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Gold nanoparticles in the engineering of antibacterial and anticoagulant surfaces☆

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ABSTRACT

Simultaneous antibacterial and anticoagulant surfaces have been prepared by immobilization of engineered gold nanoparticles onto different kinds of surfaces. The gold nanoparticle core is surrounded by a hemocompatible, anticoagulant polysaccharide, 6-*O* chitosan sulfate, which serves as reduction and stabilizing agent for the generation of gold nanoparticles in a microwave mediated reaction. The particle suspension shows anticoagulant activity, which is investigated by aPTT and PT testing on citrated blood samples of three patients suffering from congenital or acquired bleeding disorders. The amount of nanoparticles deposited on the surfaces is quantified by a quartz crystal microbalance with dissipation unit. All gold containing surfaces exhibit excellent antimicrobial properties against the chosen model organism, *Escherichia coli* MG 1655 [R1-16]. Moreover, blood plasma coagulation times of the surfaces are increased after deposition of the engineered nanoparticles as demonstrated by QCM-D.
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1. Introduction

In the past years, silver containing antimicrobial materials have reentered the focus in the engineering of medical materials. In particular, the increasing amounts of multi resistant bacteria strains, which are very often involved in hospital acquired infections (HAI, ca. 4 Mio./year in the EU), are a threat to modern societies (ECDC, 2012). While these strains are not more infectious than non-resistant ones, an effective treatment is very often difficult and leads to complications and in severe cases even to death (37,000 year^{-1} in the EU). The origins of these resistances are manifold and include careless use and prescription of antibiotics. Usually, the time span until new resistances of bacteria toward antibiotics are developed covers a few years (e.g. penicillin: mass production started in 1943, first observation of resistant strains in 1947), in a few cases even some tens of years (Davies & Davies, 2010). Nevertheless, the ability of bacteria to handle and to tolerate antibiotics after a certain period of time can be simplified in first glance as a form of evolutionary process, As a consequence, the cells develop tolerance leading to the evolution of resistances. In the beginning of antibiotic exposure, all non-resistant bacteria are killed while the resistant ones survive and multiply. In fact, resistant bacteria are much older than man-made antibiotics and the reservoir of antimicrobial strains in the environment is underestimated so far (D'Costa, McGrann, Hughes, & Wright, 2006).

However, very recently this rather general sight on the topic was related to so called persister bacterial cells. These persisters are phenotypic variants of normal cells, but they are mostly dormant and inactive. Therefore, antimicrobial agents do not kill these cells and tolerances leading to resistances are developed. In addition, the formation of biofilms buries the persister cells making them even more difficult to treat (Conlon et al., 2013).

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In contrast, there are hardly any bacterial strains known, which exhibit resistance against silver, despite the exposure of bacteria to non-inhibitory silver levels for more than 4 billion years (Alexander, 2009). This can be related to the rather unspecific mode of action, which interferes many enzymatic key processes (also those in persisters) taking place in microorganisms (Lara, Garza-Treviño, Ixtepan-Turrent, & Singh, 2011). Therefore, Ag in different kind of forms (bulk-metal, ionic, nanoparticles) is used in the coating/composition of medical materials such as wound dressings for instance (Alexander, 2009). However, one of the drawbacks of silver is its low (photo)chemical stability and it is the origin of argyria, an illness related to massive silver intake, which limits its use in several areas of medical devices. An alternative to silver is gold since it is assumed to exhibit high chemical stability and inertness under physiological conditions. This stability makes gold an attractive target in many areas of research in life sciences, biomedicine and materials sciences. Applications of gold nanoparticles as drug carriers in e.g. cancer therapy, as diagnostic tools in fluorescent tomography or as antimicrobial component have been described recently (Khan, Vishakante, & Siddaramaiah, 2013).

Since materials equipped with gold (nanoparticles, NPs) feature antimicrobial activity, they are very attractive for the design of antimicrobial surfaces. It is known that the mode of action of gold nanoparticles (AuNPs) against microorganism is very similar to those of silver NPs (Bhattacharya & Mukherjee, 2008). In addition, the use of bio- and hemocompatible polysaccharides as capping/stabilizing agents (Cheng, Betts, Kelly, Schaller, & Heinze, 2013; Sacarescu, Simionescu, & Sacarescu, 2011) for NPs allows for applying them in materials where simultaneous anticoagulant activity is required (Breitwieser et al., 2013a,b; Croes, Stobberingh, Stevens, Knetsch, & Koole, 2011; Huang & Yang, 2004).

The use of natural anticoagulants such as heparin for this purpose imposes the risk of infections in case cGMP are not strictly followed since heparin is currently obtained from vertebrates. For this reason, it is highly desirable to replace heparin against semi-synthetic anticoagulants such as sulfated celluloses and chitosans (Fasl et al., 2010; Gericke et al., 2011). Although the specific mode of interaction of heparin in the blood coagulation cascade via a pentasaccharide unit is probably hard to mimick using these semisynthetic compounds, research in this area may result in additives (either on surfaces or in solutions) enhancing the effects of heparin rather than replacing it. Another disadvantage of these semi-synthetic sulfated polysaccharides is that they do not provide broad-spectrum antibacterial activity. Even sulfated chitosans are not significantly active against a variety of microorganisms close to neutral pH values since the amine groups are blocked by the negatively charged sulfate groups (Fasl, Fras-Zemljic, Gössler, Stana-Kleinschek, & Ribitsch, 2012). As a consequence, these polysaccharides are not capable to inhibit the growth of (multi-resistant) strains, which would be highly desirable later on in materials that come into contact with blood. Since these sulfated polysaccharides have been successfully deposited on artificial blood vessels (e.g. PET), there is a large potential in case antimicrobial activity can be incorporated into this class of polysaccharides.

