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Research Paper

Exploring gold nanoparticles interaction with mucins: A spectroscopic-based study

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ABSTRACT

The interaction between two mucin types (mucin from porcine stomach – PGM and mucin from bovine submaxillary glands – BSM) and gold nanoparticles (GNPs) of various size (5, 20 and 40 nm) and functionalization (with cysteamine or thioglycolic acid) was studied under physiological conditions, in order to investigate the affinity of the nanoparticles to the proteins.

Different methods are employed to monitor the interactions: UV–vis and fluorescence spectroscopy, fluorescence lifetime, circular dichroism and transmission electron microscopy. These studies have shown the formation of a complex between GNPs and both PGM and BSM.

This aspect could be of great importance for the use of gold nanoparticles for biomedical purposes in those diseases where qualitative and quantitative mucin anomalies play an essential role in mucus composition and rheology.

1. Introduction

Gold nanoparticles (GNPs) are inert and non-toxic colloidal systems. They are easy to synthesize and have a large surface area (Azam et al., 2009) which can be exploited to easily functionalize the particles with molecules or biomarkers through thiol linkage (Ghosh et al., 2008). GNPs can be synthesized in different morphologies and size, ranging from 1 to 80 nm, therefore possessing unique physical chemical properties including tunable near-infrared (NIR) localized surface plasmon resonance (SPR) and the ability to bind amine and thiol groups (Jain et al., 2012). Gold nanoparticles have been employed for several decades in many fields (Khan et al., 2014): imaging (Giordano et al., 2016), bioengineering, biotechnology (Khan et al., 2013) and molecular biology (Jain et al., 2012) whose applications are dependent on the catalytic, electronic, magnetic and optical properties of the particles (Khan et al., 2013). The main application could be their use in cancer diagnosis and therapies (Mesbahi, 2010). In fact, it was demonstrated that GNPs contribute to enhance the generation of reactive oxygen species (ROS) such as OH, O^{2-} and $^1O_2$, under irradiation of X-rays in the diagnostic range (Ferrero et al., 2017; Strigari et al., 2017). Recently, some authors have described the preparation of gold nanoparticle (GNPs)-based vaccine candidates against the tumor-associated form of the mucin-1 (MUC1) glycoprotein (Cai et al., 2016). Moreover, very recently a group designed fluorescently encoded poly(vinyl alcohol) (PVA)-coated gold nanoparticles, functionalized with either negative ($-COO^-$) or positive ($-NH^{3+}$) surface charges, functionalized with a DC-SIGN antibody on the particle surface, enabling binding to a dendritic cell surface receptor. In this paper a 3D coculture model consisting of epithelial and immune cells (macrophages and dendritic cells) mimicking the human lung epithelial tissue was employed to assess the effects of aerosolized GNPs (Fytianos et al., 2017). These results demonstrate a potential development of pulmonary nanocarriers that can target lung. Lung offers numerous advantages as a delivery route for non-invasive drugs especially for localized therapy, *i.e.* lung cancer and treatment of airway diseases such as asthma, cystic fibrosis and chronic obstructive pulmonary disease (COPD). For all these applications, mucus penetration cannot be ignored. In fact, mucus is the first barrier that drugs must overcome to be absorbed and gain access to the circulatory system (Sigurdsson et al., 2013). The major constituents of mucus are water (95–99.5%) and high molecular weight glycoproteins called mucins. In the mucus, these large oligomeric glycoproteins form networks which are a chemical and physical barrier that not only protects the epithelial but also limits the use of oral administered and inhalatory drugs. Therefore, appropriate and physicochemical characteristics of nanoparticles and the identification of the mechanism of interaction with mucus is imperative to determine their biological behaviour in physiological and pathological environments.

