

# Ascorbic Acid-Sensitized AuNRs-functionalized nanostructured TiO<sub>2</sub> Transparent Electrodes for Photoelectrochemical Genosensing

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## Abstract

Au nanorods (NRs) modified nanostructured TiO<sub>2</sub>/ITO electrodes have been fabricated and characterized in order to develop a biosensing platform for the photoelectrochemical determination of microRNAs. The proposed method is based on the use of thiolated DNA capture-probes (CPs) immobilized onto Au NR surface. The AuNRs are chemically bound at the surface of TiO<sub>2</sub>/ITO electrodes by means of the mercaptosuccinic acid linker. Subsequently, the DNA CPs are bound to the Au NR surface through the thiolate group, and reacted with the target RNA sequence. Finally, the obtained biosensing platform is incubated with alkaline phosphatase and L-ascorbic acid 2-phosphate (AAP) enzymatic substrate, for the *in-situ* generation of ascorbic acid (AA). Such AA molecule, coordinating to surface Ti atoms, generates a charge transfer complex, that results in a shift of the UV absorption threshold toward the visible spectral region of the nanostructured TiO<sub>2</sub> forming the electrode and, hence, in the occurrence of an absorption band centered at 450 nm. The photoelectrochemical monitoring of the formation of the AA-TiO<sub>2</sub> complex, under the visible light of a commercial LED light source, allows the selective and quantitative detection of the target microRNA strands.

**Keywords:** photoelectrochemical, nanostructured TiO<sub>2</sub>, Au nanorods, nucleic acid, ascorbic acid, small RNAs

## 1. Introduction

Recently, photoelectrochemical (PEC) biosensors have emerged as particularly attractive options among the different biosensing methods. Photoelectrochemical approaches possess advantages of both optical and electrochemical methods by coupling light excitation and electrochemical detection, and thus resulting potentially very sensitive[1] due to the low background current. Moreover, while the all-optical detection methods, such as fluorescence, require complex and expensive optical imaging devices and sophisticated image recognition software, the inherent low cost of the electrochemical instrumentation makes the PEC method simple, rapid and cost effective[2]. Recently, due to the emergence of novel photoelectrochemically active species and new detection schemes, photoelectrochemistry has received increasing attention in biosensing of proteins [3], as well as in the development of hybridization assay[4–9] for the recognition of specific nucleic acid sequences.

In this work, the PEC properties of nanostructured TiO<sub>2</sub> modified ITO electrodes, decorated with colloidal Au nanorods (NRs) have been investigated for the development of a PEC biosensing platform for the determination of small RNAs, known as microRNAs, under visible light illumination. CTAB-capped AuNRs have been synthesized by means of a colloidal chemical route, which is able to control NR size and geometry. The surface of the optically transparent nanostructured TiO<sub>2</sub> electrodes has been modified by immobilizing mercaptosuccinic acid (MSA), which acts as a linker for anchoring the Au NRs. Au nanoparticles, either isotropic or anisotropic, as the NRs, are particularly suited for conjugation with biomolecules[10], preserving their biological activity. Moreover, increasing the surface-to-volume ratio, they represent a powerful tool to modify electrode materials and to construct robust and sensitive biosensors[11]. Thus, the thiolated nucleic acid capture probes (CPs) have been immobilized on the Au NRs and allowed to hybridize with the target biotinylated microRNA sequences. The enzyme alkaline phosphatase (AP) has been used as a label for the biorecognition, since it generates ascorbic acid (AA), that binding to the TiO<sub>2</sub> surface enable its photosensitization and ultimately the detection and quantization of the microRNA (Scheme 1). MicroRNAs (miRNAs) are endogenous, evolutionarily conserved, non-coding RNA molecules of about 20 nucleotides in length, functioning as posttranscriptional gene regulators. They regulate a wide range of biological functions, from cell proliferation and death to cancer progression. The importance of miRNA function suggests that their expression may provide a valuable diagnostic indicator as a cancer biomarker. However, the short nature of miRNA sequences greatly complicates the use of standard molecular biology method based upon PCR. Therefore, significant efforts have been

devoted to the development of innovative analytical methods and, recently, photoelectrochemical genosensing platforms have been extensively studied [4–9]. Different electrode materials, including Bi<sub>2</sub>S<sub>3</sub>NRs [4,5,8], graphite-like C<sub>3</sub>N<sub>4</sub>[6], or CdS modified carbon nanotubes [9] have been used, in presence of AA as electron donor. TiO<sub>2</sub> nanostructures hardly have found a direct application in PEC biosensing, since TiO<sub>2</sub> only absorbs UV-light, that is a type of radiation that can damage biomolecules and requires expensive, highly maintenance and demanding sources typically used for generating exciton in its wide band gap (ca. 3.2 eV) [12]. In order to overcome such a limitation, coupling of TiO<sub>2</sub> with narrow-band gap semiconductors or with organic photosensitizer has been performed, [13–16] in order to shift its absorption in the visible spectral range. In this work, the possibility of exploit *in situ* enzymatically generated AA for genosensing has been studied. AA is a well-known electron donor that has been widely studied in solar energy conversion systems [12,17,18]. Indeed, the surface modification of the nanostructured TiO<sub>2</sub> electrodes with AA, leads to the TiO<sub>2</sub> photosensitization, through the formation of a charge-transfer complex, that ultimately shifts the photoresponse of TiO<sub>2</sub> up to 500 nm [12,17]. In particular, a bidentate chelate binding of AA, through the two ene-diolate oxygen atoms, with the anionic oxygen as electron donor, is assumed responsible of the formation of the charge-transfer complex in the form of a chelate ring structure. Remarkably, such a photosensitization process of the AuNR surface modified nanostructured TiO<sub>2</sub> electrodes, occurs only upon AA generation from the enzymatic reaction labeling the hybridization event, thus allowing the PEC detection of the miRNA target sequence, under visible-light illumination. The photocurrent spectrum of the TiO<sub>2</sub> film sensitized by AA is characterized by a maximum located at  $\approx 450$  nm. Such a photocurrent is due to a rapid visible light-induced electron injection from AA into the conduction band of TiO<sub>2</sub>. The preliminary results of the developed genosensor for miRNA determination indicate the reliability of the proposed approach. The obtained results demonstrate that the miRNA detection can be accomplished by using a compact analytical device, equipped with a LED-light source, that allow to obtain a prototype compliant with the requirements and the characteristic of a point of care testing (POCT).

## **2. Materials and Methods**

### **2.1. Reagents**

Dithiothreitol (DTT), 6-mercapto-1-hexanol (MCH), streptavidin–alkaline phosphatase (S2890, Strept-AP, 2:1 conjugation stoichiometry), diethyl pyrocarbonate (DEPC), ascorbic acid (AA), L-ascorbic acid 2-phosphatetrisodiumsalt (AAP), mercaptosuccinic acid (MSA), bovine serum albumin (BSA), Tris–HCl, sodium borohydride ( $\text{NaBH}_4$ , ~99%), hydrogen tetrachloroaurate(III) trihydrate ( $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ , 99.9%), silver nitrate ( $\text{AgNO}_3$ , 99.9999%), were obtained from Sigma–Aldrich (Milan, Italy). Disodium hydrogen phosphate, sodium dihydrogen phosphate, magnesium and potassium chloride were purchased from Merck (Milan, Italy). NAP-10 columns of Sephadex G-25 were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Nanostructured  $\text{TiO}_2/\text{ITO}$  electrodes were purchased from Solaronix (Aubonne, Switzerland). MilliQ water (DEPC treated for RNA analysis) was used throughout this work.

