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Research paper

Physicochemical characterisation and antimicrobial phototoxicity of an anionic porphyrin in natural deep eutectic solvents

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ABSTRACT

Natural deep eutectic solvents (NADES) are a newly discovered group of eutectics which has shown promise as a solvent in antimicrobial photodynamic therapy (aPDT). The purpose of this study was to investigate preparations of an anionic porphyrin, *meso*-tetra-(4-carboxyphenyl)-porphine (TCPP), solubilised in NADES, with regard to their physicochemical and antibacterial properties. The NADES CS (pH ~ 0), ChX (pH ~ 4) and MFG (pH ~ 1) solubilised TCPP with absorption maximum ~443 nm and emission maximum ~678 nm, indicating formation of the TCPP dication. Dilution of TCPP-NADES > 1:1 (water) reduced the physical stability of the preparations. The photostability half-lives of TCPP in methanol, MFG, and CS were ~9 h, 6.9 h and 3.2 h, respectively. Nanomolar concentrations of TCPP solubilised in diluted MFG combined with $\leqslant$27 J/cm^2 blue light increased Gram-positive and Gram-negative bacterial phototoxicity, >99.98% and 96% bacterial reduction, respectively, compared to TCPP in PBS/ethanol under equivalent treatment conditions. TCPP solubilised in diluted CS was toxic to bacteria both in the absence (36–72% reduction) and presence of light. TCPP in CS, and in the CS component citric acid, induced a TCPP-concentration dependent increase in Gram-negative phototoxicity relative to controls, which was most pronounced for TCPP-CS. The mechanism behind the increased toxicity is unknown.

1. Introduction

The discovery of antibiotics has been one of the most significant advances in medicine. Unfortunately, due to misuse and over-use, development of resistance to traditional antibiotics is currently one of the greatest threats to human health worldwide. There is therefore a need of new antibacterial treatments, and if possible, with reduced risk of bacterial resistance development. Antimicrobial photodynamic therapy (aPDT), also known as photodynamic antimicrobial chemotherapy (PACT), is one such treatment modality which already has demonstrated antimicrobial efficacy against several antibiotic-sensitive and antibiotic-resistant bacteria [1–3]. aPDT results in a multi-target damaging process. The treatment combines a photosensitiser (PS), oxygen and visible light to produce reactive oxygen species (ROS) and other reactive photoproducts. Due to its multi-target mode of action, a reduced risk of resistance development compared to traditional antibiotics has been suggested [1,4].

Abbreviations: aPDT, antimicrobial photodynamic therapy; DES, deep eutectic solvents; NADES, natural deep eutectic solvents; IL, ionic liquid; ChX, choline chloride:xylitol (molar ratio 5:2); CS, citric acid:sucrose (molar ratio 1:1); MFG, malic acid:fructose:glucose (molar ratio 1:1:1); PS, photosensitiser; ROS, reactive oxygen species.

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Ionic liquids (ILs) and deep eutectic solvents (DES) have received much attention as potential "green" solvents to replace current toxic organic solvents [5]. However, their use is limited due to demonstration of toxicity in humans and to the environment, as well as high cost [5]. Further, most DES exist in the solid state at room temperature [5]. Dai et al. recently discovered that several plant metabolites changed from solid state to liquid state when mixed at certain molar ratios [6]. They found that these natural deep eutectic solvents (NADES) probably are present in living organisms and play a role as alternative solvents to water and lipids [5,6]. These NADES may be composed of two or several components including organic acids, amino acids, amines, sugars, sugar alcohols and sometimes water [5,6]. Different ratios of the components may affect the physical stability of the resulting NADES with regard to their ability to remain in the liquid state for prolonged periods [5]. The molecules in the NADES are arranged through hydrogen bonding and other intermolecular interactions, resembling liquid crystals but without a melting point. They have glass transition temperatures usually in the −60 °C to −90 °C range and decomposition temperatures above 100 °C [5]. NADES have shown high solubilisation potential of several compounds, including macromolecules such as cellulose and gluten [5], poorly water soluble drugs such as taxol [5] and griseofulvin [7], aromatic dyes and photosensitisers [8–10]. Upon dilution of the NADES with water, the intermolecular interactions responsible for the unique eutectic properties weaken. It has been reported that >1:1 dilution of NADES results in less intensive interactions within the eutectics, but also that the unique solubilising properties of certain NADES are maintained (for hours/days) even after 1:200 dilution with water [9–11].

Porphyrins are aromatic heterocycles that have been investigated as PS in photodynamic therapy (PDT) of cancer and microbial infections [12–19]. It is well documented that cationic PS efficiently photoinactivate both Gram-positive and Gram-negative bacteria, whereas neutral PS and anionic PS are predominantly phototoxic only to Gram-positive bacteria [18–25]. This observation has been associated with characteristics of the outer membrane surrounding the Gram-negative bacteria, which functions as an effective permeability barrier [24]. We have previously shown how the solubility, physical stability and bacterial phototoxicity of a neutral porphyrin, *meso*-tetraphenylporphyrin (THPP), can be enhanced upon solubilisation in NADES [9]. The current work focuses on the poorly water-soluble, anionic porphyrin *meso*-tetra-(4-carboxyphenyl)-porphine (TCPP), and aims to investigate the physicochemical and bacterial phototoxic properties of TCPP in selected NADES. Whether NADES may increase the phototoxic effect of an anionic photosensitiser has not been investigated previously. The bacteria *Escherichia coli* (Gram-negative), *Enterococcus faecalis* and *Staphylococcus aureus* (Gram-positive) were chosen as models for bacteria involved in cutaneous and oral infections.

