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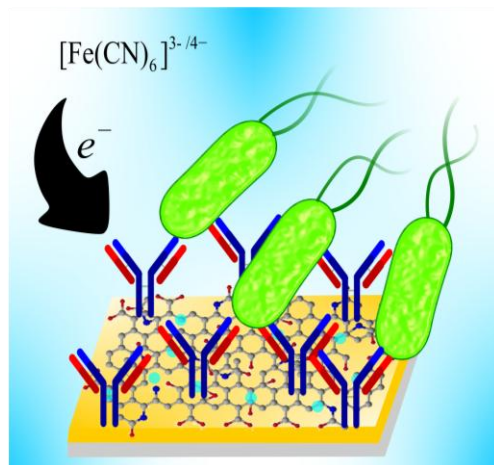
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Reduced graphene oxide/polyethylenimine based immunosensor for the selective and sensitive electrochemical detection of uropathogenic *Escherichia coli*

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Abstract

Fast, reliable and selective detection of microorganisms is of uttermost importance in clinical analysis, but also in food and water quality monitoring. In this study, we report on the construction of an immunosensor for sensitive and selective electrochemical detection of uropathogenic *Escherichia coli* (*E. coli*) UTI89 bacteria in aqueous and serum samples. We took benefit of electrophoretic deposition (EPD) to prepare, in a simple, controllable and cost effective way, gold electrodes modified with thin active layers of reduced graphene oxide/polyethylenimine (rGO/PEI). While rGO exhibits high surface area and favourable electrochemical properties, the presence of abundant $-NH_2$ groups on PEI offers a plethora of opportunities for the sensor's surface functionalization. To achieve selectivity of detection, the electrode surface was covalently modified with anti-fimbrial *E. coli* antibodies *via* amide bond formation. To minimize non-specific adsorption, the immunosensor was additionally modified with pyrene-polyethyleneglycol (pyrene-PEG) moieties prior to antibody immobilization. The detection of *E. coli* was based on the restriction of electron transfer of a redox mediator, in our case potassium ferrocyanide, to the rGO/PEI modified electrical transducer due to the formation of an immune complex. The developed immunosensor displayed a sigmoidal shape with a linear range from to $1 \times 10^1 - 1 \times 10^4$ cfu mL⁻¹ ($R^2=0.995$) according to $i(\mu A)=-16.66-20.5 \times \log[E.coli]$ (cfu

mL⁻¹) and a detection limit of 10 cfu mL⁻¹. Additionally, the sensor performed well both in aqueous and urine media, which is essential for its potential use for clinical diagnosis of pathogenic diseases. Selectivity studies showed that the immunosensor was able to discriminate between *E. coli* UTI89 wild-type strain and UTI89 Δ *fim*, without *fim* operon.

Keywords: *Electrophoretic deposition; reduced graphene oxide; polyethyleneimine; anti-fimbrial E. coli antibodies; E. coli UTI89 bacteria; electrochemical sensing.*

Introduction

Urinary tract infections (UTIs) represent the most common bacterial infectious diseases for humans, and *Escherichia coli* (*E. coli*) are the most predominant pathogens responsible for 30–50% of hospital acquired and 80–90% of community-acquired UTIs [1]. Pathogenic *E. coli* bacteria produce virulence factors, able to infect and cause disease to the host tissues. Thus, it is of high importance to detect these microorganisms at a very low level and be able to discriminate between different bacterial strains. The common techniques for bacteria identification and detection rely on conventional culturing techniques [2]. However, these techniques are time-consuming, very elaborate and necessitate microbiology laboratory to be completed. Also these standard methods take up to a full day to rule out a negative sample, and the analysis may require up to several days to confirm a positive result. As such, these techniques are not suitable for on-site monitoring. Therefore, there is continuously a huge demand for the implementation of rapid, reliable, specific and highly sensitive detection of bacteria at a low cost. In this context, biosensors occupy a central position to achieve these goals [3-5].

The last decade has witnessed an emergence of a plethora of 2D materials [6]. Particularly, graphene has attracted a big deal of interest for its outstanding properties

such as good conductivity, high surface area, ease of production and functionalization using different routes,... [6]. These properties have been exploited in various applied fields and particularly for sensing small organic molecules, metal ions and biological species [7, 8].

Detection of pathogens with graphene based sensors represents thus an appealing technology. A vast amount of work has been performed using graphene-based field effect transistors (GFETs) [9-11]. Huang *et al.* investigated CVD graphene derivatized with anti-*E. coli* antibodies for sensing *E. coli* bacteria with a detection limit of 10 cfu mL⁻¹ [11]. Chang *et al.* described a GFET comprising anti-*E. coli* modified thermally-reduced graphene oxide sheets (rGO) semiconducting channels for the selective and sensitive detection of *E. coli* O157:H7 [9]. A low detection limit of 10 cfu mL⁻¹ was achieved under optimized conditions. Chen *et al.* utilized holey rGO chemically functionalized with an antimicrobial peptide, Magainin I, as the transducer element in a GFET for *E. coli* O157:H7 detection [10]. The device exhibited a good selectivity and a detection limit of 803 cfu mL⁻¹.

Electrochemical detection of pathogens based on impedimetric [12-17] approaches is another particular appealing technique for fast and selective detection of pathogens, due to its low cost, simplicity of operation, and the possibility of miniaturization and portability [18]. Voltammetric and amperometric-based immunoassays for pathogen sensing are in addition popular label-free approaches [19-22]. Ahmed *et al.* reported on an antibody-based immunosensor for the specific detection of *S. pyogenes* pathogenic bacteria in human saliva with a linear response of 10² to 10⁵ cells [19]. The integration of graphene nanostructures has proven to be advantageous in enhancing sensitivity of electrochemical sensors. Consequently, some recent works focused on the use of graphene composite electrodes for the construction of electrochemical immunosensors for the detection of pathogenic bacteria [23].