Synthetic oligonucleotides were obtained from MWG Biotech AG (Germany), namely:

probe (DNA-SH): 5' GAA-ACC-CAG-CAG-ACA-ATG-TAG-CT – SH 3',

target miRNA 221: 5' AGC-UAC-AUU-GUC-UGC-UGG-GUU-UC – biotin 3',

non-complementary (miRNA 16): 5' UAG-CAG-CAC-GUA-AAU-A-biotin-3'

Prior to use, the thiol-modified oligonucleotides were treated with DTT. This reagent allowed reduction and cleavage of oligo dimers eventually obtained by oxidative coupling of two DNA-SH molecules (i.e. DNA-S-S-DNA). The lyophilized oligonucleotides were dissolved in a 10 mM Tris-HCl buffer solution (pH 8.3) containing 20 mM of DTT. The reaction was allowed to proceed for 2 h at room temperature. The thiolated DNA was then purified by elution through a NAP-10 column of Sephadex G-25 using 0.5 M phosphate buffer (pH 7.4). DNA-SH stock were prepared in the same buffer and stored frozen.

Human serum type AB (Sigma–Aldrich (Milan, Italy) was diluted 1:100 (v/v) in Tris buffer and filtered (0.45  $\mu\text{m}$ ).

## 2.2. Synthesis and characterization of CTAB–coated Au NRs

CTAB–coated Au NRs were prepared by using the seed-mediated growth method [19] reported in literature. The synthesis relies on two steps: firstly, a seeds solution containing CTAB-stabilized Au NPs (< 3 nm) was prepared by reducing an Au precursor,  $\text{HAuCl}_4 \times 3\text{H}_2\text{O}$  (5 mL,  $5 \times 10^{-4}$  M), with  $\text{NaBH}_4$  (0.01 M) in CTAB (5 mL, 0.2 M), and then kept under vigorous stirring for 2 h. Next, a growth solution of Au NRs was prepared: 8.5 mg of  $\text{AgNO}_3$ , 18.22 g of CTAB and 100 mg of  $\text{HAuCl}_4 \times 3\text{H}_2\text{O}$  were dissolved in 500 mL of deionized water. This mixture was kept at room temperature under continuous stirring. Then, 7 mL of a 0.0778 M aqueous solution of AA were added dropwise to the above mixture.

When the mixture becomes colorless (indicative of the reduction of Au(III) to Au(I))[19], 0.8 mL of the seeds solution was added to the growth solution and a dark-red color developed within 10 min[20]. After preparation, cycles of centrifugation were performed at 7000 rpm for 20 min, to wash the Au NRs to discard uncoordinated CTAB.

CTAB-coated Au NRs were optically and morphologically characterized by means of UV-Vis absorption spectroscopy and Transmission Electron Microscopy (TEM). UV-Vis absorption spectra of solutions of the as prepared Au NRs were recorded at room temperature, by using a Varian Cary 5000 UV-Vis-NIR scanning spectrophotometer in the 300–1200 nm range.

TEM analyses were performed with a Jeol JEM-1011 microscope, operating at 100 kV. The specimens were prepared by depositing few drops of aqueous Au NR dispersions onto a carbon-coated copper grid and then allowing the solvent to evaporate.

### **2.3. Modification and characterization of the nanostructured TiO<sub>2</sub>/ITO electrodes by Au NRs**

Commercial nanostructured TiO<sub>2</sub>/ITO electrodes were surface modified with CTAB-capped Au NRs, by using mercaptosuccinic acid (MSA) as a linker molecule. Namely, the TiO<sub>2</sub>/ITO electrodes were incubated for 24 h in a 1 M solution of MSA, and then, in a  $6 \times 10^{-9}$  M solution of CTAB-coated Au NRs at pH 9, for 3 h.

The bare TiO<sub>2</sub>/ITO electrodes and the prepared AuNRs/MSA/TiO<sub>2</sub>/ITO electrodes were morphologically characterized by Field Emission Scanning Electron Microscopy (FE-SEM) by using a Zeiss Sigma microscope, equipped with an in-lens secondary electron detector and an INCA Energy Dispersive Spectroscopy (EDS) detector. The micrographs of the bare TiO<sub>2</sub>/ITO and AuNRs/MSA/TiO<sub>2</sub>/ITO electrodes were collected at an acceleration voltage and magnification of 5.0 kV and 48.06 KX and 3.0 kV and 46.53 KX, respectively and with an aperture size of 30  $\mu$ m. Samples were mounted onto stainless-steel sample holders by a double side carbon tape and grounded by silver paste.

### **2.4. Thiolated DNA Capture-probe (CP) immobilization**

AuNRs/MSA/TiO<sub>2</sub>/ITO electrodes were exposed to 20  $\mu$ L of the thiolated oligonucleotide solution (10  $\mu$ M in 0.5 M phosphate buffer, pH =7.2). Chemisorption was allowed to proceed overnight ( $\approx$  16 h). During incubation, the electrode cell was covered with parafilm in order to prevent the evaporation of the oligonucleotide solution. The immobilization step was followed by incubation with 20  $\mu$ L of 1 mM 6-mercapto 1-hexanol (MCH) aqueous solution

for 1 hour. Such a step intends to modify AuNP surface active sites and avoid non specific adsorption of biotinylated target strands, increasing hybridization efficiency[21]. Prior to hybridization reaction, the modified electrode was washed twice with 0.5 M phosphate buffer pH 7.2 (PB).

## **2.5. Hybridization and labeling**

The probe-modified electrode was then exposed to a 20  $\mu\text{L}$  of the biotinylated target sequence in PB solution for 20 min. After hybridization, the sensor was washed twice with 10 mM Tris-HCl buffer, pH 9.8. The biotinylated hybrid obtained at the electrode surface was reacted with a 20  $\mu\text{L}$  solution containing 4 U/mL of the streptavidin-alkaline phosphatase conjugate and 10 mg/mL of BSA (blocking agent [21]) in 10 mM Tris buffer, pH= 9.8.

After 20 minutes, the sensor was washed twice with Tris buffer. The photoelectrochemical cell was then filled with 100  $\mu\text{L}$  of an L-ascorbic acid 2-phosphate (AAP) enzymatic substrate solution (10 mg/mL in 10 mM Tris buffer, pH =9.8). After 20 minutes of incubation, photocurrents were amperometrically detected.

## **2.6. Electrochemical and Photoelectrochemical Characterization and Detection**

Electrochemical measurements were performed with an Autolab PGSTAT10 computerized electrochemical system equipped with the FRA2 frequency response analyzer and controlled by GPES 4.9 software (EcoChemie). A Pt wire, Ag/AgCl wire, and AuNRs/MSA/TiO<sub>2</sub>/ITO disk served as the auxiliary, pseudo-reference, and working electrode, respectively. The electrodes were mounted in a customized cell constituted of two parts forming a Plexiglas box, as reported in Scheme 2. The modified sensor was located in a slot created on the bottom part of the cell while two screws allowed to fix the top part, where a cylindrical well housed both illumination system and solution. The electrode geometric area was 0.2 cm<sup>2</sup>. The distance of the LED from the electrode surface defines a cell volume of 100  $\mu\text{L}$ . An O-ring controlled the tightness at the electrode surface. For the PEC characterization of the bare TiO<sub>2</sub>/ITO, of the MSA/TiO<sub>2</sub>/ITO and AuNRs/MSA/TiO<sub>2</sub>/ITO electrodes, an ozone free Xenon lamp emitting in the 200-2000 nm range (450 W, Orion) coupled with a monochromator (Jobin-Yvon) was used as light source with an average emission power of 100  $\mu\text{W}/\text{cm}^2$ . A series of mirror and glasses were used in order to direct the light on the working electrode surface.

