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Sensitive detection of pyoverdine with an electrochemical sensor based on electrochemically generated graphene functionalized with gold nanoparticles

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ABSTRACT

The design and development of an electrochemical sensor for the sensitive and selective determination of pyoverdine, a virulence factor secreted by *Pseudomonas aeruginosa*, bacteria involved in nosocomial infections is presented in this work. The presence of pyoverdine in water and body fluids samples can be directly linked to the presence of the *Pseudomonas* bacteria, thus being a nontoxic and low cost marker for the detection of water pollution as well as for the biological contamination of other media. The sensor was elaborated using layer-by-layer technique for the deposition of a graphene gold nanoparticles composite film on the graphite-based screen printed electrode, from aqueous suspension. Under optimal conditions, the electrochemical signal corresponding to the pyoverdine oxidation process was proportional to its concentration, showing a wide linear range from 1 to 100 μmol L^{-1} and a detection limit of 0.33 μmol L^{-1}. This sensor discriminate with satisfactory recoveries the target analyte in different real matrices and also exhibited low response to other interfering species, proving that this technique is promising for medical and environmental applications. In addition, the proposed nanocomposite platform presented good reproducibility, high and long term stability, the sensitivity for pyoverdine remain unchanged after being stored at 4 °C for four weeks.

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*), an ubiquitous gram-negative bacterium that colonizes various samples like soil, water, humans, animals, plants, sewage and hospitals, is considered an important opportunistic human pathogen, which strongly colonizes immunocompromised patients, such as those with cancer, burns, AIDS and pneumonia. Furthermore, *P. aeruginosa* is also a leading cause of morbidity and mortality, and a chronic lung infections marker, in patients with cystic fibrosis [1]. The pathogenicity of *P. aeruginosa*, comes from its ability to produce several secreted virulence factors such as toxins (exotoxin A, exoenzymes, etc.), proteolytic or lipolytic enzymes (proteases and lipase) and siderophores (pyoverdine, pyochelin, etc.).

Siderophores are secondary metabolites produced by bacteria in order to get access to iron, which is essential for bacterial growth, being involved in respiration processes and acting as a cofactor for many enzymes involved in fundamental biological functions. The bioavailability of iron in the environment is low because of poor solubility of ferric iron at physiological pH. The chemical structures of siderophores are heterogenous and the molecular masses of most of these compounds are between 200 and 2000 Da [2,3]. Pyoverdine is the archetype of a large family of siderophores called pyoverdines, which comprise more than 60 different compounds, all produced by different strains and species of *Pseudomonas* [4]. All those siderophores have in common the structure consisting in three parts: (i) a chromophore derived from 2,3-diamino-6,7-dihydroxyquinoline, (conferring the color and fluorescence of the molecule); (ii) a peptide moiety attached to the non-aromatic ring of the chromophore; and (iii) a dicarboxylic acid, amide or α-ketoglutaric acid attached to the C-3 of the chromophore (Fig. 1). Each *Pseudomonas* specie has as characteristic the different amino acid composition and the length (usually between 6 and 12 residues) of the peptide moieties [5].

This siderophore contributes in several ways to *Pseudomonas* pathogenesis, it providing bacteria with an essential nutrient for growth (iron), and support the formation of biofilms. Moreover, the pyoverdine complex with iron serves as a signaling molecule for the expression of major extracellular virulence factors, like exotoxin A and the protease IV [6,7].

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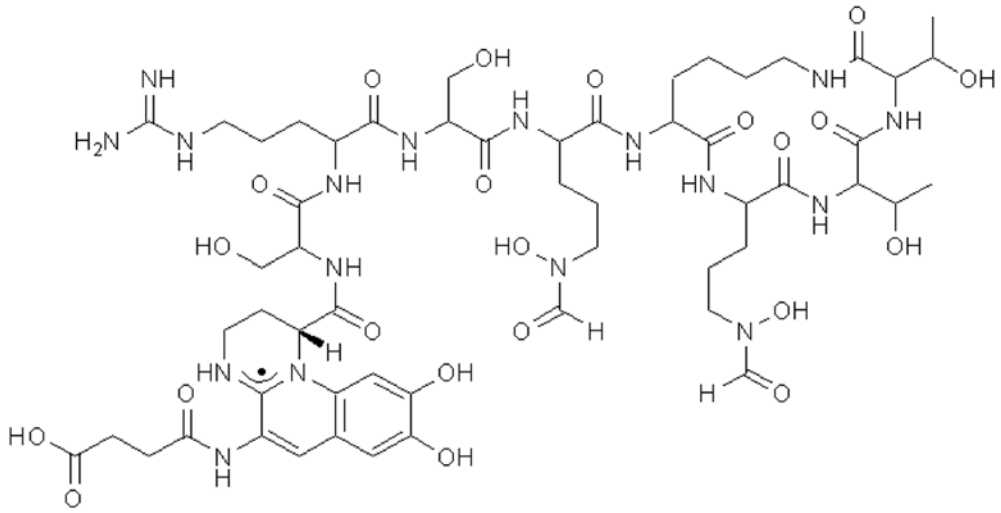

Fig. 1. The structure of pyoverdine.

