Development of photosensitiser functionalised electrospun nanofibre for microbial disinfection of waste water

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ABSTRACT

To meet the increasing demand of water use in modern society and growing industrialization, wastewater treatment to reuse is the only option. Ensuring the microbiological safety of water is one of the utmost importance. However, conventional disinfection produce undesirable toxic by-products which are difficult and costly to remove. We report on the preparation, characterisation and biological evaluation of electrospun poly (acrylic acid)/poly (vinyl alcohol) double crosslinked nanofibres functionalised with the tetra cationic photosensitiser 5, 10, 15, 20-tetrakis (1-methylpyridinium-4-yl) porphyrin tetra (p-toluene sulfonate) (TMPyP). This nanofibre shows highly efficient photo killing of antibiotic resistant gram (-ve) *E. coli* under illumination with low intensity visible light. By immobilising photosensitiser into the nanofibre this system offers toxic by-product free water. This study reports a low cost, energy efficient and eco-friendly water disinfection that will have huge socio-economic benefits to society.

Keywords: nanofibre, photosensitiser, singlet oxygen, disinfection, antibacterial.

1 BACKGROUND

Photodynamic inactivation (PDI) of microorganisms occurs where a photosensitiser (PS), preferentially associated with a microorganism, is activated with non-thermal visible light of appropriate wavelength(s) to generate singlet oxygen species that inactivate the microorganism. Thus the ability to inactivate microorganisms without inducing resistance makes PDT an appealing and useful alternative in treating wastewater. In addition, the increasing prevalence of bacterial resistance in environment is another problem for which an urgent solution is needed [1].

Ideally, since the PSs do not have to penetrate the bacterium or even come into a contact with the cell in order to be effective [2], immobilisation of the PS aims to allow both the efficient killing of microorganisms, several cycles of use, and the complete PS removal from the treated water. That prevents disinfectant accumulation in the environment.

In this paper, we describe the production, stabilisation, functionalisation and characterisation of poly (acrylic acid)/ poly (vinyl alcohol)-Glutaraldehyde (PAA/PVA-GA) double crosslinked electrospun nanofibre. At first thermal crosslinking PAA/PVA nanofibre was carried out at 150° C in a vacuum oven. Second crosslinking was done with poly (vinyl alcohol) followed by Glutaraldehyde to ensure stability of PVA nanofibre in water. Finally (PAA/PVA-GA) was functionalised with a cationic 5,10,15,20- tetrakis (1-methylpyridinium-4-yl)porphyrin(TMPyP). We propose an application of this water resistant nanofibre for photo inactivation of carbenicillin resistant gram (-ve) *E. coli* BL21 (DE3) in water. As *E. coli* is one of the indicator organisms checked during water disinfection [3]. So far, the majority of the photodynamic inactivation based water sterilisation research has been carried out using PS in solution, but to obtain disinfactant and disinfactant byproduct free water it is necessary to immobilise the PS with solid support. For photodynamic inactivation, low intensity light was used. That offers low energy consuming eco-friendly water treatment system.

2 EXPERIMENTAL

Chemicals. The following chemicals were purchased from Sigma-Aldrich at the highest acceptable purity: 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra (p-toluenesulfonate) (TMPyP), hydrochloric acid (HCl), sodium hydroxide (NaOH), glutaraldehyde(GA), LB agar, tryptone, yeast, NaCl, carbenicillin, poly (vinyl alcohol) (PVA) (Mw~146,000–186,000, 87–89% hydrolysed), poly (acrylic acid) (PAA) (Mw~450,000), All chemicals were of analytical grade.

Preparation of Electrospun poly (acrylic acid)/ poly (vinyl alcohol) Nanofibres. A needleless technology Elmarco NS lab 200 Nanospider electrospinner was used for nanofibre production. Aqueous solutions of PVA (10g) and PAA (20gml) were mixed 10:6 % (w/w) under stirring at room temperature. A cylindrical electrode was used under following conditions: voltage: 60-67 kV, electrode to substrate distance: 12-13 cm. Dry PAA/PVA nanofibres were collected onto polypropylene cloth.