For miRNA determination, AA photocurrents, at 0 V vs Ag/AgCl, were amperometrically detected, as the analytical signal, under illumination with a commercial white LED (3 mm

white LED, Nichia Corporation) emitting in the visible range 400-700 nm with maxima at 456 and 549 nm (Figure S1), respectively, and intensity in the mW/cm<sup>2</sup> order. All photoelectrochemical measurements were referred to the Ag/AgCl wire reference electrode at room temperature (25°C). It should be noted that a new CP-modified PEC biosensor was used for each tested miRNA concentration.

Cyclic Voltammetry (CV) measurements were performed at different scan rate in 0.1 M KCl solution.

Electrochemical Impedance Spectroscopy (EIS) measurements were performed with  $E_{dc}$  in the range from -0.2 to + 0.8 V, every 0.1 V with the parameters showed in Table S1. Each spectra was fitted by using ZView software.

The flat band potential,  $E_{fb}$ , and donor density,  $N_D$ , were obtained from Mott – Schottky equation [22]

$$\frac{1}{C^2} = \frac{2}{e\epsilon\epsilon_0 A^2 N_D} \left( E - E_{fb} - \frac{k_B T}{e} \right) \quad [\text{Eq.1}]$$

where  $C$  and  $A$  are the interfacial capacitance and electrode surface area, respectively,  $E$  the applied voltage,  $k_B$  is Boltzmann's constant,  $T$  the absolute temperature,  $\epsilon$  is the dielectric constant of the semiconductor,  $\epsilon_0$  is the permittivity of a free space (vacuum) and  $e$  is the electronic charge. Therefore, a plot of  $1/C^2$  against  $E$  should yield a straight line from which  $E_{fb}$  can be determined from the intercept on the  $E$  axis. The value of  $N_D$  can also be conveniently evaluated from the slope knowing  $\epsilon$  and  $A$  of the electrode. The values of  $1/C^2$  were obtained from EIS data by using the following equation:

$$\frac{1}{C^2} = \left( \frac{-Z''^2 + (Z' - R_\Omega)^2 \omega^2}{-Z''} \right)^2$$

where  $R_\Omega$  is the solution resistance,  $Z'$  and  $Z''$  the impedance values registered at the chosen angular frequencies  $\omega$  ( $2\pi f$ , with  $f = 1000, 100, 10$  Hz).

~~All the spectra recorded at each  $E_{dc}$  potential were fitted with a model circuit showed in Figure S2 in order to evaluate the capacitance  $C$  for Mott – Schottky method.~~

### 3. Results and discussion

#### 3.1. Electrochemical and photoelectrochemical characterization of the nanostructured TiO<sub>2</sub>/ITO electrodes

The TiO<sub>2</sub>/ITO electrode was electrochemically characterized in view of its application as a platform for AAPEC detection.

Electrochemical impedance spectroscopy, a valuable technique to characterize the electrochemical behavior of semiconductor electrodes in an electrolyte solution, was employed at different applied voltage bias, ranging from – 0.2 V to + 0.8 V (SCE). For all the potentials the complex plane plots (Figure 1a) show two semicircles, fitted with the equivalent circuits represented in Figure S2. In particular, the cell resistance  $R_{\Omega}$  is in series with two consecutive resistances and constant phase elements in parallels. The constant phase elements (CPE) describe the charge separation and are modelled as  $[(Ci\omega)^{\alpha}]^{-1}$ , representing a pure capacitance for  $\alpha=1$  and a non-ideal capacitance for  $0.5<\alpha<1$ .  $R_1$  and CPE<sub>1</sub> refer to the resistance and capacitance related to the TiO<sub>2</sub>-electrolyte solution interfase, while  $R_2$  and CPE<sub>2</sub> to the TiO<sub>2</sub>-ITO interfase. The values obtained from the fitting are reported in Table S2. The cell resistance is in general not influenced by the voltage variation, while the other parameters change especially at negative potentials. This phenomenon is due to the proximity with the TiO<sub>2</sub> flat band potential (evaluated in the next paragraph) for  $R_1$  and CPE<sub>1</sub> and with the tin reaction of ITO [Ref plos one] for  $R_2$  and CPE<sub>2</sub>. Moreover,  $R_1$  are an order of magnitude lower than  $R_2$ , since they are related to a solid-liquid and to a solid-solid interfase, respectively. Finally,  $\alpha_1$  is lower than 1, showing a non-ideal capacitive behavior for the electrolyte-TiO<sub>2</sub> interfase, probably ascribable to the nanoporous surface morphology of the electrode (Figure S3). The situation is different for the case of ITO-TiO<sub>2</sub> interfase, with  $\alpha_2$  very close to 1, due to a more homogeneous structure of ITO.

The study of capacitance variation at different chosen frequencies allows to describe the relationship between semiconductor and electrolyte energy levels, correlating it to the *flat band potential* ( $E_{fb}$ ) of the semiconductor through the Mott – Schottky equation (Eq. 1). [23]. Figure 1b displays the Mott–Schottky diagram of  $1/C^2$ , as a function of the applied voltage bias at different frequencies (see the experimental part for further details), from which a positive slope was observed, confirming a *n-type* semiconductor behavior. Furthermore, the plot was extrapolated to ( $1/C^2=0$ ) to estimate, by following Eq. [1], the value of  $E_{fb}$ , namely ( $-283\pm 40$ ) mV, that is in agreement with the data reported in literature [24],[25]. In addition, the carrier density (or doping level  $N_D$ ) was calculated, assuming  $\epsilon$  for TiO<sub>2</sub> equal to 173[26],  $\epsilon_0= 8.85 \cdot 10^{-12}$ F/m[27],  $A = 0.2$ cm<sup>2</sup> (0.5 cm diameter) and  $e= 1.6\cdot 10^{-19}$ C, giving  $N_D = 2.7\cdot 10^{22}$ cm<sup>-3</sup>, in accordance to the literature value for  $N_D$  that is in the range of  $10^{18}$  to  $10^{23}$  cm<sup>-3</sup>, depending on the TiO<sub>2</sub> nanocrystal film thickness and the electrolyte used[28].



The above results have been obtained considering the Mott-Schottky data linear part closer to the potential axis. Above +0.1 V a non-linear trend is evinced, already observed in the Literature [Baumanis, Bahnemann, *J. Phys. Chem. C*, 2008, 112, 19097 + Wu, Xu, Yan, *Appl. Surface Science* 386 (2016) 1-13], which could be due to different factors: non-uniform distribution of donors; dielectric relaxation phenomena due to surface structure inhomogeneities; film amorphous nature. [Baumanis, Bahnemann, *J. Phys. Chem. C*, 2008, 112, 19097]. However, the real nature of these behaviors is still to be clarified.