In a recent work by our group, we have studied the influence of the chemical composition and charge of graphene-gold interfaces on the adhesion strength of different *E. coli* strains through surface plasmon resonance (SPR) measurements [24]. Our results revealed that uropathogenic *E. coli* UTI89 interacts strongly with polyethyleneimine (PEI) modified graphene surfaces through electrostatic interactions. Given that these interactions are not specific, in this study, we propose a new strategy for rapid and specific detection of uropathogenic *E. coli* UTI89 in aqueous and serum samples using an electrochemical read out. The sensitive and

selective detection of *E. coli* UTI89 is achieved through chemical functionalization of the -NH₂ groups of PEI modified reduced graphene oxide (rGO) electrodes with anti-fimbrial *E. coli* antibodies along with poly(ethylene glycol) (PEG) modified pyrene units to inhibit non-specific adsorption of *E. coli* bacteria onto the sensor surface. The change in the peak current of potassium ferrocyanide redox probe using differential pulse voltammetry (DPV) was used for the quantitative analysis of *E. coli* UTI89 contaminated solutions. It relies on the fact that the formation of an immunocomplex between anti-fimbrial *E. coli* antibodies and *E. coli* UTI89 induces a restriction of electron transfer of potassium ferrocyanide to the electrical transducer, which scales linearly with *E. coli* UTI89 concentration. The designed pathogen chip can specifically detect *E. coli* UTI89 down to 10 cfu mL⁻¹ in a highly specific manner even in blood and urine samples.

2. Experimental section

2.1. Materials

Polyethyleneimine (PEI, Mw~25,000), 1-ethyl-3[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC·HCl), *N*-hydroxysuccinimide (NHS), phosphate buffer (PBS, 10 mM, pH 7.4), potassium ferrocyanide ([K₄Fe(CN)₆]), hexaammineruthenium(III) chloride ([Ru(NH₃)₆]Cl₃), 1-pyrenecarboxylic acid (97%, Pyr-COOH), *N,N'*-disuccinimidyl carbonate (≥95.0%, DSC), dichloromethane (CH₂Cl₂), triethylamine (≥99.5%, TEA), O-(2-aminoethyl)-O'-(2-methylethyl)heptaethylene glycol (≥90%, NH₂-PEG₈-N₃) were obtained from Sigma-Aldrich (France).

Graphene oxide (GO) powder was obtained from Graphenea, Spain.

Serum and urine samples were kindly provided by the Centre Hospitalier Universitaire of Lille.

2.2. Formation of anti-fimbrial *E. coli* modified pyrene-PEG coated rGO/PEI modified electrodes (PEG/rGO/PEI-anti-fimbrial)

2.2.1 Electrophoretic deposition of reduced graphene oxide/polyethyleneimine (rGO/PEI) on Au electrode

Graphene oxide/polyethyleneimine (GO/PEI) aqueous dispersion was obtained by stirring for 48 h at room temperature a mixture of GO (1 mg mL⁻¹) and PEI (1 mg mL⁻¹)

¹) at a volume ratio of 1/1. The Au thin film electrode was functionalized through electrophoretic deposition (EPD) of rGO/PEI using GO/PEI aqueous dispersion in a two-electrode cell by applying a DC voltage (15 V) for 4 min. The Au electrode acts as the cathode and a Pt foil (1×2 cm²) as the anode. After deposition, the interface was rinsed copiously with Milli-Q water and dried in air.

2.2.2 Functionalization of rGO/PEI interface with pyrene-PEG (PEG/rGO/PEI)

The Au/rGO/PEI interface was immersed in 5 mM pyrene-PEG (see **SI for synthesis**) aqueous solution at room temperature for 1 h. The resulting surface was extensively rinsed with Milli-Q water and dried under a stream of nitrogen.

2.2.3 Covalent immobilization of anti-fimbrial *E. coli* antibodies onto PEG/rGO/PEI electrode (PEG/rGO/PEI-anti-fimbrial)

Covalent linking of rabbit anti-fimbrial antibody onto rGO/PEI functionalized with pyrene-PEG was achieved through chemical coupling of the -NH₂ groups of PEI with the -COOH functions of anti-fimbrial *E. coli* antibodies using EDC/NHS as crosslinking agents. Briefly, the PEG/rGO/PEI coated gold interface was incubated in an aqueous solution of anti-fimbrial *E. coli* antibody solution (10 ng mL⁻¹), EDC·HCl (25 mM) and NHS (25 mM) at 4 °C for 2 h under agitation. The resulting surface was washed copiously with PBS to remove excess antibodies and unreacted reagents. The functionalized surface was then stored in PBS buffer at 4 °C before use.

2.3 Characterization

Zeta-potential measurements

The surface properties of the prepared samples were assessed by zeta-potential measurements using a Zeta-sizer Nano-ZS (Malvern Instruments Inc. Worcestershire, UK). The measurements were performed in Milli-Q water (pH 7.0) at a sample concentration of 25 µg mL⁻¹.

Scanning Electron Microscopy (SEM)

The morphology of the surface was examined by an electron microscope ULTRA 55 (Zeiss, France) equipped with a thermal field emission emitter and a high efficiency In-lens SE detector.

SEM images of pathogens were recorded using a Zeiss Compat Merlin instrument at 2 kV under high vacuum. The biological samples were fixed with 1% glutaraldehyde solution for 30 min at room temperature in the dark and then coated with a 5 nm-thick platinum film.

Optical profilometry

An optical profilometer (ZygoNewView 6000 Optical Profilometer with MetroPro software) with 1 nm height resolution was used for thickness measurements.

Electrochemical measurements

The electrochemical properties of the developed electrodes were assessed by cyclic voltammetry (CV) and differential pulse voltammetry (DPV) using an Autolab potentiostat 30 (Eco-Chemie, Utrecht, The Netherlands). A conventional three-electrodes system consisting of a working electrode (Au film), a reference electrode (silver wire), and a counter electrode (platinum mesh) was used. The following optimized conditions (modulation time: 0.05 s; interval time: 0.5 s; step potential: 5 mV; modulation amplitude: 50 mV) were used to record differential pulse voltammograms.

