased from Fluka and used for gold electrode pretreatment. Phosphate buffered saline (PBS, pH 7.4, 0.1 M) was prepared from monopotassic and dipotassic phosphate salts, sodium and potassium chlorides from Sigma Aldrich and adjusted to pH 7.4. Potassium ferricyanide  $[K_3Fe(CN)_6]$  and potassium ferrocyanide  $[K_4Fe(CN)_6 \cdot 3H_2O]$  were provided by Sigma-Aldrich. All reagents were used without further purification. Aqueous solutions were prepared using ultrapure water from a MilliQ purification system.

### 2.2. Synthesis of gold nanoparticles

AuNPs were synthesized according to the classical following procedure [19]. 250  $\mu$ L of an aqueous solution of tetrachloroauric (III) acid trihydrate  $HAuCl_4$  (2 % (w/w) was dissolved in 60 ml ultrapure in a 150 ml beaker. The solution was heated and stirred until it boiled.

In addition, 1 mL of 1 % (w/w) aqueous solution of trisodium citrate was prepared and added to the first beaker under vigorous stirring. The color of the gold chloride solution turned from pale yellow to wine-red 60s after heating was stopped, indicating the formation of a gold cluster. The mixture vessel was then allowed to cool to room temperature. The final stabilized AuNPs were characterized by UV-vis spectrometry and the results are presented in Figure 1a. The absorption peak found at around 527 nm is attributed to the characteristic plasmon band of the AuNPs.

The size of 23 nm and the morphology of AuNPs were determined by transmission electron microscopy (TEM) as shown in Figure.1b. The Transmission electron microscopy (TEM) images were obtained using a Philips CM120 with an accelerating voltage of 120 kV. AuNPs were examined after deposition of 3  $\mu$ L of diluted solutions on a formvar- coated copper grid and evaporation to dryness.



**Figure 1.** UV-vis spectrum of synthesized AuNPs suspension (a), TEM image of AuNPs (b).

### 2.3. Instrumentation

In this work, all electrochemical measurements were performed by cyclic voltammetry (CV) and square wave voltammetry (SWV) at room temperature using a Voltalab 80 model PGZ 402 analyzer instrument (Hach Lange, France) controlled with Voltamaster 4.0 software. All experiments were carried out using a 5 mL electrochemical cell connected to a conventional three electrode system with a platinum plate as an auxiliary electrode (active surface: 0.19 cm<sup>2</sup>), a saturated calomel electrode (SCE) as a reference electrode, and a gold plate electrode as the working electrode (active surface: 0.07 cm<sup>2</sup>).

Voltammetric experiments were carried out under stirring in 0.1 M phosphate buffer solution (PBS) pH 7.4 containing 10 mM Fe[(CN)]<sup>3+/4+</sup> couple. The potential was cycled from 0 mV and +800 mV with a scan rate of 100mV/s. Square wave voltammetry (SWV) was carried out between 0 mV and +800 mV with a pulse amplitude of 1 mV, a pulse width of 0.02 ms, and a potential increment of 10 mV.

AFM was used to investigate the surface topography of the PEC/AuNPs/GOD film immobilized on the clean gold surfaces. The AFM measurements were carried out using an Agilent 5500 AFM (Agilent Technologies, Palo Alto, CA, USA). Silicon tips with a nominal spring constant of 20 nm<sup>-1</sup> were used in tapping mode at a frequency of ~300 kHz.