CV of the TiO<sub>2</sub>/ITO electrodes were also recorded in the presence of 0.1 M KCl, at three different voltage scan rates, as shown in Figure 1c. All voltammograms show a large potential range where the current is only capacitive (between -0.2 to +0.8 V). In this range, the absence of faradaic phenomena on the electrode surface preserves the electrode from any modification. Figure 1d shows CV measurements performed in 0.1 M KCl in presence and absence of 10 mM AA. From the voltammograms, the potential range, where faradaic phenomena are absent, is approximately between -0.1 and +0.2 V vs Ag/AgCl.

### 3.2. Nanostructured TiO<sub>2</sub>/ITO photoanode modification with the AuNRs

CTAB-coated Au NRs were synthesized by following the procedure reported in [19], [29] and characterized by UV-Vis absorption spectroscopy and TEM investigation. Figure 2 reports a typical UV-Vis absorbance spectrum of anisotropic Au nanoparticles. In particular, it is possible to distinguish two absorption bands, the former due to light absorption along the short axis at ca. 500 nm and the latter due to absorption along the long axis at ca. 800 nm. Inset of Figure 2a shows a TEM micrograph attesting for the formation of Au NRs having average sizes of  $55 \pm 7$  and  $17 \pm 2$  nm.

Immobilization of the CTAB-coated Au NRs on the nanostructured TiO<sub>2</sub>/ITO electrode was performed by using MSA as an anchoring molecule. The -COOH moiety of MSA is expected to coordinate in a bidentate chelating bond the TiO<sub>2</sub> electrode surface, whereas -SH groups bind the Au NR surface. Figure 2b reports the SEM micrograph of a TiO<sub>2</sub>/ITO supporting electrode surface modified by Au NRs, while the SEM image of the bare nanostructured TiO<sub>2</sub>/ITO electrodes are reported in Figure S3. The secondary electron microscopy investigation shows that, after the incubation of the MSA/TiO<sub>2</sub>/ITO electrode in the solution of the CTAB-capped Au NRs, the nanoporous surface of the TiO<sub>2</sub> electrode is coated with highly bright elongated nanostructures, characterized by a morphology compatible with that observed for the CTAB-coated Au NRs in the TEM micrograph (Figure 2a, inset).

CVs recorded at AuNRs/MSA/TiO<sub>2</sub>/ITO electrode in 0.1 M KCl, in absence (i) and presence (ii) of 10 mM AA, are reported in Figure 2c. The electrochemical oxidation of AA starts at potential value higher than +0.2V. Instead, in the potential window between -0.1 - + 0.1 V faradaic phenomena are absent, confirming that the current is capacitive. The inset of Figure 2c shows an enlargement of the CV recorded at the at AuNRs/MSA/TiO<sub>2</sub>/ITO electrode (iii) in comparison to the CV recorded at the MSA/TiO<sub>2</sub>/ITO electrode (iv), confirming that the current is capacitive, in this potential window, for both electrodes. Thus, 0 V was the potential chosen for photocurrents recorded in presence of AA.

Figure 2d reports the photocurrents of the pristine TiO<sub>2</sub>/ITO, of the MSA/TiO<sub>2</sub>/ITO and of AuNRs/MSA/TiO<sub>2</sub>/ITO electrodes, respectively, as a function of the selected illumination wavelength range between 300-600 nm. The experiments were performed in Tris buffer, under the applied potential of 0 V vs Ag/AgCl. The figure shows for the bare TiO<sub>2</sub>/ITO electrodes a maximum of photocurrent at 350 nm of  $5.6 \pm 0.4 \mu\text{A}$ , which decreases of  $\approx 13\%$  down to  $4.9 \pm 0.3 \mu\text{A}$ , in the MSA/TiO<sub>2</sub>/ITO photoanode, attesting for a slightly lower photoactivity of the electrode after immobilization of the MSA linker. By contrast, the AuNRs/MSA/TiO<sub>2</sub>/ITO electrodes exhibit a significant decrease of the photoactivity in the UV range, namely of  $\approx 49\%$  at 350 nm, with a photocurrent of  $2.8 \pm 0.1 \mu\text{A}$ , that is substantially lower than that of pristine TiO<sub>2</sub>/ITO. Such a decrease could be reasonably due to a resistive behavior of the CTAB organic layer coating the Au NRs, responsible for a shielding effect towards the TiO<sub>2</sub>/ITO surface. The photocurrents of all electrodes are negligible in the visible light spectral range in the absence of AA (Figure 2d), and are not affected by the direction of the wavelength scan (data not shown).

Finally, Figure 3 reports the photocurrents of the AuNRs/MSA/TiO<sub>2</sub>/ITO electrodes, recorded at 0 V vs Ag/AgCl, at increasing concentration of AA, at different wavelengths in the 300-600 nm range. Figure 3a shows a decrease of the photocurrent value at 350 nm with the increase of the AA concentration, and concomitantly, an increase of the photocurrent in the range of wavelengths between 400- 600 nm, with a local maximum centered at  $\approx 450$  nm. The increase of the photocurrent in the visible spectral region can be attributed to the occurrence of a charge transfer complex, due to the binding of AA at the surface of TiO<sub>2</sub>/ITO electrode. It has been reported that the chelation of Ti<sup>4+</sup> with ascorbic acid, which is a well-established phenomenon that takes place via two hydroxyl groups by proton elimination, results in a sensitization process, due to the shift of the absorption threshold of the TiO<sub>2</sub> electrodes up to  $\approx 1.2$  eV toward the visible region [18]. Moreover, in concomitance to the increase of the

photocurrent at 450 nm, a decrease of the photocurrent value at 350 nm, increasing the AA concentration, was observed, thus further proving the electrode surface modification and the charge-transfer complex formation, extending the absorption towards the visible spectral range. Remarkably, the extent of the complex formation and hence the intensity of the related photocurrent, depend on the concentration of AA.

Upon exposure to AA, the surface of the sensor was regenerated with a simple washing step with demineralized water. Figure 3a (0 mM AA, clean) clearly points out that the photocurrent signal recorded after rinsing of the AuNRs/MSA/TiO<sub>2</sub>/ITO electrode with demineralized water is almost the same as that recorded before exposure to AA, thus demonstrating the full recoverability of the PEC performance of the electrode. Indeed, after photoelectron injection, AA is oxidized to dehydroascorbic acid (DHAA), that subsequently is desorbed from the electrode surface by rinsing [12].

The inter-electrode reproducibility was assessed by measuring the photocurrents at AuNRs/MSA/TiO<sub>2</sub>/ITO electrodes, under UV illumination at 0V. A RSD% of 22% ( $3.5 \pm 0.8 \mu\text{A}$ ,  $n=3$ ) was obtained.

Further experiments were performed in order to evaluate the AA concentration-dependent behavior of the photocurrent, under illumination of a commercial white LED light. To perform the measurements, the photoanode was mounted on *ad hoc* developed electrochemical cell housing both illumination system and solution (Scheme 2). The results show the linear dependence of the photocurrent (Figure 3b), recorded under the white LED light source illumination, as a function of the AA concentration, investigated in the range of 0.1 mM-10 mM in Tris Buffer.