2.4 Bacterial culture, incubation and detection based on anti-fimbrial *E. coli* antibodies modified PEG/rGO/PEI interface

Three bacterial strains are used in this study, including *E. coli* UTI89 (wild-type strain), *E. coli* UTI89 *Δfim* (without fim operon) and *S. aureus*. A single *E. coli* or *S. aureus* colony from LB/BHI agar plate complemented with the appropriate antibiotics was inoculated overnight in LB/BHI medium at 37 °C with moderate shaking. The pre-culture was diluted 50-fold and kept under moderate shaking for another 3-4 h, until the OD_{600nm} had reached 0.6 – 1. The bacteria were re-suspended in sterile Milli-Q water and adjusted to the required concentration (1×10^1 - 1×10^8 cfu mL⁻¹).

A series of bacteria cell concentrations ranging from 1×10^1 to 1×10^8 cfu mL⁻¹ in 100 μL volume were incubated on anti-fimbrial *E. coli* antibodies functionalized PEG/rGO/PEI electrode for 30 min to achieve bacterial cells immobilization. After incubation, the functionalized electrode was copiously rinsed with Milli-Q water to remove excess planktonic cells and then kept in PBS buffer or growth medium at 4°C for further analysis. Finally, the electrochemical response of the 10 mM [Fe(CN)₆]^{3-/4-} probe in 0.1 M KCl was recorded using DPV.

3. Results and Discussion

3.1. Formation of reduced graphene oxide/polyethylenimine (rGO/PEI) modified interfaces

Figure 1A outlines the strategy employed for the preparation of functionalized electrodes for specific detection of *E. coli* UTI89 by electrochemical means. It is based on the functionalization of a gold (Au) electrode by electrophoretic deposition of rGO/PEI, followed by non-covalent modification with pyrene-PEG (see **SI for synthesis**) and covalent linking of *E. coli* UTI89 anti-fimbrial antibodies to the amine groups of PEI. We opted for the electrophoretic deposition (EPD) concept, as anodic EPD of rGO using GO as a starting material was reported to result in highly stable interfaces that can be produced in a cost-effective manner and good reproducibility. As such, EPD is well adapted for the functionalization of different electrode surfaces for designing sensing platforms [25]. GO aqueous solutions exhibit a negative ($\zeta = -41.3 \pm 0.8$ mV) zeta potential and the GO platelets migrate towards the anode upon application of a DC voltage [26]. For a more complete reduction of GO to rGO, cathodic EPD deposition and a matrix with positive zeta potential is required. This can be achieved by charging of GO with a cationic polymer with repeated amine groups such as polyethylenimine (PEI). A 1/1 mixture of GO and PEI results in GO/PEI nanosheets with $\zeta = +36.4 \pm 1.3$ mV, being well adapted for cathodic EPD.

Figure 1B shows the morphology of a thin Au electrode before and after electrophoretic coating with rGO/PEI. Upon the application of +15 V_{DC} for 4 min, the Au electrode is coated with a homogeneous film of rGO/PEI nanoplatelets of about 4 nm in thickness, as determined by profilometry measurements.

Figure 1C exhibits the differential pulse voltammograms of the thin film Au electrode before and after coating with rGO/PEI through electrophoretic deposition using $[\text{Fe}(\text{CN})_6]^{4-}$ as the redox probe. As compared to bare Au electrode, functionalization with a thin rGO/PEI film resulted in a significant increase of the redox current. The enhanced current of the Au electrode modified with rGO/PEI thin film correlates most likely with the good electronic properties of rGO/PEI, increased surface area and favourable electrostatic interactions between the negatively charged redox probe and the positively charged rGO/PEI interface. Indeed, using a positively charged redox probe led to a small increase of the redox current for the rGO/PEI

interface when compared to bare Au electrode (see SI, Figure 1A).