#### 2.4. Preparation of polysaccharide complex PEC /AuNPs/GOD mixture

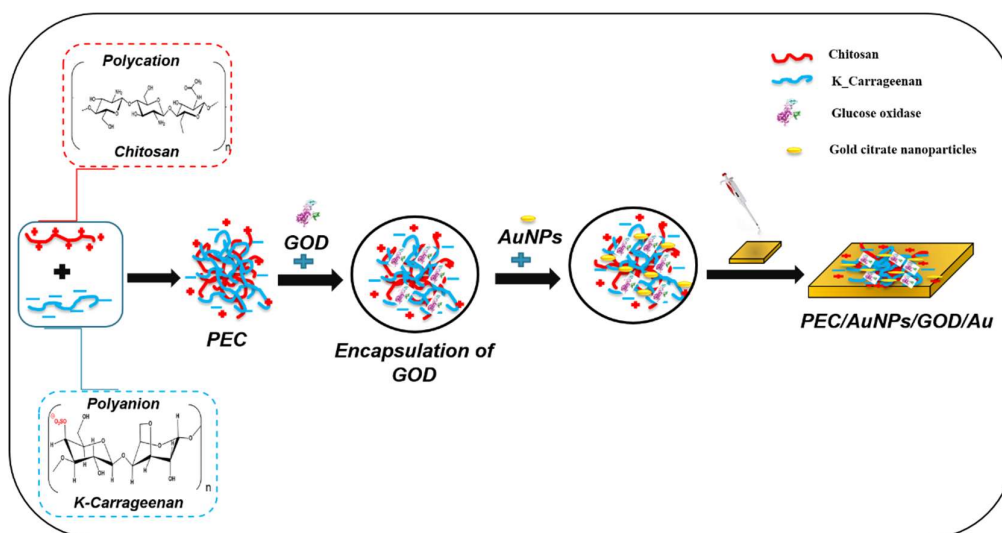
The polyelectrolyte complex (PEC) with oppositely charged chitosan/κ-carrageenan showed a high encapsulation efficiency of 79% at charge ratios of 3. The kappa-carrageenan solution (1 mg/2 ml of water) was mixed with a chitosan solution to complete the coacervation process. The chitosan was prepared with 3 mg in 2 ml liquid composed of 1.4 mL of HEPES buffer solution at pH 7.4 completed with 0.6 mL of acetic acid. The mixture was allowed to stabilize at room temperature for 15 min. Then, for the encapsulation of the enzyme, 5 mg of glucose oxidase (GOD) was added to the 1ml polyelectrolyte complex solution and mixed again. Another 15 min of stabilization at room temperature was then allowed. 20 μl of AuNPs ( $8 \times 10^4$  AuNPs) was added to 1 ml of the PEC/GOD and mixed again, followed by 15 min of stabilization. Thereafter the final mixture was kept for 24 hrs at 4°C.

#### 2.5. Elaboration of the glucose biosensor

Gold substrates were fabricated by the French RENATECH network (LAAS, CNRS Toulouse). They were constructed using standard silicon technologies. (100)-oriented, P-type (3–5 Ω.cm) silicon wafers were thermally oxidized to grow an 800 nm-thick field oxide. Then, a 30 nm-thick titanium layer followed by a 300 nm thick gold top layer were deposited by evaporation under vacuum.

Before functionalization of the working electrode, the surface of a gold microelectrode (1.2×1.2 cm<sup>2</sup> square plates) was cleaned for 5 min in acetone in an ultrasonic bath, then in ultra-pure water. Finally, it was dipped for 5 min at room temperature in a piranha solution (H<sub>2</sub>SO<sub>4</sub>:H<sub>2</sub>O<sub>2</sub> = 3:1 v/v), then cleaned in ultra-pure water, and dried under a nitrogen flow.