### 3.3. PEC Hybridization assay

Integration of the AuNRs/MSA/TiO<sub>2</sub>/ITO photoanode into a PEC biosensor for small RNA detection was achieved via a surface functionalization process of Au NRs by a thiolated CP. The scheme of the assay is shown in Scheme 1. The capture-probe-modified electrode was reacted with the analyte, the biotinylated target miRNA. Then, the biotinylated hybrid was exposed to the alkaline-phosphatase-streptavidin conjugate and phosphorylated ascorbic acid (AAP). AAP acts as a substrate of the biocatalytic reaction leading to the generation of AA. Such a product of the enzymatic reaction was photoelectrochemically monitored by using amperometry under white LED light illumination. The immobilization of the CP has been monitored by the photocurrent measurements. Indeed, the binding of CP on the

AuNRs/MSA/TiO<sub>2</sub>/ITO electrode is confirmed by the decrease of the photocurrent response, generated in presence of AA (Figure 4a). Such a decrease in the photoelectrochemical current intensity is ascribed to both enhanced steric hindrance and electrostatic repulsion of the negatively charged DNA strands against the diffusion of AA to TiO<sub>2</sub>, thus impeding the charge complex formation. The photocurrent has been monitored also after binding of the target and of the alkaline-phosphatase-streptavidin conjugate to CP (CP/10 nM target miRNA/ALP) (Figure 4b). In the absence of enzymatic substrate, the electrode exhibited a response (curve ii) lower than that of the bare electrode (curve i), in agreement with the literature reports [30], [31], and due to the bio-layer architecture that increased the steric hindrance effect and decreased the electron transfer efficiency. The addition of the enzyme substrate, AAP, causes AA generation and, accordingly, an increase in the photocurrent current intensity at 450 nm (iii), confirming the presence of confined ALP, and thus the feasibility of the functionalization protocol. In order to exclude any spontaneous hydrolysis of AAP, the biosensor was washed with 0.5 M PB, and exposed for 30 min to a 0.5M PB solution in absence and in presence of 10 mg/mL AAP. Phosphate ions present in solution naturally inhibit the enzyme alkaline phosphatase, avoiding AA production. Photocurrents generated in the experiments (curve iv) are comparable to those obtained in absence of AAP (the enzymatic substrate), thus ruling out the occurrence of the spontaneous hydrolysis. This photocurrent variation can be, thus, exploited for detecting the hybridization reaction. The analytical performances of the photoelectrochemical assay were evaluated by designing a calibration experiment (Figure 4c-d). The obtained CP-modified PEC biosensor was tested for different miRNA concentrations, under the conditions reported in the materials and methods section, demonstrating a linear response, with an estimated limit of detection (LOD) of approximately 20 fmole in 10  $\mu$ L of sample solution, calculated using a criterion of the background signal plus three times its standard deviation, and a sensitivity of 1.85 nA/nM. The relative standard deviation (RSD) for five consecutive measurements of 10 nM target miRNA was 5% (n=5) with  $\approx$ 20 nA average anodic current. A typical calibration curve is shown in Figure 4d with relative amperograms in Figure 4c.

The selectivity of the photoelectrochemical genosensor has been investigated by using a miRNA sequence (miRNA 16) that markedly differs from miRNA 221 (the target sequence). Therefore, it can be regarded as a fully non-complementary sequence. Figure 4d shows clearly that the photocurrent response, due to the nonspecific sequence, is lower than that achieved with the specific binding of the target sequence. Reciprocal selectivity of miRNA221 and miRNA222 (a sequence

structurally closely related to miRNA 221, frequently overexpressed in the same human malignancies) was studied in a previous work[32].

Preliminary results on real matrixes, performed by analyzing AA spiked samples, demonstrated the possibility to perform the photocurrent measurements in diluted serum samples. Thus, further research will be aimed to optimize the experimental conditions in order to improve the analytical performance and carry out analysis on real samples. Recently, a procedure to label microRNAs with biotin for the development of an electrochemical genosensor was reported [32]. This procedure should be further implemented and coupled to the proposed photoelectrochemical assay to analyze miRNAs in serum samples. Finally, the possibility to use nanoarchitectures rich in enzyme labels[33], [34] will be evaluated in order to increase the sensitivity.

#### 4. Conclusions

Nanostructured TiO<sub>2</sub>/ITO electrodes were modified and characterized for miRNA detection in a photoelectrochemical enzymatic amplification route. After preliminary electrochemical and photoelectrochemical characterizations of bare TiO<sub>2</sub>/ITO electrodes, showing their *n-type* semiconductor properties, AuNRs were immobilized on the TiO<sub>2</sub>/ITO electrode surface by using MSA as a linker molecule. The modified electrode based on AuNRs/MSA/TiO<sub>2</sub>/ITO was further functionalized with CP for subsequent target hybridization. For this purpose, the ALP catalytic activity was exploited for the *in situ* AA generation, starting from AAP. The AA coordination to the TiO<sub>2</sub> surface enabled the semiconductor sensitization, thus ultimately allowing the quantitative determination of the target sequence in the visible spectral range. Such a novel procedure has been demonstrated effective in a genosensing mechanism for the specific determination of a model target miRNA sequence, miRNA 221, a sequence present in many types of lung and breast cancer. Photocurrent at different wavelengths were evaluated in order to demonstrate the generation of a photocurrent in the visible region, avoiding expensive, dangerous and high maintenance UV light sources. A calibration plot in the 0-50 nM range of miRNA was performed, by using a commercial white LED with a power emission in the mW/cm<sup>2</sup> range. The enzyme amplification produces a sensitivity of 1.85 nA/nM, with an estimated LOD of 20 fmole and an average RSD of 5%. LOD estimated should be considered high for many specific applications, but the possibilities to develop new assay scheme with nanoarchitectures rich in enzyme molecules could further increase the sensitivity of the method. This method is not limited to only hybridization assay, but it can also be extended to other applications and other affinity assay. The investigated assay,

demonstrates the possibility to develop a simple detection scheme with commercial semiconductor electrode and commercial low cost light sources.

