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Casein modified gold nanoparticles for future theranostic applications

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ABSTRACT

The synthesis and characterization of gold nanoparticles (AuNPs 20 nm sized) modified with k-casein derived peptides in order to monitor the peptide effect as bacterial adhesion inhibitor, thanks to the carrier/concentrator effect of the AuNPs is here presented. Some aspects related to the stability of AuNP/peptide conjugates for a potential application in the design of an electrochemical biosensor for pathogen bacteria detection are also discussed. This peptide based nanoparticle assay takes advantage of the dual character of the AuNPs: as carrier of the biorecognition molecule and also as electrocatalytic label, allowing the evaluation of the pathogen bacteria–peptide interaction in a simple and rapid way through the chronoamperometric monitoring of the hydrogen evolution reaction on screen-printed carbon electrodes. The developed proof of concept theranostic system may open the way to therapeutic and biosensing applications with interest for various fields.

1. Introduction

Nanomaterials (NMs) play an important role in current sensing and biosensing technologies. These materials are showing improvement of the performance of biosensing systems in general i.e. for the detection of proteins (De la Escosura-Muñiz et al., 2010a), cells (Perfézou et al., 2011), heavy metals (Aragay et al., 2011) and that of electrochemical sensing devices particularly. In addition to the biosensing applications (De la Escosura-Muñiz et al., 2008; Merkoçi, 2010; Pérez-López and Merkoçi, 2011), the use of nanoparticles (NPs) as carriers of biomolecules and their application in biomedical and nutritional technologies, between others, is an emerging research field in the last years. It is well known that NPs exhibit physical properties that are truly different from both small molecules and bulk material (Dreaden et al., 2012). The special properties of NPs are due to their high ratio between surface area/total volume and the high surface energy, allowing a strong biomolecules adsorption. These biomolecules can be used in order to carry the nanoparticles to the target organ and/or the biomolecules by themselves can exert therapeutic effects (Mohanraj and Chen, 2006). Gold nanoparticles (AuNPs) are specially suitable for such applications, due to their low toxicity (Xue et al., 2011) and good biocompatibility with peptides, proteins, DNAs, etc. (Xu et al., 2006). These nanomaterials can be synthesized reproducibly, modified with seemingly limitless chemical functional groups, and in certain cases, characterized with atomic-level precision, (Giljohann et al., 2010).

Thanks to the combination of all these properties, AuNPs have been extensively proposed for applications in several fields such as the environmental and the food/agriculture one (Huang et al., 2011; Dykman and Khlebtsov, 2012; Jans and Huo, 2012) being especially relevant their application for biomedical and therapeutic purposes (Merkoçi, 2010). Functional nanomaterials show different modalities for treatment of common diseases such as cancers (Peer et al., 2007; Barreto et al., 2011; Yang et al., 2012), abnormal blood vessel growth (Chauhan et al., 2012), and infectious diseases (Blecher et al., 2011).

Among potential target diseases for functional nanomaterials are the enteric diseases, in both humans and animals. While infants diarrhea is a major cause of mortality in developing countries, enteric diseases is also a leading cause of mortality in piglets and a major cause of economic losses in the pig industry (Madec et al., 2000). Enterotoxigenic *E. coli* (ETEC) K88 is the main bacterial cause of diarrhea in piglets around weaning and the adhesion of ETEC to the intestinal mucosa is a prerequisite step for its colonization. Specifically, *E. coli* F4, K88 serotype expresses fimbrial adhesions which specifically identifies affinities to glycoproteins, sialoglycoproteins or glycosphingolipids in the membrane of host cells (Sharon and Gallagher, 2009; Shoaf-Sweeney and Hutkins, 2009). Recently, different authors have suggested that the dietary inclusion of some receptor analogs, on the basis of their glycoside composition, would be a practical strategy to reduce the number of some

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intestinal pathogens that bind to the animal cells via carbohydrate-specific adhesions (Lane et al., 2010).

The casein glycomacropeptide (CGMP), a glycoprotein originated during cheese manufacture, has shown promising effects on the interaction with the microbiota through the activity of carbohydrate moieties present in the molecule (sialic acid content of around 4.2%, (Nakano et al., 2007)). Some authors have reported that CGMP binds the cholera toxin of *Vibrio cholera* (Kawasaki et al., 1992) and inhibits the adhesion of pathogenic *E. coli* to the mucosal surface or its growth in vitro (Malkoski et al., 2001) and *in vivo* and even it has anti-cariogenic properties (Aimutis, 2004). In addition CGMP may also interfere with the host cells, and has been shown to affect both innate and adaptive immunity, modulating the immune/inflammatory response by the activation of macrophages, down-regulation of IL-6 and up-regulation of IL-10 (de Medina et al., 2010).

In the present study, we have combined the ability of the AuNPs to act as, as both carriers/electrocatalytic labels, with the properties of k-casein derived peptides to bind specific bacteria fimbriae adhesions. These peptides modified nanoparticles are used for the evaluation of the interaction between the peptides and the K88 fimbriae bacteria (K88) and found with interest for future potential applications not only for biosensing purposes but also for therapeutic ones. Such a theranostic platform can also be extended to other biotechnological applications including food, health and pharmaceutical fields.

2. Experimental section

2.1. Apparatus and electrodes

Zeta potential of the AuNPs and peptide/AuNPs conjugates was determined with a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd., UK) according to the manufacturer's recommendations.