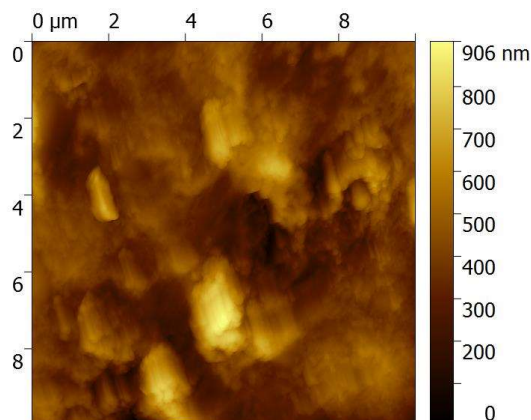
Subsequently, the gold electrode was incubated in an ethanolic solution of 4-ATP (10 mM) for 12 hrs at 4°C in order to form an anchoring layer at the electrode surface. Then the substrate was rinsed with ethanol to remove the unbonded thiols and dried under nitrogen stream. After that, 10 μl of the homogenized mixture [PEC/AuNPs/GOD] was dropped onto the working electrode surface. The modified electrode was then placed for 20 min in saturated glutaraldehyde vapor (cross-linker) and was stored for 24 hrs at 4°C. All the modified electrodes were immersed in PBS (0.1 M, pH 7.4) to examine their water stability. The different steps of preparation the glucose biosensor are summarized in Figure 2.



**Figure 2.** Schematic drawing of complexation of oppositely charged polysaccharides coupled with gold nanoparticles and elaboration of PEC/AuNPs/GOD/Au.

The morphology of the PEC/AuNPs/GOD) film deposited on the gold slides was exhibited using AFM (Figure 3). Some figures, due to stratification of the film, appear on the surface. This

phenomenon is due to the interaction of PEC with gold nanoparticles, leading to the self-organization effect [17, 18].



**Figure 3.** AFM image of a gold electrode modified with PEC/AuNPs/GOD/Au.

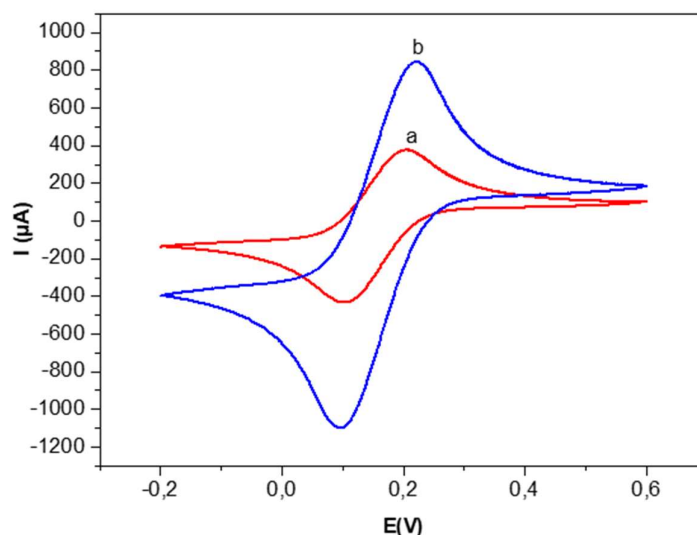
### 3. Results

#### 3.1. Electrochemical characterization of the modified gold electrode

First, cyclic voltammetry (CV) was used to investigate the electrochemical properties of the gold electrodes modified by the complex of polysaccharide mixed with glucose oxidase (**PEC/GOD/Au**) before and after AuNP doping.

The modified gold electrode was electrochemically characterized in 0.1M phosphate buffer solution at pH 7.4 in 1 mM of  $[\text{Fe}(\text{CN})_6]^{3/4-}$  solution. The separation between anodic and cathodic peak potentials ( $\Delta E_p = E_{pa} - E_{pc}$ ) as well as the intensity of the peaks ( $I_{pa}$  and  $I_{pc}$ ) are correlated to the electron transfer properties of the electrodes.

The cyclic voltammograms before and after doping with AuNPs are presented in Figure 4. Both oxidation and reduction peaks of the  $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$  couple are clearly detected, the observed value of  $\Delta E_p$  is 0.09 mV and anodic peaks 397  $\mu\text{A}$ . After AuNPs doping (Figure 4b) of the biocomposite film (CPE/AuNPs/GOD),  $\Delta E_p$  slightly increases from 0.09 mV and 0.12 mV and the anodic peak greatly increases from 397  $\mu\text{A}$  to 996  $\mu\text{A}$ , indicating an increase of the electron transfer rate in the presence of Au nanoparticles.