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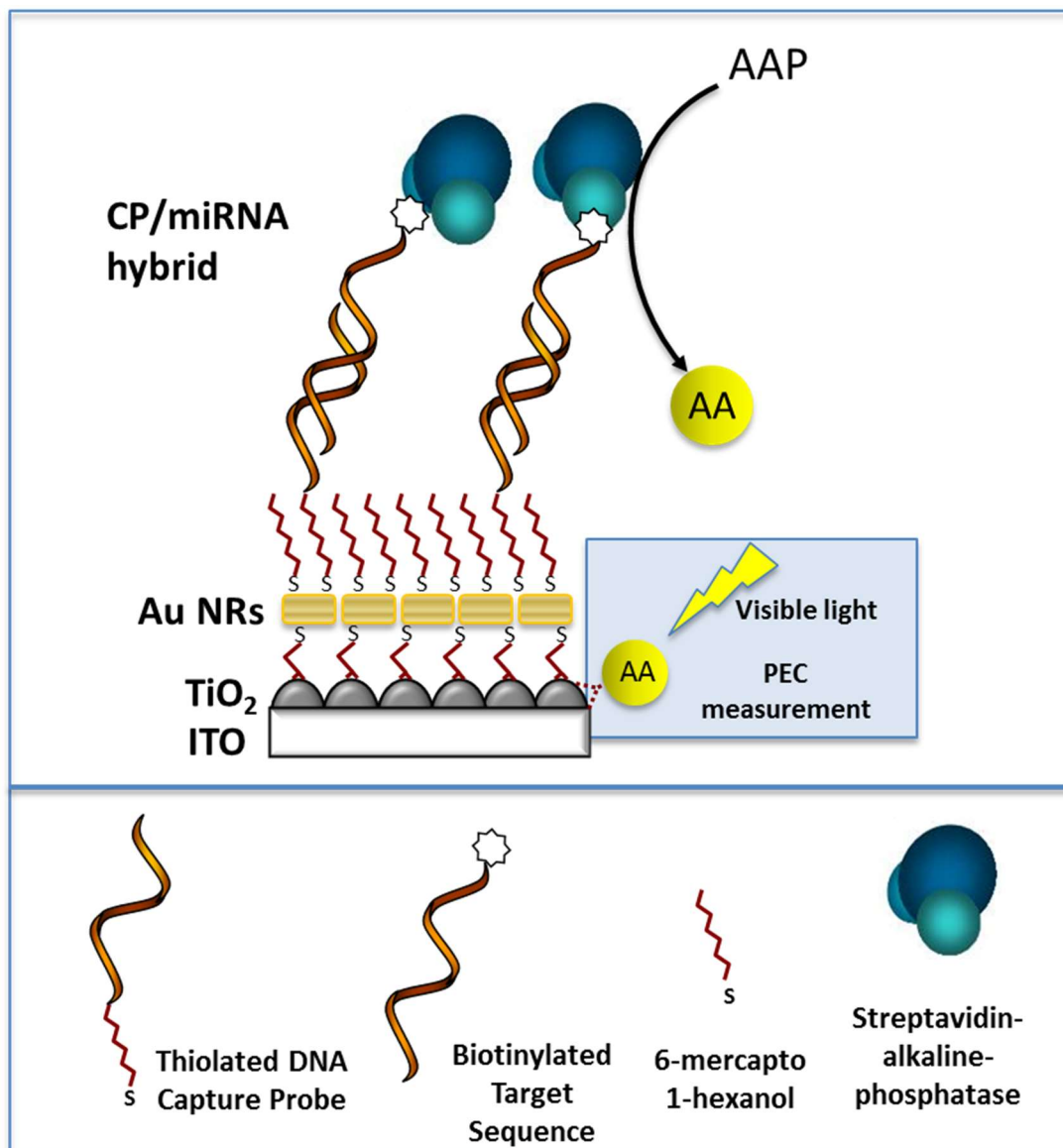
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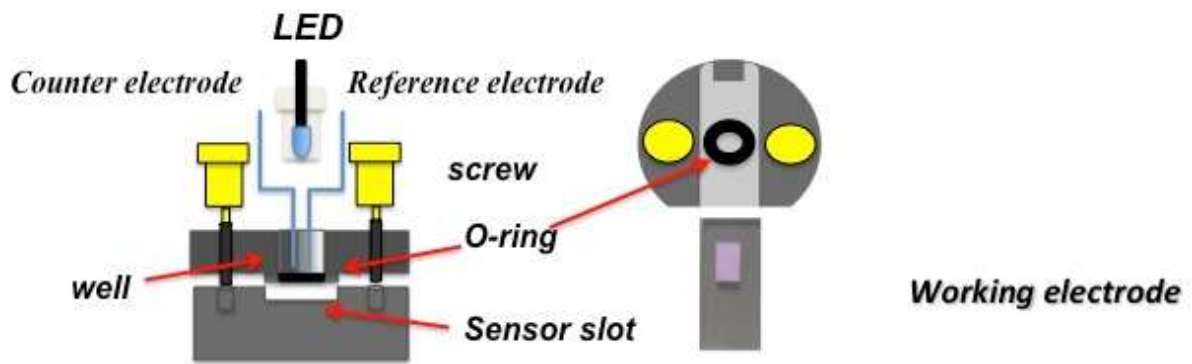
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Scheme 1: Scheme of the assay: AuNRs/MSA/TiO<sub>2</sub>/ITO electrodes are, first, exposed to a solution containing the thiolated oligonucleotide, followed by an incubation with mercaptohexanol. The probe-modified electrode is then exposed to a solution of the biotinylated target sequence. The biotinylated hybrid obtained at the electrode surface is reacted with a solution containing the streptavidin-alkaline phosphatase conjugate. The enzyme catalytic activity converts L-ascorbic acid 2-phosphate (AAP) in ascorbic acid (AA). AA, through the formation of a charge-transfer complex, shift the onset of photocurrents in

the visible spectral region.