Crosslinking of PAA/PVA nanofibre. For thermal crosslinking, PAA/PVA nanofibre were heat treated in a

Gallenkamp vaccum oven attached to Edwards high vacuum pump model E2M5, at 140°C for 30 minutes to 1 hour. After heat treatment the nanofibre was mechanically strong enough. To prevent leaching of uncross linked PVA into water electrospun PAA/PVA nanofibrous mat was immersed into an aqueous solution composed of isopropanol (30 mL), water (3.3 mL), concentrated HCl (0.3 mL), and the GA solution (5% w/v, 3.7 mL) for 1 h; Water stability of nanofibre was confirmed by mass-spectral analysis.

Mass spectrometry. Nanofibre washings (in water) were diluted to 50% (v/v) acetonitrile, and analysed by Z-spray nanoelectrospray ionisation mass spectrometry (IMS) using a quadrupole- IMS-orthogonal time-of-flight (TOF) MS (Synapt HDMS, Waters UK Ltd.) using in-house fabricated gold/palladium coated nanospray capillaries.

Neutralisation and Ion Exchange with TMPyP. Electrospun PAA/PVA nanofibre material was treated with 20 mL of 1.5N NaOH to completely replace OH^+ with Na^+ at r.t. for 30 minutes. Finally, the materials were washed with deionised water until the pH value reached 6−7 and stored at r.t. in a dry place.The ion exchanged PAA/PVA nanofibre material (2 cm^2) was immersed in 2 mL of a 3 mM aqueous solution of TMPyP in deionised water overnight in the dark, with shaking.

Generation of singlet oxygen generation by developed TMPyP functionalised PAA/PVA nanofibre was confirmed by observing decreasing manner in absorbance (318 nm) of 2-amino-3-hydroxypyridine when reacted with singlet oxygen [5, 6]. Two assay solutions were prepared for this experiment: (a) TMPyP was used as control and a stock solution of TMPyP was made and dissolved with 200 mM aqueous 2-amino-3-hydroxypyridine, final conc of TMPyP was 1µM (b) 1cm2 piece of TMPyP functionalised PAA/PVA was taken in solution of 2-amino-3-hydroxypyridine (200 mM). Both were put in a conventional quartz cell with a light path length of 1 cm. Both were illuminated with a Schott cold light KL 2500 LCD, 250 W for 1 minute at a distance of 30 cm. The fluence rate was 32 mW cm-2. Changes in the absorbance before and after photo-irradiation were measured at 318 nm.

Estimation of TMPyP bound to PAA/PVA nanofibre

Unbound concentration of TMPyP was measured first at 420nm. Then, from the difference of the initial concentration and unbound TMPyP concentration, the bound TMPyP was estimated. The concentration of loaded TMPyP into PAA/PVA nanofibre was 10.92x 10-6mole/ cm^2.

Bacterial culture and transferring to water

For the photodynamic inactivation assay E.coli Carbenicillin resistant BL21 (DE3) strains were used. Fresh culture of BL21 (DE3) in carbenicillin (100mg/ml) amended Luria Broth (LB) respectively. They were allowed to grow for 4 h at 37°C under gentle shaking (140 rpm). Then the both fresh culture were transferred to (dd) sterile water followed by three times washing of LB.

The light source for PDI experiments was a Schott KL 2500 LCD (Schott Ltd., Stafford, UK) which provides a cool white light. Fluence rate of illumination during photoinactivation experiments were measured using a light meter (Clas Ohlson, UK). Stationary and flow models were adopted for PDI using TMPyP functionalised PAA/PVA nanofibre. Visible light was used and fluence rates (radiant exposure) were 32 mW cm^{-2}.

Photo-oxidation of model bacterial species BL21 (DE3) using TMPyP functionalised PAA/PVA nanofibre

bacterial stock cells (10^7 to 10^8/ml) after six fold serially dilluted in (dd) water and incubated with photosensitiser functionalised nanofibre in a 24 well plate for 30 minutes in the dark.. After incubation, the cells with nanofibre discs were illuminated from 30 cm above for 90 minutes at 32 mW/cm2.