The proof of concept of using mucin to mimic mucus is widely

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reported in the literature also by Hanes's group, who stated that reduced mucin-binding *in vitro* has been shown to correlate with rapid penetration of nanoparticles through mucus (Xu et al., 2015). Mucins are large transmembrane *O*-glycosylated proteins composed for 75% of carbohydrate and 25% of amino acid (Kesari et al., 2015). Mucins are the most abundant macromolecules in mucus, about 20 types have been identified, and are responsible for the biochemical and biophysical properties of mucus (Kesari et al., 2015). An abnormal viscosity and excessive amount of mucus is involved in many respiratory diseases such as cystic fibrosis, asthma and pulmonary diseases (Pontremoli et al., 2015). Even though structural and physico-chemical properties of mucins vary depending on their origin, the glycoproteins share common features. Generally, mucin molecules consist of hydrophilic and hydrophobic parts: a highly glycosylated central protein core and one or two terminal peptide regions which are either non-glycosylated or carry only a few carbohydrate moieties. In particular, mucin from porcine stomach (PGM) and mucin from bovine submaxillary glands (BSM) are described with reference to a bottlebrush model: a copolymer of relatively rigid (glycosylated) and relatively flexible (bare) moieties (Bansil and Turner, 2006). Other differences between the two mucins resides in the carbohydrate content of BSM in comparison with PGM having the higher content (*i.e.*, in BSM it is about 61–69 wt% while in PGM is about 83–86 wt%), and also the content of sialic acid in PGM and BSM differs. In fact, the sialic acid content in PGM is about 2–3 wt% leading to a weakly charged molecule, while BSM possesses a much higher content of sialic acid (about 30 wt%).

In the human body, GNPs have a good distribution and can easily get to the target cells, where they can carry out their action. Once GNPs enter the body, they are exposed to various molecules and can contact and adsorb many proteins such as ubiquitin, serum albumin, tumor necrosis factor (TNF), fibrinogen (Canoa et al., 2015) and cytochrome C. Many studies have investigated these interactions that can induce structural and physiological changes on the biomolecules (Dobrovolskaia et al., 2009); in particular, ubiquitous proteins are able to interact with GNPs and form a protein corona that influences the distribution of nanoparticles in the human body (Wang et al., 2015). The interest in studying the interaction that occurs between nanoparticles and mucins will provide a better understanding of the complex nature of mucin-particle interactions in terms of the surface characteristics of the nanoparticles. For this purpose a plethora of nanoparticles has been studied like silica, chitosan and the synthetic biodegradable polymer poly (lactic-*co*-glycolic acid) (PLGA) and its PEGylated derivative (PEG–PLGA), Natural or synthetic polymeric nanoparticles were studied in order to define rules for obtaining mucus penetrating nanoparticles. (Sunoqrot et al., 2017; Griffiths et al., 2015). Recently the effect of *b*-cyclodextrin and/or pluronic 127 coating on magnetic nanoparticles at mucin interface was also investigated (Boya et al., 2017). In our previous paper we studied the interaction between Carbon Nanotubes (CNTs) and mucin from porcine stomach Type III (PGM) and it was demonstrated that the superficial functionalization of the material may play a role on the binding (Barbero et al., 2016). Following these reasons, we studied the interaction between gold nanoparticles and mucin from two commercial mucins, porcine stomach Type III (PGM) and mucin from bovine submaxillary glands (BSM), in order to define their affinity with GNPs. We analysed the interaction with GNPs of different size (5 nm, 20 nm and 40 nm) and positively and negatively charged GNPs, under physiological conditions. Normally, to overcome *in vivo* delivery barriers, GNPs are modified with functional moieties such as stabilizing materials, targeting ligands, and bioresponsive linkers. However, it should be said that extensive functionalization may also cause unwanted toxic side effects. For this reason, we also decided to investigate the interaction of mucins with GNPs that present simple functionalization moieties like cysteamine and glycolic acid. The complex GNPs-mucin was characterized by UV–vis and fluorescence spectroscopy, circular dichroism, and transmission electron microscopy (TEM).