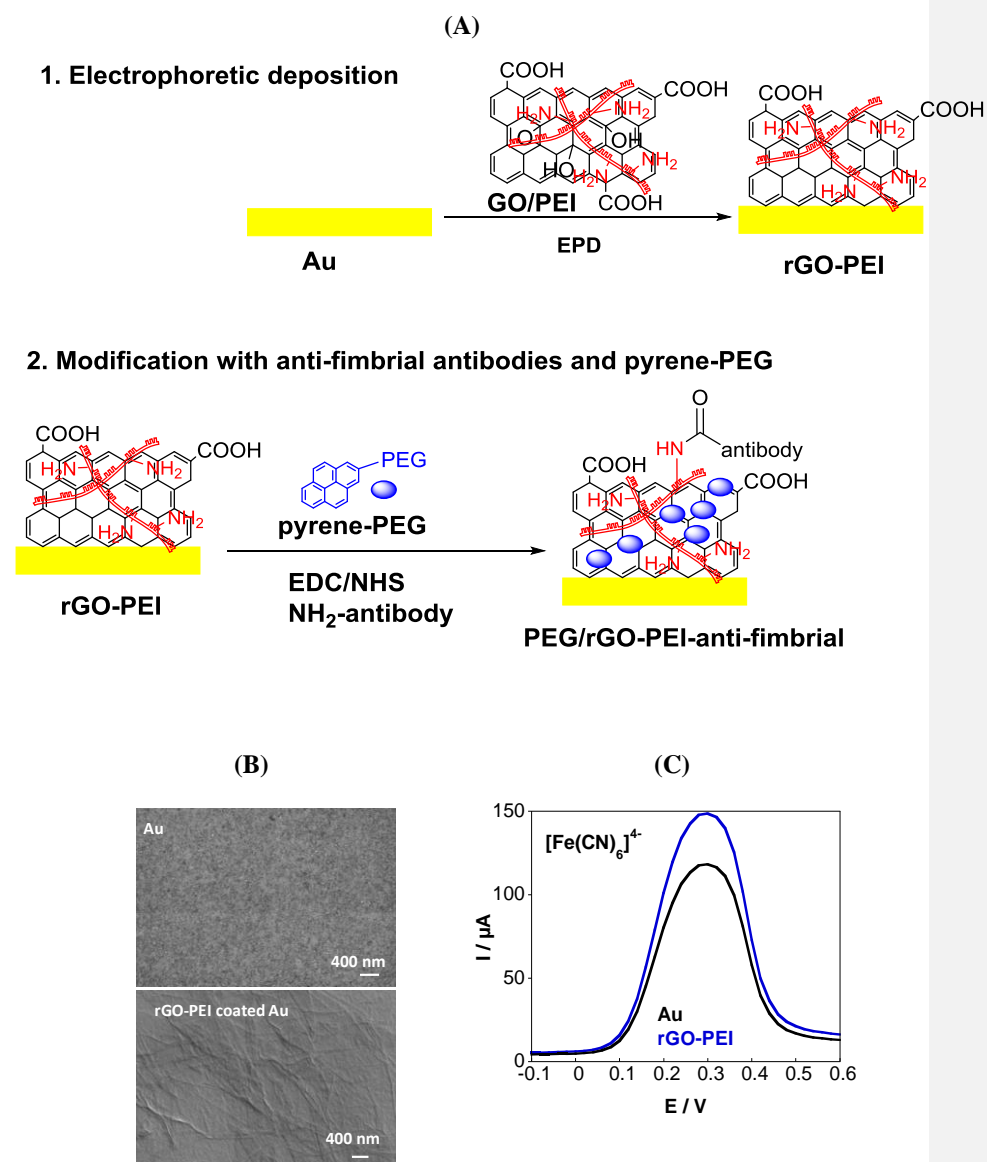


Figure 1: (A) Different steps employed for the construction of the immunosensor for the selective electrochemical detection of *E. coli* UTI89; (B) Scanning electron microscopy (SEM) micrographs of Au electrodes before and after electrophoretic coating with rGO/PEI nanoflakes upon application of 15 V_{DC} for 4 min; (C)

Differential pulse voltammograms of Au (black) and Au coated with rGO/PEI (blue) recorded in $[\text{Fe}(\text{CN})_6]^{4-}$ (10 mM)/KCl (0.1 M).

To determine whether rGO/PEI coated Au electrodes are adaptable for bacteria sensing, the interfaces were first immersed into *E. coli* solutions. **Figure 2A** shows the change in the redox current of the rGO/PEI coated Au interface using $[\text{Fe}(\text{CN})_6]^{4-}$ as the redox probe upon incubation for 30 min in *E. coli* UTI89 solution of different concentrations. After an insignificant increase in the peak current for *E. coli* solution of 10^2 cfu mL⁻¹, a small but steady decrease in the redox current is observed upon increasing the *E. coli* concentration. The interfaces proved however to be not highly specific towards *E. coli* UTI89, especially at low concentrations, where a comparable change in electrochemical signature is observed upon incubation with *E. coli* UTI89 ΔFim , a modified *E. coli* UTI89 strain deprived bacteria fimbrials (**Figure 2B**). SEM images of an rGO/PEI coated Au interface incubated with *E. coli* UTI89 for 30 min confirmed *E. coli* UTI89 adsorption on the interface (**Figure 2C**).

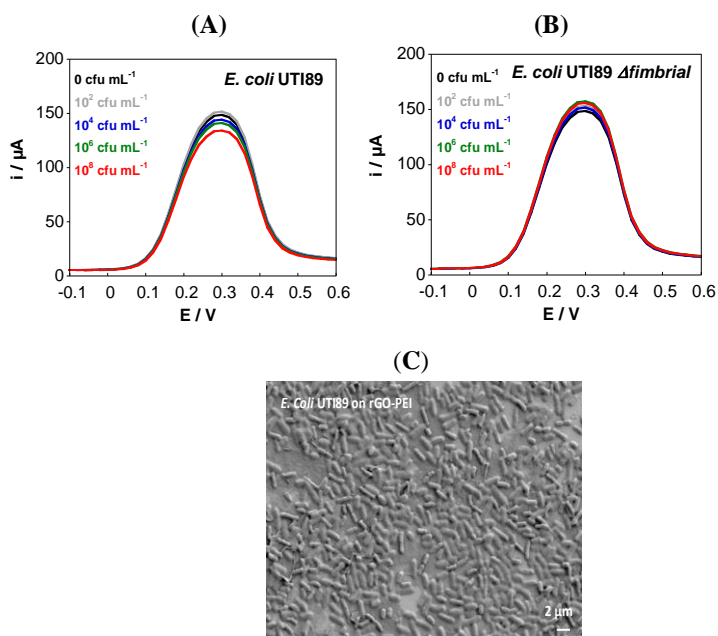


Figure 2: Differential pulse voltammograms recorded in $[\text{Fe}(\text{CN})_6]^{4-}$ (10 mM)/KCl (0.1 M) on rGO/PEI coated Au electrodes before (black) and after incubation for 30 min with *E. coli* UTI89 (**A**) and *E. coli* UTI89 Δ fimbrial (**B**) solutions of different concentrations; (**C**) SEM image of rGO/PEI coated Au electrode incubated with *E. coli* UTI89 (10^8 cfu mL⁻¹) for 30 min.