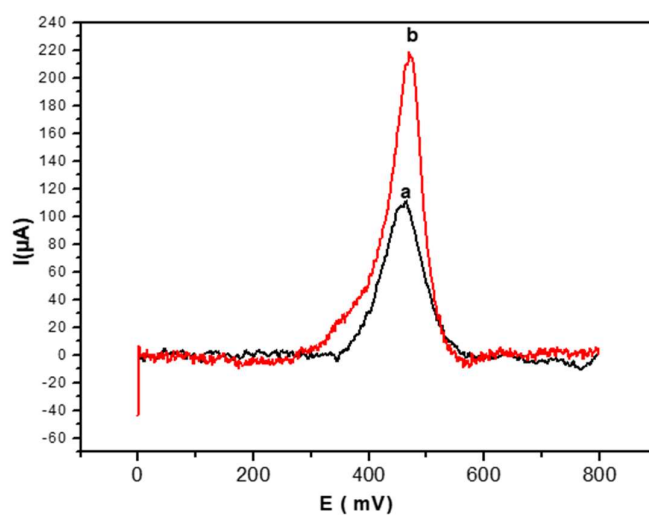


**Figure 4.** CVs curves of the PEC/GOD modified gold electrode in the absence (a) and the presence of AuNPs (b). Measurements in 10 mM  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  PBS solution (0.1M, pH 7.4) and scan rate 100 mV/s.

### 3.2. SWV response of the glucose biosensor

The SWV response of the glucose biosensor was studied at a concentration of 0.2 mM of glucose. As seen in Figure 5, the SWV anodic peak obtained by the electrode modified by PEC/GOD/Au is (curve 5a) 460 mV and the peak intensity value is 111  $\mu\text{A}$ .

After AuNPs doping of the biofilm, the intensity of the anodic peak (curve 5 b) increases from 111  $\mu\text{A}$  to 217  $\mu\text{A}$ , and the peak potential value also shifts towards positive values, from 460 mV to 470 mV.



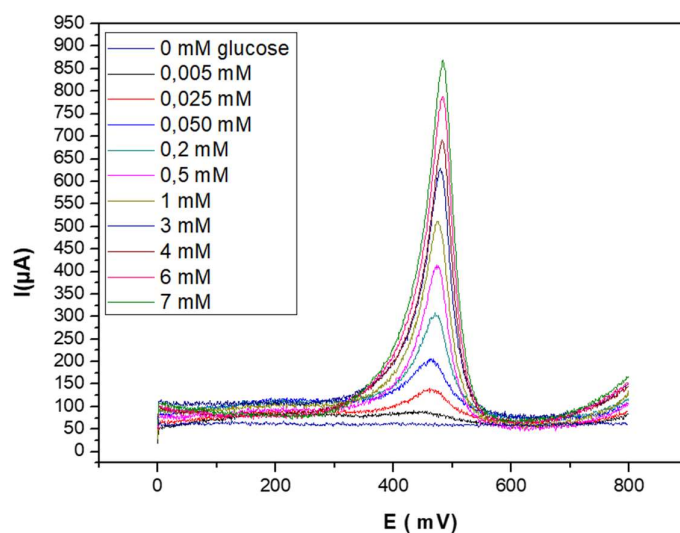
**Figure 5.** SWV anodic peak for glucose detection (0.2 mM) obtained with the PEC/GOD modified gold electrode in the absence (a) and the presence (b) of AuNPs. SWV performed at 100 mV/s in PBS solution (0.1M, pH 7.4).