2. Materials and methods

2.1. Reagents

$HAuCl_4$ was purchased from Sigma Aldrich with a purity ≥ 99.9%, the reducing agents trisodium citrate and $NaBH_4$ produced by Sigma Aldrich had a purity ≥ 99% and ≥ 98% respectively.

Cysteamine and thioglycolic acid were purchased from Sigma Aldrich with a purity ≥ 98% and were used to functionalize gold nanoparticles. The solutions employed for the synthesis were prepared by dissolving the reagents in Millipore water.

Mucin from porcine stomach (PGM) (Type III, bound sialic acid 0.5–1.5%, partially purified powder) was purchased from Sigma Aldrich. PGM stock solution (1 mg/mL) was prepared by dissolving the protein in PBS (phosphate buffer solution, 2 mM, pH = 7.4).

Mucin from bovine submaxillary glands (BSM, Type I-S) was purchased from Sigma Aldrich. BSM stock solution (1 mg/mL) was prepared by dissolving the protein in PBS (phosphate buffer solution, 2 mM, pH = 7.4). Both solutions were sonicated for few seconds to enhance solubility in water.

2.2. Synthesis and functionalization of GNPs

Gold colloidal nanoparticles, with three different sizes 5, 20, 40 nm, were synthesized with the Turkevich's method by varying the ratio between trisodium citrate in order to obtain various diameters (Daniel and Astruc, 2004).

To synthesize GNPs with a size of about 5 nm, 10 mL Millipore water solution of the gold salt (0.5 mM) were added to 10 mL Millipore water solution of trisodium citrate (0.6 mM); then 0.6 mL of a water solution of $NaBH_4$ (0.1 M) was added to the solution.

15.4 mL NaOH 20 mM and 19.6 mL Millipore water were added to 5 mL of a 25mM $HAuCl_4$ solution in Millipore water. The solution was heated for 30 min at 110 °C. 1.2 mL Millipore solution of trisodium citrate 170 mM are then added and the sample was again left for 15 min at 110 °C. GNPs with a diameter of 20 nm were obtained.

To obtain GNPs with 40 nm of diameter, the citrate to gold salt mole ratio was 1.36 and 1.5 mL of 1% trisodium citrate was added to 250 mL $HAuCl_4$ 0,25 mM in Millipore water solution.

Gold nanoparticles with a diameter of 20 nm were functionalized with cysteamine (Cys) and thioglycolic acid (Tga) to shape GNPs with a positive and a negative charge, respectively. To 10 mL GNPs 1 mM (197.00 μg/mL) were added 500 μL Millipore water solution of cysteamine 0.26 mM. To 10 mL GNPs 1 mM were added 15 μL Millipore water solution of thioglycolic acid 0.26 mM.

All the GNPs were purified by centrifugation at room temperature for 5 min at 12,000 rpm.

The GNPs dimension was checked by UV–vis spectroscopy and TEM (see Fig. S1 SI).

2.3. GNP-protein complex formation and purification

1.5–4.5 mL of GNPs (1 mM, 197.00 μg/mL) were added to a 0.5 mL solution of mucin protein (1mg/mL) in 2 mM sodium phosphate buffer, pH 7.2 and the mixture was stirred for 1 h at room temperature. The reaction mixture was then loaded on a Sephadex G-25 gel-permeation column. The protein complexes were eluted with a sodium phosphate buffer and the elution was monitored by UV/vis spectroscopy.

2.4. Instrumentation

2.4.1. UV–vis and fluorescence spectroscopy

UV–vis measurements were recorded using a UH5300 Hitachi spectrophotometer. Increasing concentrations of GNPS ranging 10 μM − 200 μM (from 1.97 μg/mL to 39.00 μg/mL), 10 μM − 400 μM (from 1.97 μg/mL to 78.00 μg/mL) and 10 μM − 450 μM (from 1.97 μg/