3.3. Formation of anti-fouling anti-fimbrial *E. coli* antibodies modified rGO/PEI Au electrodes

The selective detection of bacteria was achieved through covalent functionalization of the Au/rGO/PEI interface with antibodies against the major pilin protein FimA of the *E. coli* UTI89 type-1 fimbriae using the classical NHS/EDC coupling chemistry (**Fig. 1A**). Indeed, surface plasmon resonance (SPR) investigations showed specific and concentration-dependent interactions of the IgG polyclonal antibodies with purified type-1 fimbriae immobilized onto a CM5 sensor chip, using the standard EDC/NHS procedure (see SI, **Figure S2**). The best fit could be obtained using a Langmuir 1:1 binding model, with a global K_d value of 6 ± 4 μM . The same surface functionalization strategy was employed for the electrochemical sensing interface using EDC/NHS chemistry, resulting in amide bond formation between the free $-\text{NH}_2$ functions present on the rGO/PEI matrix and the available $-\text{COOH}$ groups of the antibody (**Figure 1A**). To minimize anti-fouling issues, prior to antibody immobilization, the rGO/PEI coated Au electrode was immersed into pyrene-PEG to block free rGO sites. In fact, this has proved to be more efficient when compared to post-functionalization of antibody modified rGO/PEI interfaces with pyrene-PEG; this is most likely due to the restriction of π - π stacking and H-bonding interactions of pyrene moieties with rGO by the anti-fimbrial *E. coli* antibodies immobilized on the rGO/PEI surface.

3.4. Electrochemical detection

The electrochemical response of the anti-fimbrial *E. coli* antibody modified interfaces was further investigated. While pyrene-PEG does not influence the electron transfer, its presence shows excellent anti-fouling properties (**Figure 3A**). Linking antibodies against the FimA major pilin of *E. coli* UTI89 type-1 fimbriae to the

interface results in a partial blocking of the electron transfer of $[\text{Fe}(\text{CN})_6]^{4-}$ seen in the decrease of the redox current (**Figure 3B**).

SEM imaging of pyrene-PEG modified rGO/PEI after immersion in *E. coli* UTI89 at a high concentration of 1×10^8 cfu mL^{-1} for 2 h indicates clearly the absence of pathogens on the surface and the good anti-fouling properties of pyrene-PEG modified electrodes (**Figure 3C**). This image contrasts that obtained when anti-fimbrial were immobilized on the surface (**Figure 3D**).

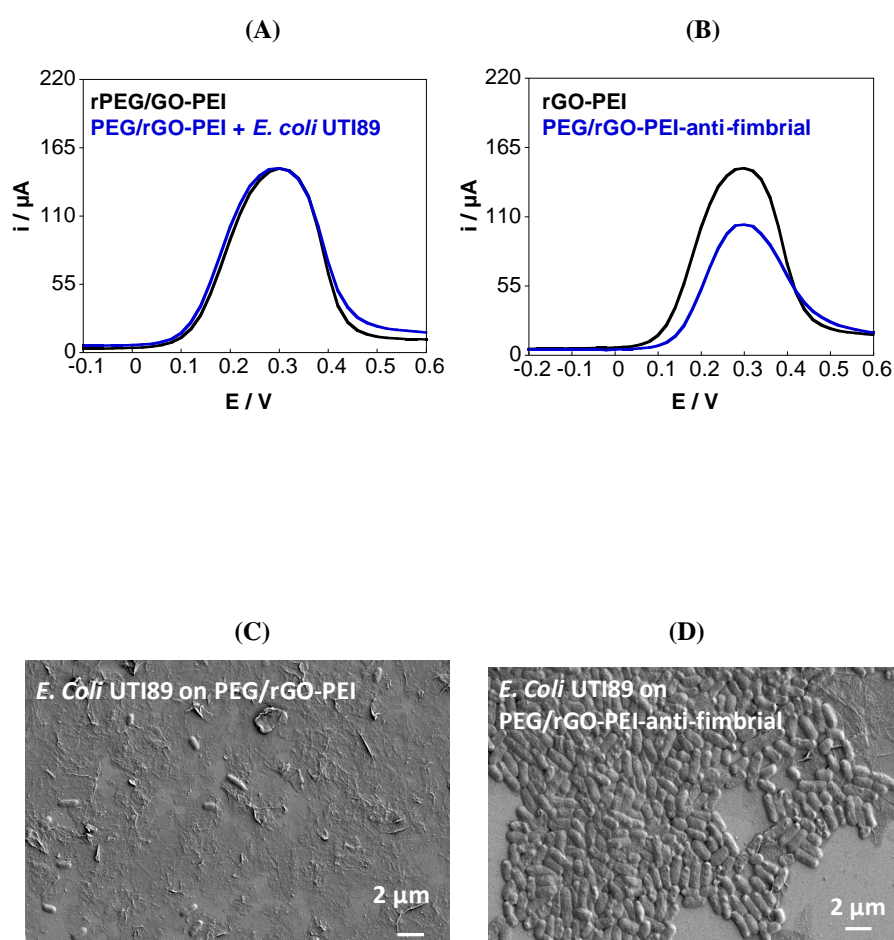


Figure 3: (A) DPVs of PEG/rGO/PEI modified Au electrodes before and after incubation for 2 h in *E. coli* UTI89 (10^8 cfu mL^{-1}) solution containing $[\text{Fe}(\text{CN})_6]^{4-}$ (10 mM)/KCl (0.1 M); (B) DPVs of PEG/rGO/PEI-anti-fimbrial modified Au electrodes before and after incubation for 2 h in *E. coli* UTI89 (10^8 cfu mL^{-1}) solution

containing $[\text{Fe}(\text{CN})_6]^{4-}$ (10 mM)/KCl (0.1 M); SEM images of PEG/rGO/PEI (C) and PEG/rGO/PEI-anti-fimbrial (D) modified Au electrodes after 2 h of incubation with *E. coli* UTI89 wt 10^8 cfu mL⁻¹.