4 sets of control wells were observed, set (1) was neither sensitised with a photosensitiser nor exposed to the light source. Set (2) and (3) were incubated in solution of photosensitiser with and without exposing to light, Set (4) was incubated functionalised nanofibre discs in dark.

After illumination the survival of the bacteria was determined by counting the numbers of CFU. Determination of dark toxicity was performed using the same protocol but the final bacteria-photosensitiser suspension was wrapped in foil and kept in a dark. Experiments were repeated in triplicate.

Reuse of ENF and Proof of sterilisation. Previously used nanofibres were incubated in a conical flask, covered with aluminium foil containing 20 ml of freshly prepared LB broth. This was placed in a shaking incubator at 120 rpm and 37°C for 72 h. No bacterial growth was observed in the nutrient media. The same nanofibres were used up to 10 times for PDT of BL21 (DE3).

Scanning Electron Microscopy (SEM) The nanofibre morphology was studied with a SEM (FEI Quanta 200F FEG-Scanning Electron Microscope) with an operating voltage of 15 kV. Prior to measurements, the nanofibrous mats were sputter coated with a 4 nm-thick Pt film and observed using a secondary electron detector at high vacuum.

3 RESULTS AND DISCUSSION

Preparation and Morphology of the Nanofibre Materials. The structure of the original electrospun PAA/PVA nanofibre materials was visualised by SEM (**Figure 1**). Neutralisation of the original electrospun nanofibre materials using NaOH led to the formation of cation exchange functionality on the nanofibre surfaces. The nanofibrous character of the materials was not changed by this treatments and functionalisation (Figure 2, B-D) or by long-term storage in water. Morphologies of the electrospun PAA/PVA nanofibre was observed using SEM.

Crosslinking of PAA/PVA and PVA-GA. As both PAA and PVA are water-soluble, the electrospun

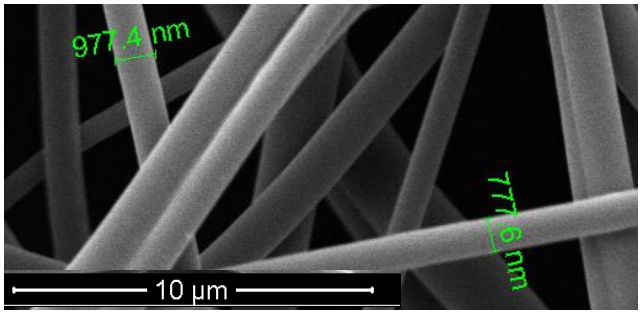

Figure 1: SEM images of PAA/PVA nanofibre.

PAA/PVA nanofibres could be dissolved in water immediately. To retain their unique fibre structure in water, the PAA/PVA nanofibrous mats were heated at 140°C for 1 hour to form intermolecular crosslinking. And to crosslink the unreactive PVA the nanofibre was 2nd time crosslinked with glutaraldehyde (GA). After treatment with GA solution, mass spectral analysis revealed no peak of PAA and PVA suggesting that both crosslinking was successful.

FTIR spectra were recorded using a Nicolet 5700 FTIR spectrometer (Thermo Nicolet Corporation,) at ambient conditions. Chemical crosslinking between PVA and PAA occurred through an ester formation between the –OH groups in PVA and –COOH groups in PAA.

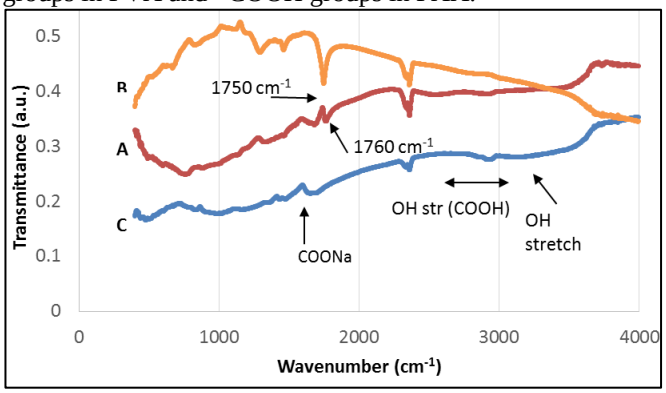

Figure 2: FTIR spectra of before (A), after (B) heat treated and post neutralised (C) by NaOH PAA/PVA crosslinked nanofibre.