Scheme 2: Scheme of the photoelectrochemical cell

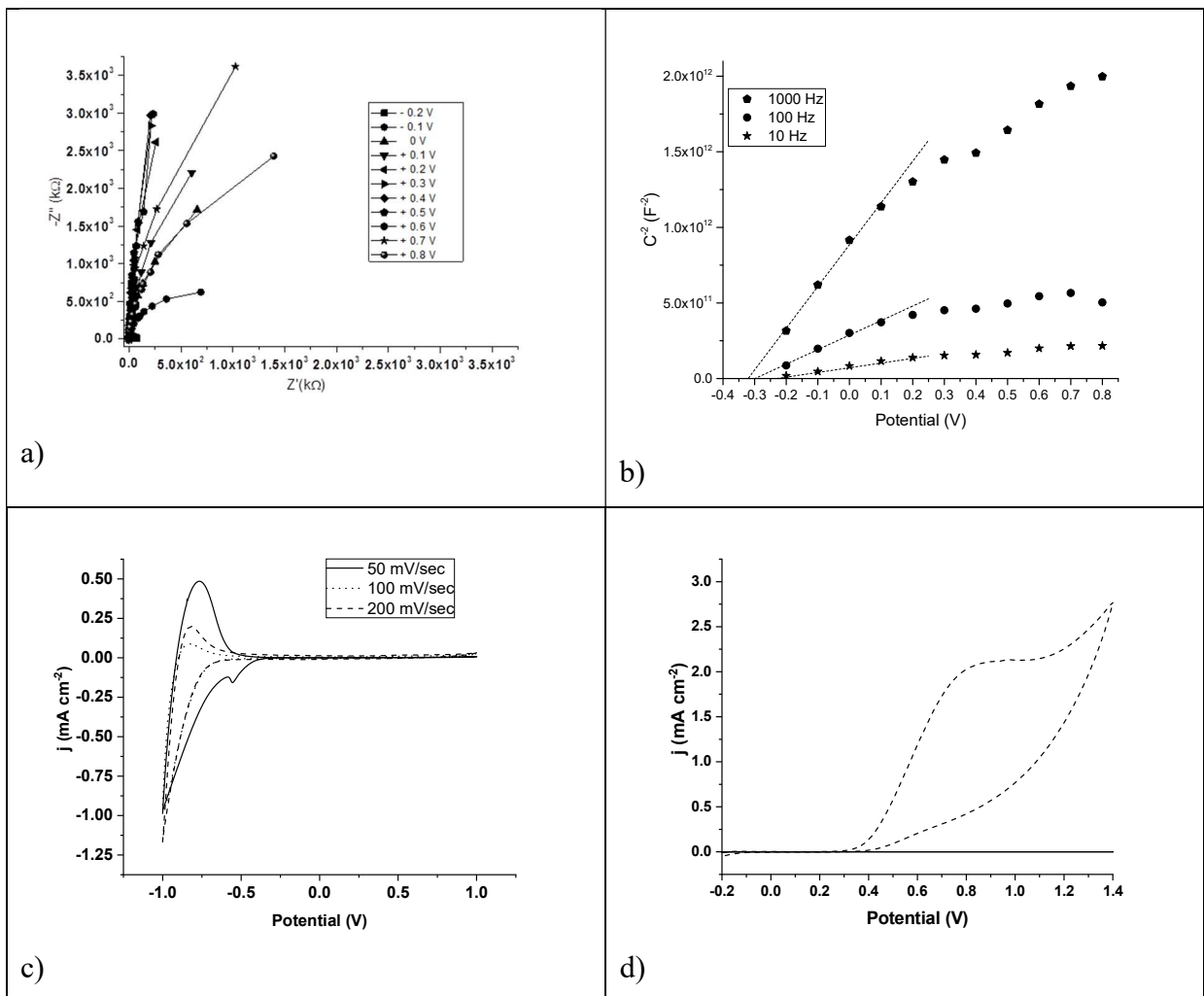


Figure 1: Electrochemical Characterization of the  $\text{TiO}_2/\text{ITO}$  electrode: a) Nyquist plots recorded in  $0.1 \text{ M KCl}$  at  $\text{TiO}_2/\text{ITO}$  electrode from  $-0.2$  to  $+0.8 \text{ V}$ ; experimental data are expressed as points, while lines represent the obtained fitting; b) Mott-Schottky plot for  $\text{TiO}_2/\text{ITO}$  electrode in  $0.1 \text{ M KCl}$  solution, at  $1000$ ,  $100$ ,  $10 \text{ Hz}$ , respectively; c) CVs recorded from  $-1.0$  to  $+1.0 \text{ V}$  vs.  $\text{Ag}/\text{AgCl}$ , at different scan rate, in  $0.1 \text{ M KCl}$  at  $\text{TiO}_2/\text{ITO}$  electrode; d) CVs recorded from  $-0.2$  to  $+1.4 \text{ V}$  vs.  $\text{Ag}/\text{AgCl}$ , at  $50 \text{ mV/s}$ , in  $0.1 \text{ M KCl}$  in presence (dashed line) and absence (solid line) of  $10 \text{ mM AA}$ .

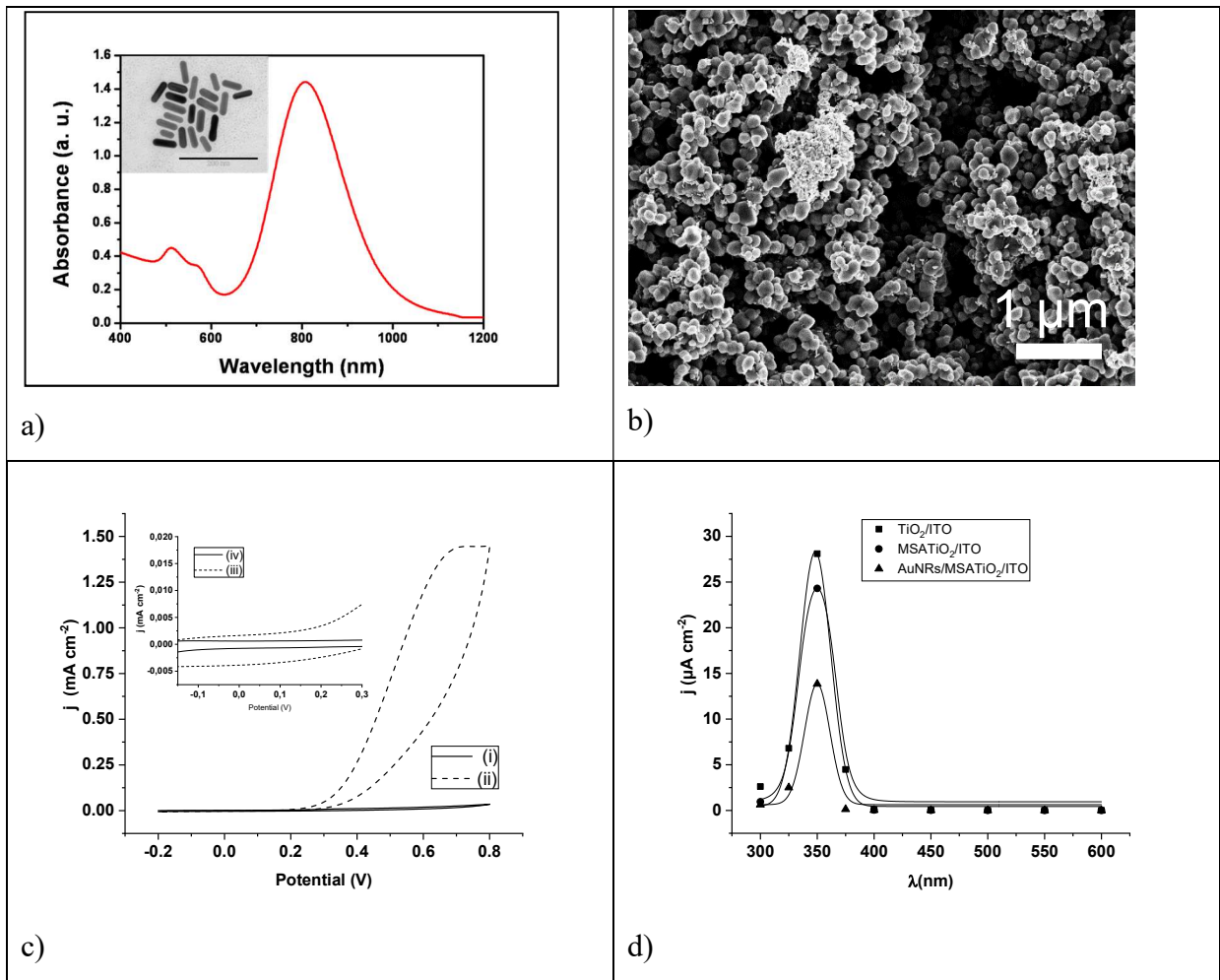


Figure 2: UV-vis-NIR absorption spectroscopy, SEM and electrochemical characterization of the AuNRs/MSA/TiO<sub>2</sub>/ITO electrode: a) AuNRs absorption spectrum, recorded from 400 to 1200 nm in toluene. The inset show the TEM micrograph of synthesized Au NRs; b) SEM micrograph of the AuNRs/MSA/TiO<sub>2</sub>/ITO electrode; c) CVs recorded at AuNRs/MSA/TiO<sub>2</sub>/ITO electrode in 0.1 M KCl, scan rate 50 mV/s in absence (i) and presence (ii) of 10 mM AA, in dark (the inset show an enlargement of the CVs recorded at AuNRs/MSA/TiO<sub>2</sub>/ITO (iii) and MSA/TiO<sub>2</sub>/ITO electrode (iv), respectively, in the range -0.1 to + 0.3V, scan rate 50 mV/s); d) Photocurrents recorded at AuNRs/MSA/TiO<sub>2</sub>/ITO, MSA/TiO<sub>2</sub>/ITO, TiO<sub>2</sub>/ITO electrodes in 10 mM Tris buffer, pH 9.8 at 0 V vs Ag/AgCl, under illumination from 300 to 600 nm.