The sensitivity of the PEG/rGO/PEI-anti-fimbrial electrode towards the selective detection of *E. coli* UTI89 in aqueous solution was performed in a wide range of bacteria concentrations. **Figure 4A** depicts the change of the DPV peak current of $[\text{Fe}(\text{CN})_6]^{4-}$ redox probe upon incubation of the electrode for 30 min with *E. coli* UTI89. In absence of bacteria (control), the current value was 144 ± 3 μA (I_0). Once UTI89 bacteria (100 cfu mL⁻¹) was added, the electrochemical response of the system was altered due to electron transfer restriction of $[\text{Fe}(\text{CN})_6]^{4-}$ to the electrical interface and the current decreased to 120 ± 3 μA (I_c) and even further with an increasing concentration of UTI89 cells in solution. To demonstrate the sensor's performance to transduce the biorecognition reaction between the antibody and the FimA carrying pathogen into measureable signals, the change in current as a function of pathogen concentration was plotted. As seen from the calibration curve in **Figure 4B**, where the change in the peak current $I_0 - I_c$ vs. $\log [E. coli \text{ UTI89}]$ is plotted, with I_0 being the initial current in the absence of pathogens, a linear range from 1×10^1 to 1×10^4 cfu mL⁻¹ ($R^2 = 0.995$) according to $i(\mu\text{A}) = -16.66 - 20.5 \times \log[E. coli] \text{ (cfu mL}^{-1}\text{)}$ is obtained. The system displays a detection limit for UTI89 below 10 cfu mL⁻¹ from five blank signals (95% confidence level).

(A)

(B)

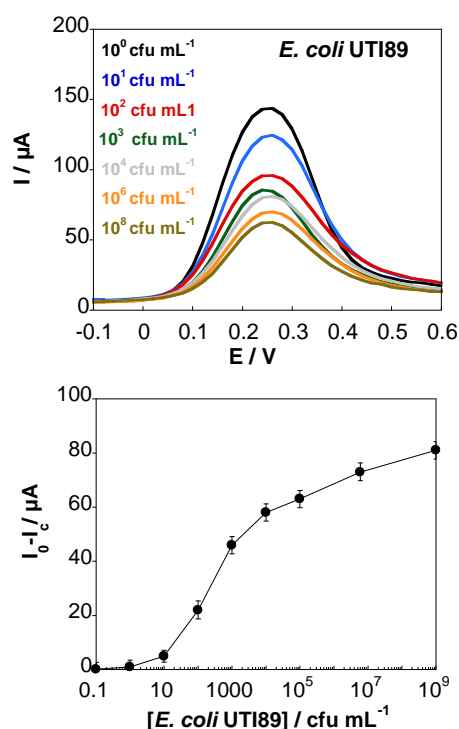


Figure 4: (A) Change in oxidative current of DPVs in $[\text{Fe}(\text{CN})_6]^{4-}$ (10 mM)/KCl (0.1 M) upon incubation for 30 min in aqueous solutions of *E. coli* UTI89 of different concentrations (10^1 - 10^8 cfu mL $^{-1}$) using the developed PEG/rGO-PEI-anti-fimbrial modified Au electrodes; (B) Respective calibration curve for *E. coli* UTI89 solutions.

Table 1 compares the electroanalytical performance of our sensor to other pathogen electrical or electrochemical sensing platforms. The performance of the developed sensor is comparable or slightly lower to those described in the literature. However, the linear range recorded for our sensor is quite large as compared to that reported for other systems with the benefit of the present sensor of being easy to implement and versatile. In fact, the PEI contains plenty of $-\text{NH}_2$ groups that can be easily coupled to various functional groups for designing different sensing platforms. Additionally, the electrophoretic deposition method used for the functionalization scheme is a fairly rapid and a low cost process employed for a broad range of industrial processes.

Table 1: Selected examples of *E. coli* immunosensors reported in the literature.

| Method | Interface | pathogen | LOD cfu mL ⁻¹ | Linear range | Ref. |
|----------------|---|---------------------------|-----------------------------|--|--------------|
| EIS | Ec _{AB} /rGO-CysCu/ Au electrode/ | <i>E. coli</i> O157:H7 | 3.8 | 10 ¹ -10 ⁸ | |
| EIS | Anti- <i>E. coli</i> antibodies/ SAM/ Au electrode | <i>E. coli</i> | 10 | 10 ¹ -10 ³ | |
| EIS | Anti- <i>E. coli</i> antibodies/IDA microelectrode | <i>E. coli</i> O157:H7 | 6 x10 ⁶ | 4.36x10 ⁵ - 4.36x10 ⁸ | |
| EIS | Anti- <i>E. coli</i> antibodies/ SAM/ Au electrode | <i>E. coli</i> | 10 ³ | 3x10 ³ -3x10 ⁷ | |
| EIS | Anti- <i>E. coli</i> antibodies/ Glu/PANI/Au electrode | <i>E. coli</i> O157:H7 | 100 | 10 ² - 10 ⁷ | |
| EIS | thiolated antibodies/Au SPEs | <i>E. coli</i> | 3.3 | 5 - 10 ⁸ | |
| Amperometric | Anti- <i>E. coli</i> antibodies / Au NPs / FeDC modified SPCEs | <i>E. coli</i> O157:H7 | 600 | 10 ² - 10 ⁷ | |
| Capacitance | Graphene + pyrene-maleimide +anti- <i>E. coli</i> antibodies | <i>E. coli</i> O157:H7 | 10-100 | 10-10 ⁷ | [25] |
| GFET | CVD graphene + anti- <i>E. coli</i> antibodies | <i>E. coli</i> | 10 | 10-10 ⁴ | [9] |
| GFET | rGO + anti- <i>E. coli</i> antibodies | <i>E. coli</i> O157:H7 | 10 | 10-10 ³ | [7] |
| GFET | holey reduced graphene oxide functionalized + antimicrobial peptide | <i>E. coli</i> | 803 | - | [8] |
| Potentiometric | rGO+aptamer | <i>S. aureus</i> | 1 | 10-10 ³ | [26] |
| DPV | rGO-PEI+anti- fimbrial antibody | <i>E. coli</i> UTI89 | 100 | 10 ¹ -10 ⁷ | This work |

Comment [RJ1]: Pandey, Chandra Mouli, et al. "Highly sensitive electrochemical immunosensor based on graphene-wrapped copper oxide-cysteine hierarchical structure for detection of pathogenic bacteria." *Sensors and Actuators B: Chemical* 238 (2017): 1060-1069.