In this paper, we aim at addressing this problem by encapsulating gold nanoparticles in an anticoagulant sulfated chitosan polymer. There are several questions that must be envisaged starting from the applicability of the chosen polysaccharide for nanoparticle preparation, the anticoagulant activity of nanoparticles in suspensions and their behavior, when they are adsorbed onto surfaces in terms of antibacterial and anticoagulant activity. Since an extensive study on all of the parameters would go beyond scope of a single paper, selected tests will be performed in order to assess the suitability of the chosen approach in order to prepare *simultaneous* antibacterial and anticoagulant surfaces. In order to tackle these questions, the paper is structured as follows: (i) Detailed characterization of the nanoparticles size and charge in order to validate the presence of a sulfated chitosan shell around a gold core. (ii) Determination of the anticoagulant activity of the gold nanoparticle suspension using aPPT and PT tests on patients suffering from clotting diseases. (iii) Immobilization of the nanoparticles onto cellulosic surfaces and quantification using a quartz crystal microbalance. (iv) Determination of antibacterial activity against a model organism (gram negative one, *Escherichia coli* MG 1655 [R1-16]) and estimation of anticoagulant activity of blood plasma on the surfaces.

2. Experimental

2.1. Materials

$HAuCl_4 \cdot 3H_2O$ (>99.99%) was obtained from Alfa Aesar (Karlsruhe, Germany). For the dissolution of $HAuCl_4 \cdot 3H_2O$, MilliQ water was used ($\Omega \leq 18{\cdot}2\ \mu\Omega$). Sulfated chitosan was prepared from hydrolyzed chitosan (M_w ca. 20 kDa, degree of deacetylation according to producer: 85%, prepared from low molecular weight chitosan (source: crab shells, Batch No. 13604PC) from Sigma Aldrich by refluxing in 1 M HCl under nitrogen atmosphere, followed by adjustment to pH 5.8 using 2 M NaOH, subsequent dialysis and lyophilization) and $ClSO_3H$ under reflux in DMF according to a literature procedure (Fasl et al., 2010). Sulfated chitosan with a DS_S of 0.86 and a M_w of ca. 10 kDa was obtained after sulfation. The molecular weight was determined by using an Ubbelohde viscosimeter with a diameter of 0.24 mm which was placed in a thermostat ($T = 20\,^{\circ}C$). The reduced viscosity η_{red} was determined according to the relation $\eta_{red} = \frac{(t_{sample}/t_{solvent})-1}{c_{sample}}$ whereby t_{sample} is the time which was needed for the different concentrations to pass the marks, $t_{solvent}$ is the time needed for the water to pass the marks and c_{sample} is the corresponding concentration in mg/ml. The reduced viscosity was plotted as a function of the sample concentration and extrapolated to zero concentration. The intercept of the linear plot is the intrinsic viscosity $[\eta]$. The calculation of M_w was performed using the *Mark–Houwink*–Sakurada equation

$$[\eta] = K \times M_w^a$$

The used values for the coefficients K (4.97×10^{-5}) and a (0.77) were determined by Vikhoreva et al. (2005) for sulfated chitosan with a sulfur content of 14–15%.

The degree of deacetylation (83%) of the sulfated chitosan was determined by conductometric titration: 2 ml of 1% (w/w) aqueous sulphated chitosan solution was pipetted into a titration vessel. 1 ml of 100 mM HCl was also added to adjust the pH to about pH 3. At this pH value all amino groups protonate into positively charged NH_3^+ groups which behave as a weak acid in the analytic solution and can be titrated with a base titrant (KOH). The vessel was then filled up with distilled water to a volume of 40 ml. A Mettler Toledo T70 titrator, with a 10 ml burette, was used for the incremental addition of 100 mM KOH as the titrant. Increments of 0.1 ml were added in a timed interval within 10–60 s. An equilibrium criteria of 0.1 μS/10 s was set. The equivalence points were evaluated with the "slope" method. The equivalence points correspond to the crossing points of the titration curve slopes.

EDC·HCl (1-ethyl-3,3-dimethylaminopropyl carbodiimide hydrochloride, 98%) was obtained from Sigma. CMC (carboxymethyl cellulose, M_w 90,000, DS_{COONa}: 0.7) and PEI (high molecular weight) were purchased from Sigma Aldrich. Trimethylsilyl cellulose with a DS_{Si} of 2.55, (M_w = 175,000, M_n = 36,000, kindly provided by the Centre of Excellence for Polysaccharide Research, University of Jena) was used for cellulose model film preparation (Koehler, Liebert, & Heinze, 2008). Blood

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