The detection of pyoverdine in clinical and environmental samples can be thus considered a useful method to prove the presence of *P. aeruginosa*. Indeed, the ability to quickly detect the presence of bacteria and its secreted virulence factors like pyoverdine, in patient samples is of outmost importance to ensure patient further protection. All siderophores are very difficult to be detected because of their complicated structures and low concentrations in real samples [8]. For this reason, a large amount of sample is usually needed and specific techniques for extraction and enrichment are also required [9]. Classical treatment methods used for this purpose, such as chromatographic and extractive ones [10], presents some drawbacks like complex procedure and poor reproducibility. Solid phase extraction could be also applied to the extraction of siderophore this method implying the need of small quantity of samples and present acceptable reproducibility and efficiency [11]. Spectrophotometric techniques have been widely adopted for the analysis of most siderophores including the specific detection and quantification of pyoverdine in bacterial culture, by UV–Visible spectrophotometry [12]. In fact, these methods based on the spectral properties of the siderophore are generally complex, involving sample preparation, bacterial culture and incubation, as well as data processing, steps that often determine the substantial increase in the total duration of the analysis. Iso-electrofocusing may be considered as faster fingerprinting method for the identification and classification of *Pseudomonas spp*., for epidemiological purposes [13]. Hyphenated methods such high performance liquid chromatography-electro spray ionization mass spectrometry (HPLC-ESI-MS) and high performance liquid chromatography-tandem mass spectrometry coupled with solid phase techniques (SPE-HPLC-MS) were applied [9,14] but the majority allow only qualitative analysis or cannot cover all types of siderophores. Pyoverdine was used as fluorescence biomarker, many optical biosensors for detecting pathogenic bacteria and other analytes being elaborated based on the measurement of its fluorescence emission [15–20]. Pyoverdine can be also employed as capture probe for the identification of *Pseudomonas* spp. via Raman spectroscopy [21].

Thus the necessity to elaborate simple methods for the sensitive and selective detection of pyoverdine became crucial for the development of current analytical tools. Furthermore, the elaboration of miniaturized sensors, compatible with real-time response, and easier to use for in situ analysis has emerged as a priority [7].

Electrochemical sensors became of interest in the last decades due to their sensitivity, versatility, easiness in use, possibility of miniaturization, feature of decentralize analysis, as well as the opportunity for surface modification improving the selectivity and specificity. The surface modification could be achieved by using different types of nanomaterials like C-based nanomaterials (graphene, carbon nanotubes) [22] or metallic (nanoparticles or nanorods) [23,24].

Graphene, a crystalline allotrope of carbon formed from sp^2-bonded carbon atoms, has become one of the most exciting topics of research in the last few years. Graphene is currently making an important impact in the electrochemistry field due to its high surface area, excellent chemical stability, and unique electronic and mechanical properties [25]. The two-dimensional structure of this material is particularly attractive for the attachment of metallic nanoparticles. Gold nanoparticles (AuNPs) have the advantages of good biocompatibility, high catalytic activity, and fast electron transfer rate [26]. Recently, several researchers showed that the deposition of metal nanoparticles on the graphene surface can significantly improve the performances of the graphene-modified electrodes [27,28].

Herein, we report on the deposition of a composite material based on graphene and AuNPs onto graphite screen-printed electrodes (SPEs), via layer-by-layer deposition from aqueous suspension. This platform was applied for the elaboration of a sensor for pyoverdine detection in several complex real samples (serum, tap water and saliva). The combination of graphene generated by electrochemical exfoliation and AuNPs at the electrode surface resulted in a very simple fabrication procedure with excellent analytical performance in terms of sensitivity, reproducibility and linear range. To the best of our knowledge, this is the first approach of detecting pyoverdine as a possible marker for *Pseudomonas* spp. presence in different matrices, by using the signal due the electrochemical oxidation of pyoverdine.

2. Experimental part

2.1. Materials

All reagents were of analytical grade and used without further purification. All solutions were prepared using double distilled water and ultrapure water (Milli-Q, Millipore; 18 MΩ cm^{-1}). Sulfuric acid, nitric acid, hexachloroauric acid, ethanol and sodium ascorbate were purchased from Merck Chemicals. Sodium chloride, disodium hydrogen phosphate, sodium dihydrogen phosphate, potassium ferrocyanide ($K_4[Fe(CN)_6]$), potassium ferricyanide ($K_3[Fe(CN)_6]$) and graphite rods (6 mm diameter, 99.995% purity) were supplied by Sigma-Aldrich. Pyoverdines-Fe complex from *Pseudomonas fluorescens* was purchased from Sigma. A stock solution of 860 μmol L^{-1} was prepared in ultrapure water (MilliQ) and stored at −20 °C. Solutions of different concentrations were daily prepared in pH 7.4 phosphate buffer saline (PBS) of 0.02 mol L^{-1} and stored for a short time at 4 °C. Several analytes were