Comment [RJ2]: Maalouf, Rita, et al. "Label-free detection of bacteria by electrochemical impedance spectroscopy: comparison to surface plasmon resonance." *Analytical chemistry* 79.13 (2007): 4879-4886.

Comment [RJ3]: Yang, Liju, Yanbin Li, and Gisela F. Erf. "Interdigitated Array Microelectrode-Based Electrochemical Impedance Immunosensor for Detection of Escherichia coli O157: H7." *Analytical chemistry* 76.4 (2004): 1107-1113.

Comment [RJ4]: Geng, Ping, et al. "Self-assembled monolayers-based immunosensor for detection of Escherichia coli using electrochemical impedance spectroscopy." *Electrochimica Acta* 53.14 (2008): 4663-4668.

Comment [RJ5]: Chowdhury, Ankan Dutta, et al. "Label free polyaniline based impedimetric biosensor for detection of E. coli O157: H7 Bacteria." *Sensors and Actuators B: Chemical* 171 (2012): 916-923.

Comment [RJ6]: Escamilla-Gómez, Vanessa, et al. "Gold screen-printed-based impedimetric immunobiosensors for direct and sensitive Escherichia coli quantisation." *Biosensors and Bioelectronics* 24.11 (2009): 3365-3371.

Comment [RJ7]: Lin, Yueh-Hui, et al. "Disposable amperometric immunosensing strips fabricated by Au nanoparticles-modified screen-printed carbon electrodes for the detection of foodborne pathogen Escherichia coli O157: H7." *Biosensors and Bioelectronics* 23.12 (2008): 1832-1837.

It is well known that the amount of antibody linked to the surface can have important consequences onto the performance of the sensor. The amount of antibody grafted onto the PEG/rGO/PEI interface proved to be indeed of great importance for the sensing performance. **Figure 5A** depicts in the form of a bar diagram the oxidative

current recorded upon incubation with *E. coli* UTI89 when different concentrations of anti-fimbrial *E. coli* antibodies were immobilized onto the sensor's surface. An interface modified with 10 ng mL⁻¹ anti-fimbrial antibodies results indeed in an optimal sensing current to *E. coli* UTI89 with the largest current drop.

The specificity of this electrode architecture for the sensing of FimA carrying *E. coli* UTI89 over $\Delta fimA$ deprived *E. coli* is evidenced in **Figure 5B**, showing a far lower change of the current response compared to that of *E. coli* UTI89 (**Figure 4A**).

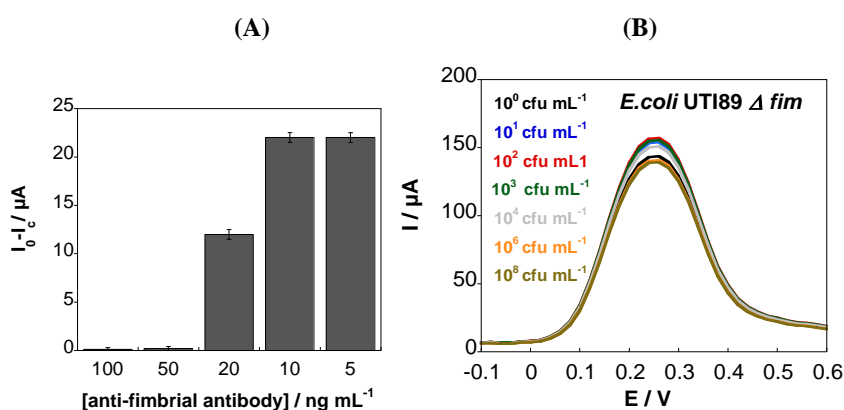


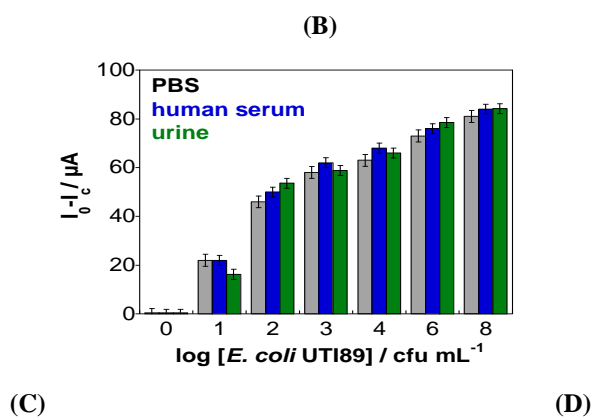
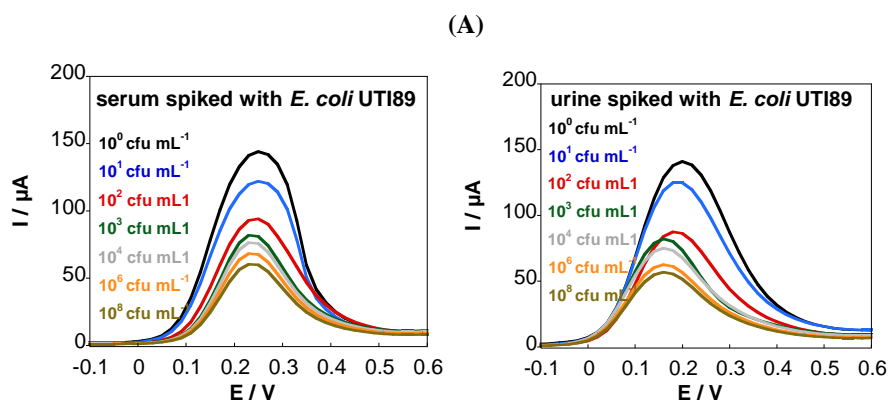
Figure 5: (A) Influence of anti-fimbrial antibody concentration on the current response of PEG/rGO/PEI-anti-fimbrial modified Au electrodes in [Fe(CN)₆]⁴⁻ (10 mM)/KCl (0.1 M); The current was recorded upon incubation with 100 cfu mL⁻¹. (B) DPV of PEG/rGO/PEI-anti-fimbrial modified Au electrodes measured in [Fe(CN)₆]⁴⁻ (10 mM)/KCl (0.1 M) before and after incubation for 30 min in aqueous solutions of *E. coli* UTI89 Δfim (10¹-10⁸ cfu mL⁻¹).