Optical characterizations of the AuNPs and peptide/AuNPs conjugates were performed using a Transmission Electron Microscope (TEM) Jeol JEM-2011 (Jeol Ltd, Japan) and a Gemini SpectraMax M2e Multi-Mode Microplate Reader (Molecular Devices, CA, USA).

The electrochemical transducers used were homemade screen-printed carbon electrodes (SPCEs), consisting of three electrodes: working electrode, reference electrode and counter electrode in a single strip fabricated with a semi-automatic screen-printing machine DEK248 (DEK International, Switzerland). The reagents used for this process were: Autostat HT5 polyester sheet (McDermid Autotype, UK) and Electrodag 423SS carbon ink, Electrodag 6037SS silver/silver chloride ink and Minico 7000 Blue insulating ink (Acheson Industries, The Netherlands). (See the detailed SPCE fabrication procedure and pictures of the obtained sensors in the supplementary material, Fig. S1).

An ultrasonic bath (JP Selecta, Spain) was used for the bacteria/peptide/AuNP conjugate pre-treatment before the electrochemical measurements.

The electrochemical measurements were taken using a CompactStat potentiostat (Ivium Technologies, The Netherlands) connected to a PC. All the measurements were carried out at room temperature with a working volume of 50 μL, which was enough to cover the three electrodes contained in the SPCEs connected to the potentiostat by a homemade edge connector module.

2.2. Reagents and solutions

Caseinoglycopeptide (CGP) was purchased from Sigma-Aldrich (Spain) and dissolved in 0.01 M PBS, pH 6.8. Lacprodan (CGMP-10) was purchased from Arla Foods Ingredients (Denmark) and its solutions were prepared in 0.01 M PBS, pH 7.4.

Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$, 99.9%) and trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) reagents used for the gold nanoparticles preparation were also purchased from Sigma-Aldrich (Spain). KH_2PO_4 and K_2HPO_4 reagents used for the preparation of the phosphate buffer solutions (PBS) were acquired from Fluka (Spain).

An *E. coli* K88 fimbriae bacteria (K88) isolated from a colibacillosis outbreak in Spain serotype (O149:K91:H10 [K-88]/LT-I/STb) was provided by the *E. coli* Reference Laboratory, Veterinary Faculty of Santiago de Compostela (Spain). A non-fimbriated *E. coli* (F4-, F6-, F18-, LT1-, ST1-, ST2+, Stx2e-) (NF) isolated from the feces of a post-weaning piglet was donated by the Department of Animal Health and Anatomy of the Universitat Autònoma de Barcelona (Spain). *E. coli* K88 was cultured in unshaken Luria Broth (Sigma, St Louis) at 37 °C while the *E. coli* NF was cultured shaking the media. Cultures were serially passage every 48 h, at least three times.

All chemicals were used as received and all aqueous solutions were prepared in Milli-Q water.

Safety precautions necessary to work with this bacterium were followed during all the preparation and measurements steps in the lab.

2.3. Methods

2.3.1. Preparation of gold nanoparticles and modification with peptides

20 nm gold nanoparticles (AuNPs) were synthesized by reducing tetrachloroauric acid with trisodium citrate, a method pioneered by Turkevich et al. (1951). A total of 200 mL of 0.01% $HAuCl_4$ solution were boiled with vigorous stirring. 5 mL of a 1% trisodium citrate solution were added quickly to the boiling solution. When the solution turned deep red, indicating the formation of gold nanoparticles, it was left stirring and cooling down. In this way, a dispersed solution of AuNPs was obtained. The size of the obtained AuNPs follows a Gaussian distribution as evaluated by TEM analysis, being the main value (and its deviation) of 20.5 ± 2.2 nm (see Supplementary Material, Fig. S2). In order to facilitate the reading these AuNP are mentioned as "20 nm" NPs along the text.

The conjugation of AuNPs with CGP was performed adapting the procedure reported by Liu and Guo, (2009) where it is described that the critical micelle concentration of casein is around 1.0 mg/mL in 0.01 M PBS pH 6.8. Considering this, different mixtures of CGP in the above mentioned buffer and AuNPs solution (from the obtained stock solution of around 3 nM concentration) were prepared at different ratios. Concretely, three different concentrations of CGP (1, 0.1 and 0.01 mg/mL) and three different CGP/AuNPs concentration ratios (1:1, 1:3 and 3:1) were assayed. The incubations of the CGP/AuNPs solutions were performed in a final volume of 500 μL at 20 °C for 30 min with gentle mixing. Finally, in order to remove the excess of CGP, the conjugate was centrifuged 1× at 14,000 rpm (25 °C) and re-suspended in 500 μL of 0.01 M PBS pH 6.8.

In the case of the CGMP-10 peptide, a similar procedure was followed but using 0.01 M PBS, pH 7.4 as buffer and only a fixed condition: a 0.01 mg/mL concentration of peptide and a CGMP-10/AuNP concentration ratio of 3:1.

2.3.2. TEM analysis with negative staining

3 μL of uranyl acetate were added to 3 μL of the peptide/AuNPs conjugates previously placed on the carbon grill. After 1 min, the excess of acetate was removed with filter paper and dried at room temperature. For comparison purposes, this pre-treatment was also applied for the AuNPs without peptides modification.