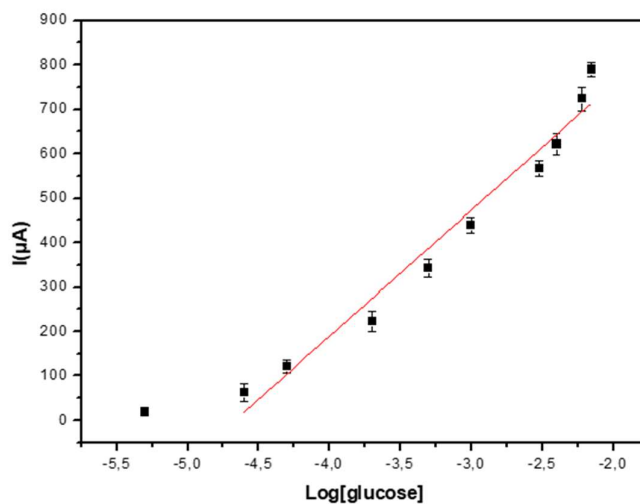
### 3.3. SWV response of PEC/AuNPs/GOx/Au biosensor when the concentration of glucose increases

The electroanalytical characterization of glucose biosensors was performed by using SWV as the detection technique, recording the current intensity at the PEC/AuNPs/GOD/Au modified electrode at different concentrations of glucose, after 5 min of contact. This time was sufficient to achieve a stable signal for each injected concentration. As seen in Figure 6, the biosensor signal increased with glucose concentration in the 0 mM–7 mM range. The sensitivity of the technique, corresponding to the slope of the linear part of the curve, was  $283.9 \mu\text{A}/\log[\text{glucose}]$ .

The calibration curve shown in Figure 7 presents a limit of detection (LOD), calculated as the concentration of glucose giving a signal equal to three times the standard deviation on the blank (in absence of glucose), of  $5 \mu\text{M}$ , and a dynamic linear range between  $10 \mu\text{M}$  to  $7 \text{ mM}$ .



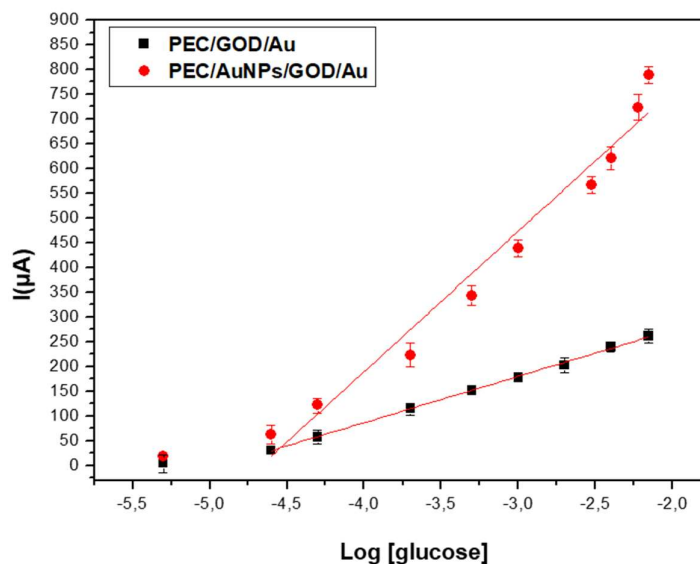
**Figure 6.** SWV curves obtained for PEC/AuNPs/GOD modified gold electrode upon injection of increasing concentrations of glucose (0–7 mM), in PBS solution (0.1M, pH 7.4).



**Figure 7.** Calibration curve of the PEC/AuNPs/GOD/Au biosensor (in PBS 0.1 pH 7.4).

### 3.4. Analytical performance of the glucose biosensor

#### Comparison of biosensor response recorded for glucose in absence and presence of AuNPs



**Figure 8.** Comparison of the SWV responses of PEC/AuNPs/GOD/Au biosensor recorded for glucose (5  $\mu$ M – 7 mM) in absence (■) and presence of AuNPs (●).

As shown in Figure 8, the maximum SWV response for 7 mM of glucose, obtained with a PEC/AuNPs/GOD modified electrode, is 791  $\mu$ A, compared to 261  $\mu$ A for the PEC/GOD modified one [7]. The presence of these gold nanoparticles improves the performance of the glucose sensor based on PEC/GOD, due to the electrocatalytic properties of AuNPs.