The reproducibility of PEG/rGO/PEI-anti-fimbrial modified gold electrodes for *E. coli* UTI89 sensing, expressed in terms of the relative standard deviation, was determined to be 2.3 % at *E. coli* UTI89 bacteria concentration of 10⁴ cfu mL⁻¹ (n=5). The long-term stability of the PEG/rGO/PEI-anti-fimbrial electrode upon storage in

PBS at 4 °C for 2 weeks was also evaluated. A loss of 2.8 % in the peak current was observed at *E. coli* UTI89 bacteria concentration of 10^4 cfu mL⁻¹.

3.5. Measurements in real samples

The reliability and potential application of the developed sensor for bacteria sensing in real samples were examined. *E. coli* UTI levels in urine and human serum samples spiked with *E. coli* UTI at different concentrations were determined and the current response was compared to that obtained in PBS. **Figure 6A** depicts the electrochemical signal obtained from spiked serum and urine, showing the same trend as in PBS, i.e. a decrease of current with increasing *E. coli* UTI89 concentration. **Figure 6B** compares the current responses for *E. coli* UTI89 detected on the PEG/rGO/PEI-anti-fimbrial modified Au electrodes in PBS or spiked human serum and urine. The differences in detected *E. coli* UTI89 concentrations are within the error of the analysis technique, pointing towards the possibility to analyze real samples with the developed sensor.



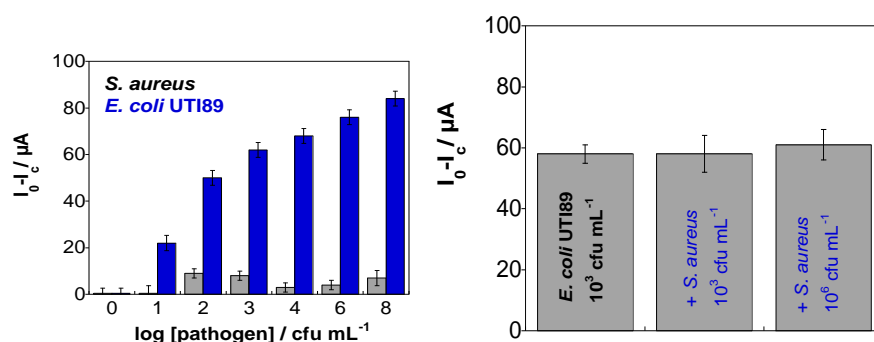


Figure 6: (A) DPV of PEG/rGO/PEI-anti-fimbrial modified gold electrodes recorded in $[\text{Fe}(\text{CN})_6]^{4-}$ (10 mM) before and after incubation for 30 min in human serum and urine samples spiked with different concentrations of *E. coli* UTI89 (10^1 - 10^8 cfu mL^{-1}); (B) Comparison between the electrochemical signature of *E. coli* UTI89 in PBS (grey), in human serum (blue) and when analyzed in urine (green) samples; (C) Recorded oxidative current in $[\text{Fe}(\text{CN})_6]^{4-}$ (10 mM) in human serum spiked with different concentrations of *S. aureus* (10^1 - 10^8 cfu mL^{-1}); (D) Comparison between the electrochemical signature of human serum spiked with 10^3 cfu mL^{-1} *E. coli* UTI89 and in presence of *S. aureus* at 10^3 and 10^6 cfu mL^{-1} .

The specific detection of pathogenic *E. coli* in complex media remains a challenging task. To study if the developed electrode is indeed suitable for specific *E. coli* UTI89 sensing in serum, we first tested serum samples spiked with *S. aureus*. No change in electrochemical signal was observed even for high *S. aureus* concentrations, suggesting the high specificity of the anti-fimbrial modified electrode toward *E. coli* UTI89 (Figure 6C). The developed sensor was finally exploited to detect *E. coli* UTI89 in human serum samples spiked with 10^3 cfu mL^{-1} *E. coli* UTI89 and in addition with *S. aureus* (10^3 and 10^6 cfu mL^{-1}). From Figure 6D it becomes evident that the presence of *S. aureus* is not interfering significantly with the detected current response of *E. coli* UTI89 on PEG/rGO/PEI-anti-fimbrial modified gold electrodes.

Conclusion

We have demonstrated the potential of polyethyleneimine modified reduced graphene oxide nanosheets electrophoretically deposited onto gold electrode for the sensitive and selective electrochemical detection of *E. coli* UTI89. Covalent

functionalization of the Au electrode with anti-fimbrial *E. coli* antibodies resulted in a sensing device highly sensitive to *E. coli* UTI89. Using differential pulse voltammetry measurements and $\text{Fe}(\text{CN})_6^{4-}$ as a redox probe, a detection limit of 10 cfu mL^{-1} was achieved. As *E. coli* UTI89 concentrations of 10^4 cfu mL^{-1} are correlated to highly infected patients, the low detection limit will allow to give indication at early stages of eventual urinal infections. The interfaces proved to be also adequate for sensing in human serum and urine samples as well as in the presence of other bacteria species, making the approach of high clinical interest. Furthermore, the concept of using a rGO/PEI modified electrode for covalent modification with pathogenic antibodies is general and can be easily adapted to any other pathogen species, making the approach very versatile.

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