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Antimicrobial activity of menthol modified nanodiamond particles

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ABSTRACT

Advances in nanotechnology have seen the development of several microbiocidal nanoparticles displaying activity against biofilms. These applications benefit from one or more combinations of the nanoparticle properties. Nanoparticles may indeed concentrate drugs on their surface resulting in polyvalent effects and improved efficacy to fight against bacteria. Nanodiamonds (NDs) are among the most promising new materials for biomedical applications. We elucidate in this paper the effect of menthol modified nanodiamond (ND-menthol) particles on bacterial viability against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria. We show that while ND-menthol particles are non-toxic to both pathogens, they show significant antibiofilm activity. The presence of ND-menthol particles reduces biofilm formation more efficiently than free menthol, unmodified oxidized NDs and ampicillin, a commonly used antibiotic. Our findings might be thus a step forward towards the development of alternative non antibiotic based strategies targeting bacterial infections.

1. Introduction

Bacterial infections are one of the most serious risks to public health. Extensive efforts have been devoted to develop rapid and sensitive methods for the detection, identification of the pathogen strain at hand and for destruction or inhibition of pathogens and their actions [1–4]. Despite considerable recent progress in the understanding of the mechanisms underlying bacterial infections, and in the development of nanostructured materials displaying antibacterial properties and activity against biofilms [1,5–8], the quest to design and fabricate new antibacterial nanostructures remains a high research priority. Nanoparticles have been considered as affective solution to fight against bacterial infections [4,9–15]. Such nanostructures allow the concentration of antibacterial agents and functions on their surface to deliver polyvalent effects. Some materials such as silver are themselves antibacterial and can result in enhanced efficacy [16–18]. Although recent studies using silver nanoparticles appear promising [19], the antimicrobial activity is highly dependent upon their size and shape, with smaller particles demonstrating greater activity [20,21]. More recently concerns about the cytotoxic effects of silver nanoparticles against human cells have been voiced [22]. Moreover, practical applications of Ag NPs are often hampered by the aggregation and loss of antibacterial activity [23].

Among the potential alternatives to these compounds are nanodiamond particles (NDs). One of the advantages of NDs over other carbon-based materials such as fullerenes and carbon nanotubes is that they are completely inert, optically transparent, biocompatible and can be functionalized in many ways depending on their intended ultimate application [24–31]. Although their *in vivo* toxicity depends on their particular surface characteristics [32], ND particles do not induce significant cytotoxicity in a variety of cell types [32–35] and have been used in a variety of biomedical applications. More recently, the antibacterial activity of diamond particles [36–38] and their potential to interfere with biofilm formation [1] have been highlighted. The underlying anti-adhesive strategy proposed was based on the interfering with type 1 fimbriae-mediated mannose recognition events [39]. Such biofilm disrupting activity had not been observed previously for other glyco-nanoparticles (glyco-NPs) such as glycofullerenes, gold-based glyco-NPs or for other multivalent mannose-derived molecules [2,40]. Wehling et al. have concluded that the bactericidal activity of diamond particles is directly linked to their surface chemistry, being thus the driving force of antibacterial effects [37].

In this paper, we investigate the antimicrobial properties of ND particles modified with menthol (Fig. 1A) and their potential to inhibit the

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growth of Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria.

Menthol, also known as mint camphor, is a naturally occurring cyclic terpene alcohol of plant origin, which has been used since antiquity for medicinal purposes [41–43]. It is a major constituent in the essential oils of *Mentha canadensis L.* (cornmint) and peppermint possessing well-known cooling characteristics and a residual minty smell of the oil remnants from which it was obtained. Several studies have additionally demonstrated that next to the effect of sensory parameters, menthol has antibacterial and antifungal activities [44,45]. However, the antimicrobial activity was reported to be lower compared to antibiotics such as penicillin [46]. More recently, Imbert and co-workers showed the *in vitro* activity of terpenes, including that of menthol, against *Candida* biofilms [47]. We show here that ND-menthol particles affect moderately *S. aureus* and *E. coli* growth in a concentration dependent manner. In addition, the ND-menthol particles are found to inhibit *S. aureus* and *E. coli*-driven biofilm growth significantly.

2. Experimental

2.1. Materials

Hydroxyl-terminated nanodiamond (ND-OH) particles were purchased from International Technology Center (Raleigh, NC, USA) and exhibit a primary average particle size of 4.0 nm. (1*R*,2*S*,5*R*)-(−)-menthol, 4-dimethylaminopyridine (4-DMAP), succinic anhydride, hexane (Hex), dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), ethyl acetate (EtOAc), *N*,*N*′-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich and used as received.

All microbiological media, equipment and the ampicillin were purchased from Fisher Scientific (UK).

2.2. Synthesis of menthol derivative (**2**)

2.2.1. Succinic acid mono-(2-isopropyl-4-methyl-cyclohexyl) ester (**1**)

To the solution of menthol (0.8 g, 5 mmol) in chloroform, 4-DMAP (0.31 g, 2.5 mmol) and succinic anhydride (1.02 g, 10 mmol) were added. This mixture was stirred overnight under reflux. After solvent evaporation, the crude product was purified using flash column chromatography with Hex:EtOAc as eluent to give white crystals 1.1 g (84%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.2–11.1 (broad, 1H) 4.77–4.68 (m, 1H), 2.67–2.55 (m, 4H), 2.05–1.95 (m, 1H), 1.91–1.79 (m, 1H), 1.74–1.62 (m, 2H), 1.55–1.33 (m, 2H), 1.1–0.8 (m, 10H), 0.78–0.7 (d, 3H).