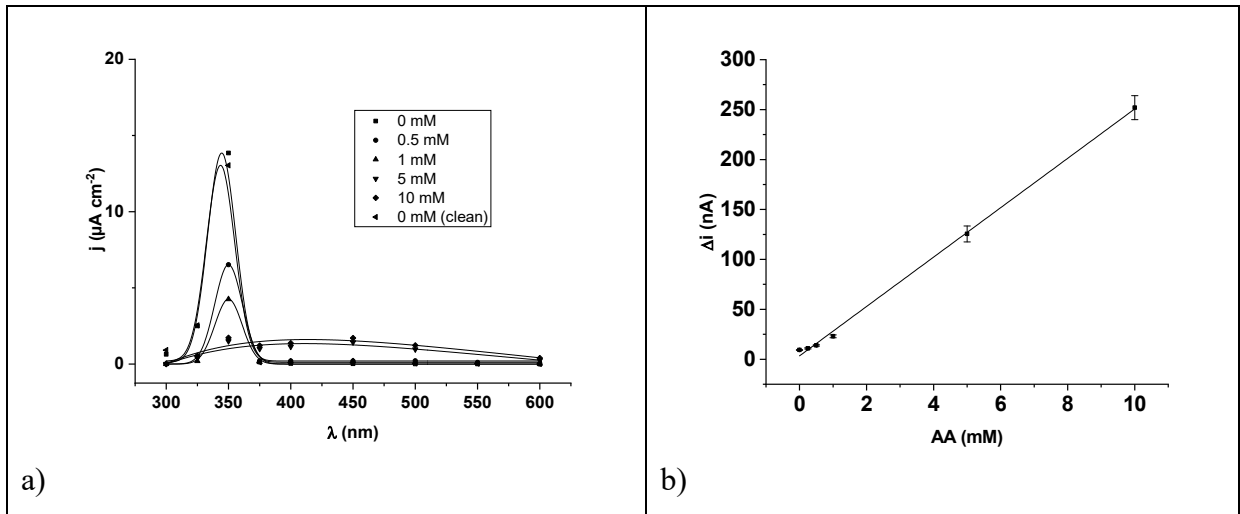


Figure 3: Evaluation of AA concentration on photocurrent values: a) Photocurrent response in presence of different concentration of AA corresponding to 0, 0.5, 1, 5, 10 mM, respectively. Photocurrent recorded in 0 mM AA after awashing step with demineralized water (-◄-) was also reported. Excitation light from 300 to 600 nm. The AuNRs/MSA/TiO<sub>2</sub>/ITO electrode was exposed to increased concentration of AA in 10 mM Tris buffer (pH=9.8); b) The PEC calibration plot of AA, recorded in 10 mM Tris buffer (pH=9.8), at a constant potential of 0 V and using a commercial white LED light.



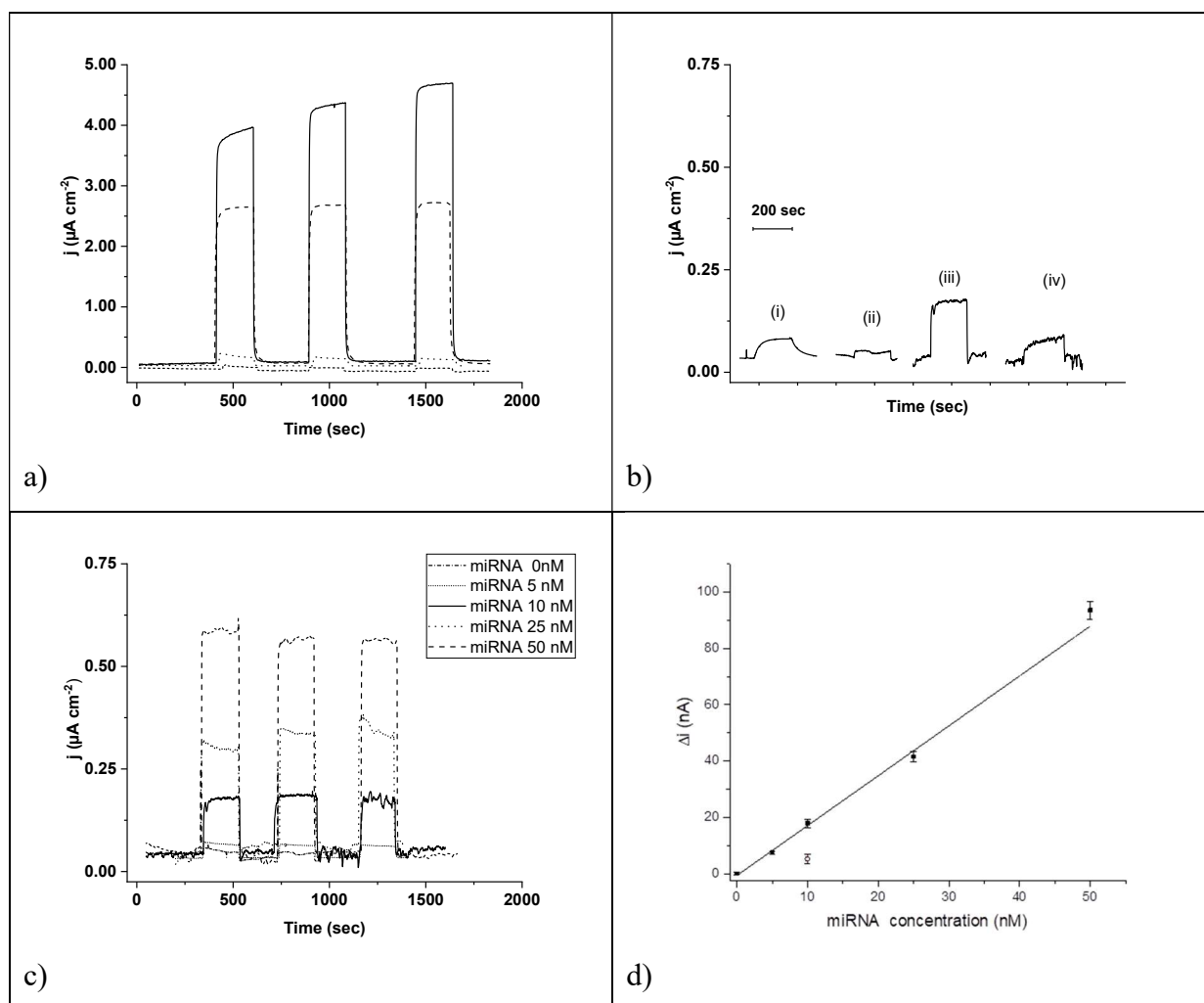


Figure 4: Photoelectrochemical characterization of the genosensor: a) Photocurrents recorded at the bare AuNRs/MSA/TiO<sub>2</sub>/ITO electrode in presence (solid line) and absence (dotted line) of 10 mM AA in 10 mM Tris pH=9.8, and photocurrents recorded at CP-modified-AuNRs/MSA/TiO<sub>2</sub>/ITO electrode in presence (dashed line) and absence (short dashed line) of 10 mM AA in 10 mM Tris pH=9.8; b) photocurrents recorded at the bare AuNRs/MSA/TiO<sub>2</sub>/ITO electrode (i) in 10 mM Tris (pH=9.8) and at ALP-10nM miRNA-CP-modified-AuNRs/MSA/TiO<sub>2</sub>/ITO electrode in presence (iii) and absence (ii) of 10 mg/mL AAP in 10 mM Tris pH=9.8, and at the same electrode after washing and in presence of 10 mg/mL AAP in 0.5M PB (iv); c) Photocurrents recorded in presence of increased concentration of microRNA target (0, 5, 10, 25, 50 nM). Data are recorded at 0 V vs Ag/AgCl in 10mM Tris (pH=9.8) in presence of 10 mg/mL AAP. Further details are reported in the material and methods section. d) Corresponding calibration plot, where the photocurrent value of a non complementary microRNA sequence was also reported (●). Each point represents

the mean of at least three measurements and the error bars the corresponding standard deviation.