The analytical performance of the glucose biosensor based on PEC and glucose oxidase, with and without AuNP doping, is summarized in Table 1. The relative standard deviation is calculated from three different biosensors. The AuNP doping improves the sensitivity of glucose detection and inter-sensor reproducibility.

**Table 1.** Analytical performance of the glucose biosensor based PEC/GOD, with and without AuNP doping.

Biosensor	LOD for glucose	Sensitivity $\mu$ A/log[glucose].	RSD
PEC/GOD/Au (Biosensor without AuNPs)	5 $\mu$ M	93.7	6%
PEC/AuNPs/GOD/Au (Biosensor with AuNPs)	5 $\mu$ M	283.9	4.5%

As shown in Table 2, the analytical characteristics obtained in this study were compared with those of some typical glucose biosensors based on AuNPs and another type of polysaccharide. The results obtained are in the same range as the best voltammetric/ amperometric glucose biosensors



based on relevant AuNPs matrices with polysaccharides, some of them being limited in the dynamic range.

**Table 2.** Comparison of the proposed biosensor with voltammetric/amperometric glucose biosensors based on GOD and polysaccharides coupled with AuNPs.

	Detection limit	Dynamic range	reference
PEC/AuNPs/GOD/Au	5 $\mu$ M	10 $\mu$ M-7mM	This work
Chitosan/PPy/AuNPs/ITO	3.1 $\mu$ M	0 – 3.2 mM	[21]
Chitosan-GOD/AuNPs/GC	13 $\mu$ M	50 $\mu$ M-1.3 mM	[20]
Au/ dithiol/Au/cystamine/GOx	8.2 $\mu$ M	20 $\mu$ M-5.7mM	[22]
Amperometric detection (GOx-AuNP) <sub>n</sub> /Au	2.7 mM	0.005–2.4 mM	[23]
CS/ $\beta$ -G-GOD/AuNPs– CS/PB–CS/Au	1.56 $\mu$ M	6.25–93.75 $\mu$ M	[24]
(GCE/CHI–AuNPs)	370 $\mu$ M	400 $\mu$ L to 10.7 mM	[25]

#### Reproducibility and shelf life of the PEC/AuNPs/GOD based glucose biosensor

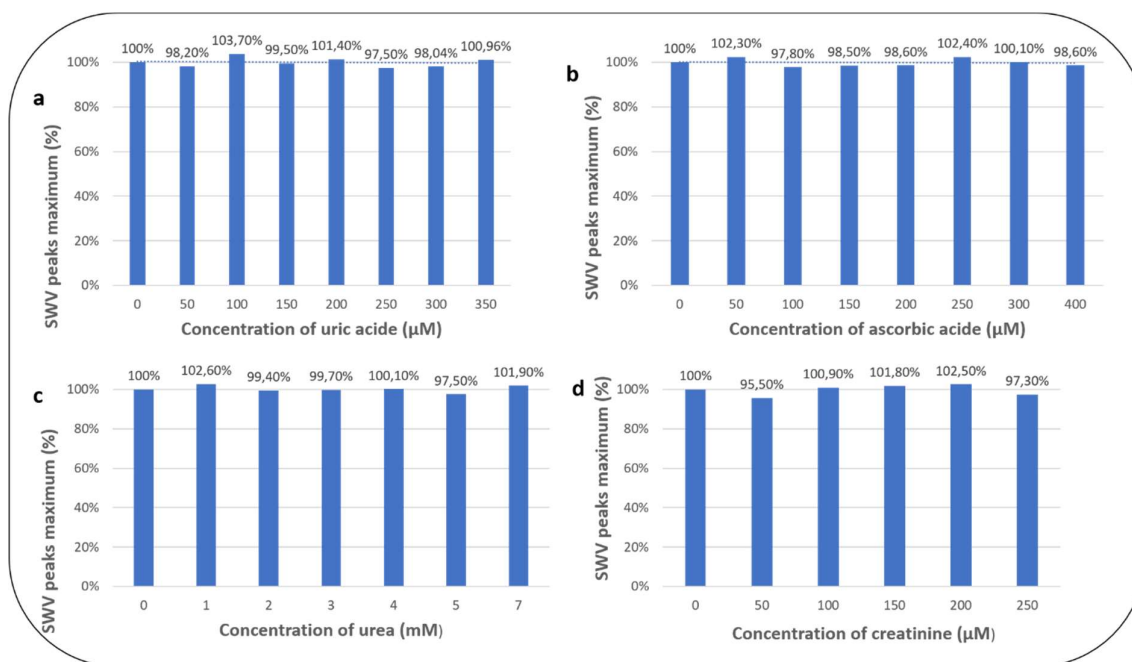
Three independent electrodes were used to measure reproducibility at different glucose concentrations. The resulting relative standard deviation indicates good reproducibility sensor to sensor (4.5%).

