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Preparation of bio-deep eutectic solvent triggered cephalopod shaped silver chloride-DNA hybrid material having antibacterial and bactericidal activity

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ABSTRACT

2.5% *w*/*w* DNA (Salmon testes) was solubilized in a bio-deep eutectic solvent [(bio-DES), obtained by the complexation of choline chloride and ethylene glycol at 1:2 molar ratio] containing 1% *w*/*w* of silver chloride (AgCl) to yield a AgCl decorated DNA based hybrid material. Concentration dependent formation of AgCl crystals in the DES was observed and upon interaction with DNA it gave formation of a cephalopod shaped hybrid material. DNA was found to maintain its chemical and structural stability in the material. Further, AgCl microstructures were found to have orderly self assembled on the DNA helices indicating the electrostatic interaction between Ag^+ and phosphate side chain of DNA as a driving force for the formation of the material with ordered microstructural distribution of AgCl. Furthermore, the functionalized material exhibited excellent antibacterial and bactericidal activity against both Gram negative and Gram positive pathogenic bacteria.

1. Introduction

Natural deep eutectic solvents (NADESs) or bio-DESs are emerging as solvents alternative to ionic liquids (ILs) for number of applications such as nanomaterial syntheses, [1] biomass processing, [2–4] organic syntheses, [5] metal and metal oxide processing [6] and many more in various fields [7].

Ionic and nano colloidal silver are the silver derived formulations used widely as anti-microbial agents for applications such as wound care as well as against food borne pathogens in the field of health-care and food products [8,9]. However, substantial research efforts are being made to improve the antimicrobial efficiency of AgCl [10].

Unique properties such as molecular recognition [11], suitable mechanical strength and flexibility [12] etc., make deoxyribonucleic acid (DNA) suitable as templates for applications such as tailored growth of silver nanoparticles, targeted attachment of functionalized silver nanowires etc., [13]. DNA templates are proven to be suitable for metallization [14]. Rudiuk et al. (2003) has described the outstanding versatility of DNA-protein assemblies to generate metal nanostructures having various sizes and branching [15]. Further DNA in its composite form is also useful for various applications in bioanalysis and biomedicine [16].

Based on our success in dissolving DNA in a DES, obtained by the complexation of choline chloride (as hydrogen bond acceptor) and ethylene glycol (as hydrogen bond donor) at 1:2 molar ratio at elevated temperature (Chol · Cl — EG 1:2) [17], later on we were able to fabricate a DNA based material functionalized with protonated layered dititanate sheets ($H_2 \cdot Ti_2O_5 \cdot H_2O$) and Fe_3O_4 having both antimicrobial and magnetic properties [18].

Human pathogens are getting more and more importance day by day for acquiring multi drug resistance (MDR). Therefore, more effective therapeutic strategies are required to overcome these problems. Micro and nanoparticles are emerging as new class of therapeutic agents with superior antimicrobial properties. The silver containing antimicrobial agents suffers from problems such as cytotoxicity to human cells, leaching out of silver from engrafted materials and hence creating difficulties for their applications in dermatology formulations. This promotes modification of silver with biodegradable polymers for minimizing the drawbacks [19–21].

Although in our previous work we were able to prepare a DNA based material having antimicrobial properties but the dose required to inhibit the growth of the bacteria was quite high [18] and hence constant endeavours were made to prepare DNA based antimicrobial material having improved efficacy. In this research work, we employed the DES consisting of the mixture of choline chloride and ethylene glycol for the preparation of a hybrid material with DNA and AgCl microstructures as two components. The antibacterial as well as bactericidal activity of this hybrid material was assessed against eight different pathogenic

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bacterial strains (both Gram positive and Gram negative). DNA stability in the material and behaviour of AgCl in the DES and its structural distribution in the material was also studied in this research work.

2. Experimental section

2.1. Materials

DNA (Salmon Milt) was purchased from Tokyo Chemical Industry Co., Ltd, Japan. Choline chloride (AR grade) and AgCl (Extra Pure grade) was purchased from SD Fine chemicals, Mumbai, India. Ethylene Glycol was procured from Merck Chemicals, Mumbai. All chemicals were used as received.

To test antibacterial efficacy of the synthesized DNA structures, eight different bacterial strains were used. Six of them were Gram negative bacterial strains namely; *Vibrio cholerae* N16961, *Escherichia coli*, *Shigella flexineri*, *Shigella boydii*, *Salmonella enterica*, *Pseudomonas fluorescens* and two of the strains were Gram positive namely *Bacillus subtilis* and *Bacillus licheniformis*. All strains were stored at −80 °C in Luria Bertani broth, Miller (HiMedia, India) supplemented with sterile 20% glycerol. Standard ampicillin was procured from Himedia Laboratories, Mumbai.

2.2. Synthesis of deep eutectic solvent

Chol · Cl — EG 1:2 was synthesized and characterized as described previously [17]. In a typical reaction, choline chloride and ethylene glycol was mixed in the molar ratio of 1:2 and was heated at 80 °C until a transparent solution was obtained. The water produced was evaporated using rotary evaporators.