2. Materials and methods

2.1. Materials

The following chemicals were obtained: TCPP (4,4′,4″,4‴-(porphine-5,10,15,20-tetrayl)tetrakis(benzoic acid) (Fig. 1a), purity 75%), D-(−)-fructose and sucrose (Fluka Analytical) (Sigma-Aldrich Co. LLC, St. Lois, MO); choline chloride (Biochemica) (AppliChem GmbH, Darmstadt, Germany); citric acid monohydrate (for analysis) and maleic acid (for synthesis) (Merck KGaA, Darmstadt, Germany); D-(+)-glucose (anhydrous) (VWR, Radnor, PA); DL-malic acid and xylitol (Alfa Aesar GmbH & Co KG, Karlsruhe, Germany); glycerol (85%) (Apotekproduksjon AS, Oslo, Norway); Nile red (Life technologies, Eugene, OR); phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS) (Lonza Group Ltd., Verviers, Belgium).

2.2. Preparation of NADES

The two or three components of each deep eutectic solvent investigated (Table 1) were dissolved in warm water (~50 °C) and evaporated at 45 °C for 15 min with a rotatory evaporator. The liquid obtained was transferred to polypropylene tubes with a tight cap. The water content of each NADES was determined by Karl Fischer titration (C20 Coulometric KF Titrator, Mettler Toledo Inc., Schwerenbach, Switzerland). The pH in undiluted NADES and after dilution 1:1 and 1:200 with PBS was measured using a pH 526 MultiCal® pH meter (WTW GmbH, Weilheim, Germany) (Table 1).

2.3. Solubility test

A screening was performed where the NADES (Table 1) were mixed with an excess amount of TCPP (Fig. 1) and stored protected from light at 22 °C for 14 d with regular mixing 3 times daily on a vortex mixer. TCPP was soluble in several of the NADES (Table 1). Six of the NADES, representing a variety of constituents in the following molar ratios (abbreviation in brackets): citric acid:sucrose (CS, 1:1), choline chloride:citric acid (ChC, 2:1), choline chloride:-maleic acid (ChM, 2:1), choline chloride:xylitol (ChX, 5:2), citric acid:xylitol (CX, 1:1) and malic acid:fructose:glucose (MFG, 1:1:1), were selected for quantitative analysis. The solubility of TCPP in MilliQ-water, PBS, 2.7 M citric acid and 2.1 M malic acid (the same concentration of the acids as in undiluted CS and MFG, respectively) was also quantified (n = 3).

The solubility of TCPP was determined as follows: Triplicate samples of the NADES described above were prepared containing an excess amount of TCPP in 1.5 ml Eppendorf tubes. Preliminary studies showed that no TCPP adsorbed to the plastic tubes when dissolved in these NADES. The tubes were agitated on an Eppendorf Thermomixer Compact (at 1400 rpm; Eppendorf AG, Hamburg, Germany) protected from light at 22 °C for 72 h, and centrifuged (6918*g*, 60 min, 22 °C; Centrifuge 5430R, Eppendorf AG). A defined volume of the supernatant was withdrawn and diluted 1:200 with methanol and then 1:1 with the mobile phase prior to quantification by HPLC at detection wavelength 415 nm. A reversed-phase HPLC analysis was conducted with gradient elution with a mobile phase consisting of acetonitrile (A) and 1% acetic acid in MilliQ water containing 0.02% trimethylamine (B). The initial composition of the mobile phase was 50% A and 50% B (0–0.5 min), reduced to 25% B after 0.5 min and gradually increased to 50% B again after 9 min. An Ultra Biphenyl 3 μm column (100 × 2.1 mm, Restek Corporation, Bellefonte, PA) was used. The retention time of TCPP was approximately 3.5 min at flow 0.3 ml/min and a column temperature of 30 °C.

The NADES CS and MFG were selected for stability and phototoxicity studies representing solutions with different constituents and polarity in which TCPP was highly soluble. In addition, the NADES ChX was included in the phototoxicity study to represent a pH-neutral NADES with lower polarity than CS and MFG.

2.4. Absorption spectroscopy

Absorption spectra were recorded between 190 and 700 nm on a Shimadzu UV-2101 PC UV–vis scanning spectrophotometer (Kyoto, Japan) using a quartz cuvette with 1 cm light path. Absorption measurements were performed on samples of 1.3 μM TCPP in methanol, 50% (v/v) formic acid, and 5% (v/v) ammonia. Spectra