Stability is also an important characteristic for the performance of a biosensor. This property was studied over three weeks by using one single glucose biosensor. The results show that the sensor remained stable by providing the same detection range and sensitivity during that period, and also a good relative standard deviation RSD=4.5%. The glucose biosensors based on the polyelectrolyte complex doped with AuNPs were stored at 4 °C in a refrigerator when not in use.

#### Selectivity

In the presence of different analytes which are classical interfering species in glucose determination: uric acid (Figure 9a), ascorbic acid (Figure 9b), urea (Figure 9c) and creatinine (Figure 9d), the PEC/AuNPs/GOD based biosensor was tested for the detection of 0.2 mM of glucose, to demonstrate the selectivity of this biosensor.

The percentage of variation of the peak maximum for the detection of 0.2 mM glucose concentration in the presence of the interfering substances is presented in Figure 9: 99.91% for uric acid, 99.78% for ascorbic acid, and 100.17% for urea and 99.66% for creatinine. These results show that the detection of glucose with PEC/AuNPs/GOD film limits the interference of the electroactive species.



**Figure 9.** SWV peak maximum (%) for the detection of 0.2 mM glucose concentration in presence of different concentration of possibly interfering substances (a) uric acid (b) ascorbic acid (c) urea and (d) creatinine.

### Real sample analysis

The applicability of the glucose biosensor based on PEC/AuNPs/GOD modified gold electrode was investigated. The recovery of glucose was determined in spiked saliva samples after diluting it (10%) in PBS (pH 7.4).

**Table 3.** Glucose detection in real spiked saliva samples.

Added concentration of glucose	Recovery %
50 μM	106
0.2 mM	97

As seen in table 3, the proposed biosensor showed excellent reliability and accuracy for glucose detection in real samples.

#### 4. Conclusion

This study describes a fast and easy way to elaborate chitosan/kappa-carrageenan (PEC)-gold nanoparticle films using a SWV and CV electrochemical methods for the glucose biosensor response.

In addition, the resulting PEC film containing gold nanoparticles can be used to construct biosensors by assembling enzymes on the surface of the film. The immobilized enzymes retain good bioactivity (283.9  $\mu\text{A}/\log[\text{glucose}]$ ) and stability. The obtained biosensor presents good selectivity and is applicable to real biological samples. The film fabrication technique demonstrated in this work and its application to biosensors is readily applicable to the fabrication of other PEC/metal nanoparticle films and biosensors based on nanobiocomposites.

**Author Contributions:** For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used “conceptualization, N.J. and H.M.; methodology, N.J., M.B.; validation, I.R.; formal analysis, A.B., F.B., G.R.; investigation, I.R., M.B.; data curation, I.R.; writing—original draft preparation, I.R.; writing—review and editing, N.J.; supervision, H.M.; funding acquisition, N.J.”.

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**Conflicts of Interest:** The authors declare no conflict of interest

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