2.3. Synthesis of AgCl functionalized DNA material in deep eutectic solvent

In a typical reaction, 10 mg of AgCl powder was added in 1 g of Chol · Cl — EG 1:2 (1% *w*/*w*) with gentle stirring for 1 h at 80 °C. After ensuring the complete dispersion of AgCl in the solvent, the mixture was cooled to room temperature and 25 mg of salmon milt DNA was added in to the solution (2.5% *w*/*w*) with constant stirring until the mixture became visibly transparent. After the complete dissolution of DNA, the composite formed in the solution was precipitated in cold isopropanol (IPA), which was washed several times with IPA and finally vacuum dried.

2.4. Determination of bacteriostatic and bactericidal concentration of AgCl decorated DNA material

All bacterial strains were grown in 3 ml of Luria-Bertani broth, Miller at 37 °C in 120 rpm using single colony from LB agar plate. After 12 h of growth, OD at 600 nm was adjusted to 1.0. Subsequently, cultures were diluted 200 fold with LB broth [approximate concentration 10^5 Colony Formation Unit (CFU)/ml] and incubated with and without Chol · Cl — EG 1:2 at 37 °C in 120 rpm for 2 and 4 h respectively. Dose dependent assay was performed by using different concentrations of AgCl functionalized DNA material such as 1, 10, 25, 50, 100, 200, 400 (μg/ml). Positive control was considered as only bacterial culture without the test material. Tests were performed by a broth dilution method [22] and minimum inhibitory concentration (MIC) of the material was determined. After incubation the culture was serially diluted with sterile 0.9% physiological saline and 100 μl of solution was spread on LB agar plate. The number of colonies (CFU/ml) was counted after an overnight incubation at 37 °C. Standard antibiotic ampicillin was used to compare the antibacterial and bactericidal efficacy of the test material in both Gram positive and Gram negative bacteria. All experiments were performed in triplicates and the mean values with SD were calculated. Optical microscopic images of bacterial strains were taken after Gram staining procedure to check the morphological changes induced by the material.

The percentage of reduction of viable cells was calculated using the following equation:

$$\%\ \text{Reduction} = [(a-b) \times 100]/a$$

where 'a' is the number of viable cells (CFU mL^{-1}) in the control specimen and 'b' is the number of viable cells in the specimens containing the AgCl decorated DNA material or ampicillin.

2.5. Halo zone sensitivity test of AgCl

Antibacterial sensitivity test for AgCl was done by Halo zone test method [23]. In brief, all eight bacterial strains were subcultured on nutrient agar (Hi Media, India) and incubated overnight at 37 °C. A single colony of each bacterium were inoculated on nutrient broth and incubated for 4 h at 37 °C in 120 rpm. Further bacterial cultures were suspended to the same media to produce a final concentration of 10^5 CFU/ml of culture and were used to streak on agar plates. Sterile swab was used for this purpose and moistened with the bacterial suspension and streaked on agar plates. Due to the inadequate solubility of AgCl in water, the suspension of the compound in water was used for the experiments. The suspension was put on the surface of agar plates in different quantities (1 μg, 10 μg, 25 μg, 50 μg, 100 μg and 200 μg) and incubated at 37 °C for a day. The susceptibility of the tested bacterial strains to AgCl was determined by the size of the transparent halo circle developed around the AgCl specimen after incubation.

3. Result and discussion

AgCl was added separately in to Chol · Cl — EG 1:2, aqueous choline chloride solution, ethylene glycol and water at elevated temperature (80 °C). When the solution was cooled to room temperature (25 °C), formation of AgCl crystals of different morphology was observed in the DES. However, no crystal formation of AgCl was observed in ethylene glycol, aqueous choline chloride solution and water under similar condition. To study more precisely the crystal formation phenomenon in the DES, different quantities of AgCl (0.1–1% w/v) was added to the DES at 80 °C and the morphology of the crystals was monitored under optical microscope (100× magnification) at room temperature. It was observed that, butterfly shaped crystals of AgCl was formed with 0.6% AgCl in the DES, which got transformed to different shapes with increasing AgCl concentration up to 1% w/v as shown in Fig. 1. No crystal formation for AgCl in the DES was observed below 0.3% w/v of AgCl. Formation of fully grown hexapods was obtained with 1% w/v AgCl in the DES. When DNA (2.5% *w*/*w*) was added to the dispersion, the bio-macromolecule was found to bind with the hexapods of AgCl and the morphology of DNA looked like a bullet.

It should be noted that, AgCl is known for its very low solubility in water (0.05% w/v). However 1% w/v of AgCl was found to be soluble (no insoluble particle was visible with naked eye) in the DES as shown in Supporting Fig. S1. In a typical experiment 1% w/v of AgCl was added separately in to the DES, 1 M aqueous choline chloride, ethylene glycol and water and heated for 1 h at 80 °C and cooled to room temperature. As seen in the photographs, at beginning colloidal type solutions were observed in all the solvents except for the DES. Upon 1 h of standing at room temperature AgCl got precipitated out from all the solutions except the DES indicating good compatibility of AgCl with the DES.

3D-AgCl microstructures are very important to enhance its efficiency for applications such as photocatalysis [24] and hence substantial research endeavours are being made to synthesis AgCl structures having new morphologies. A 3D AgCl hierarchical super structure was synthesized by a wet chemical oxidation method, where Ag plates were added to a solution of $NaClO_2$, NaCl and citric acid [24]. AgCl microstructures having shapes of octapods, hexapods etc., were synthesized by the addition of NaCl in the solution of $[Ag(NH_3)_2]^+$ [25]. In most of the reports it