2.2.2. Succinic acid 2,5-dioxo-pyrrolidin-1-yl ester 2-isopropyl-4-methyl-cyclohexyl ester (**2**)

To the solution of acid (**1**) (0.5 g, 1.9 mmol) in dichloromethane, *N*,*N*′-dicyclohexylcarbodiimide (0.4 g, 1.9 mmol) and *N*-hydroxysuccinimide (0.22 g, 1.9 mmol) were added and the mixture was stirred overnight at room temperature. After filtration and solvent evaporation a white powder was obtained, yield: 95%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.77–4.68 (m, 1H), 2.85–2.81 (s, 4H), 2.54–2.46 (m, 4H), 2.01–1.94 (m, 1H), 1.88–1.76 (m, 1H), 1.73–1.62 (m, 2H), 1.55–1.33 (m, 2H), 1.22–0.82 (m, 10H), 0.75–0.7 (d, 3H).

2.3. Preparation of menthol-modified NDs (ND-menthol)

Menthol derivative (**2**) (10 mM), was dissolved in 5 mL water and added to a suspension of ND-OH particles in water (10 mg in 5 mL). The solution was stirred at room temperature for 24 h under nitrogen. The resulting ND-menthol particles were isolated by centrifugation at 10,000 rpm, purified through four consecutive wash/centrifugation cycles at 10,000 rpm with ethanol, and water and finally oven dried at 50 °C for 24 h.

2.4. Characterization

2.4.1. FTIR spectroscopy

Fourier transformed infrared (FTIR) spectra in transmission mode were recorded using a ThermoScientific FTIR instrument (Nicolet 8700) with a resolution of 4 cm^{-1}. Dried ND powder (1 mg) was mixed with KBr powder (100 mg) in an agate mortar. The mixture was pressed into a pellet under 10 ton load for 2–4 min, and the spectrum was recorded immediately. Sixteen accumulative scans were collected. The signal from a pure KBr pellet was subtracted as a background.

2.4.2. Particle size measurements

ND suspensions (20 μg mL^{-1}) in water were sonicated. The particle size of the ND suspensions was measured at 25 °C using a Zetasizer Nano ZS (Malvern Instruments S.A., Worcestershire, U.K.) in 173° scattering geometry and the zeta potential was measured using the electrophoretic mode.

2.4.3. Transmission electron microscope (TEM)

TEM measurements were performed in a FEI Tecnai G2 20 equipped with EDS micro-analysis, Gatan energy filter (EELS), electron precision and tomography.

2.4.4. Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA) measurements were made in Al_2O_3 crucibles in an atmosphere of nitrogen at a heating rate of 10 °C min^{-1} using a TA Instruments Q50 thermogravimetric analyzer.

2.5. Antimicrobial assays

2.5.1. Bacterial biofilm formation

Overnight cultures of *E. coli* (NCTC 8196) and *S. aureus* (NCTC 6571) were prepared by inoculating 10 mL of nutrient broth and incubating at 37 °C for 18 h on a rotary shaker (Stuart Scientific) at 120 rpm. After incubation, the cultures were diluted to OD_{600} of 0.1 in nutrient broth at a final volume of 100 mL, and a 96-well plate was set up as follows. Each assay sample required 6 wells, with another 6 wells for a negative control/blank, which consisted of 125 μL of sterile nutrient broth. Next, 110 μL of culture plus 15 μL of PBS was pipetted into the next 6 wells to act as the positive growth control. In the remaining wells, the assay was set up as follows: 110 μL of culture and 15 μL of the appropriate NDs and ampicillin solutions. Sterile nutrient broth and NDs and sterile nutrient broth and ampicillin were also set up in sets of six wells each to act as controls. The 96-well plate was incubated at 37 °C for 24 h with no agitation. After 24 h, the plate was removed from the incubator, and the supernatant of each well was removed by pipetting. The plates were subsequently allowed to dry for 15–20 min in a Class Two Microbiological Hood. Next, 125 μL of PBS was transferred into each well in order to remove any non-adhering cells. The PBS was then removed by pipetting, and the plates were again allowed to dry in the Class Two Microbiological Hood for 15 min. All wells were stained by adding 150 μL of 0.9% crystal violet in sterile reverse osmosis water, and the plates were incubated at room temperature for 15 min. After this time, the stain was removed by pipetting, and excess dye was washed off by manually adding and then removing 165 μL of PBS. To solubilize the crystal violet, 175 μL of 95% ethanol was added to each well, and the plates were incubated at room temperature for 30 min. The bacterial cell density was determined by the optical density of each well at 595 nm, using the negative control wells as blanks.

2.5.2. Viability of bacterial biofilms

The LIVE/DEAD® *Bac*Light™ assay kit was used to determine the effect of ND-menthol (100 μg/mL) on bacterial cell viability. Overnight cultures of *E. coli* and *S. aureus* were prepared by inoculating 10 mL of nutrient broth and incubating at 37 °C for 18 h on a rotary shaker (Stuart