Even though FTIR spectrum shows a considerable amount of –OH groups left in the nanofibre (3300-3600cm^{-1} region), it is clearly seen the –C=O stretching vibration at 1750 cm-1 becomes stronger after heat treatment. The peak around 1750 cm-1 in spectrum (B) clearly shows that a carbonyl group such as a ketone produced by the degradation of PVA and carbonyl of the ester linkage is forming the crosslinking. The transmittance peak around 1670 cm-1 is attributed to the sodium carboxylate formed as a result of ion exchange of the residual carboxyl group in the PAA/PVA blend nanofibre.

Confirmation of water resistance of nanofibre by mass spectrometry. After heat treatment, MS was carried out for several conditions. From MS analysis of the washes there appears to be polymer related peaks from PVA traces (72 and 44 D unit mass). But presence of PVA was still observed. Interestingly there is a common presence of another entity with unit mass 82, which is coming from PVA as a common by product of PVA hydrolysis. Although sodium acetate was get rid off by pre-washing of PVA in methyl alcohol befor electrospinning. And nanofibre was treated with glutaraldehyde (GA) solution to crosslink the unreacted PVA with GA. Complete insolubility of PAA and PVA was confirmed from Mass-spectral analysis.

Confirmation of singlet oxygen production. Singlet oxygen generation by water resistant PAA/PVA crosslinked ENF was determined.

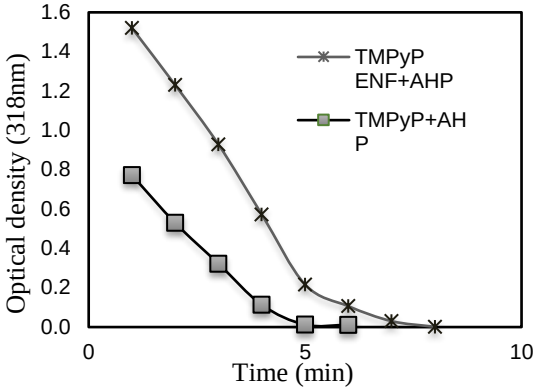

Figure 3: Absorbance of 2-amino-3hydroxy pyridine (AHP) at 318 nm. showing decrease of absorbance due to singlet oxygen quenching when aqueous solution of 2-amino-3hydroxy pyridine was illuminated with free TMPyP and TMPyP functionalised PAA/PVA nanofibre in 2-amino-3hydroxy pyridine assay solution respectively.

The absorbance of 2-amino-3-hydroxy pyridine (AHP) decreased to zero within 5 minutes when AHP was illuminated with free TMPyP in assay solution. Whereas a slower decrease of absorbance was observed with TMPyP functionalised PAA/PVA nanofibre illuminated with AHP. This shows that singlet oxygen generation by TMPyP functionalised PAA/PVA nanofibre was slower than free TMPyP.

Photo oxidation of *E. coli* BL21 DE3 using TMPyP functionalised PAA/PVA nanofibres was used to investigate microbial inactivation of carbenicillin resistant *E. coli* BL21 (DE3) by photodynamic inactivation (Figure 4). PAA/PVA-GA nanofibre showed complete inactivation of *E. coli* BL21 (DE3) giving 7.5 log CFU/ml reduction. Illumination was carried out for 90 m at 32 mW/cm^2. The light intensity was as low as 3% of sunlight. Efficient photodynamic inactivation at low intensity of light suggested that it could be used in countries with limited daylight like the UK and other European countries. This indicates that by increasing the intensity or using sunlight it is possible to reduce the time of illumination and thus can lower energy consumption. Although the rate of singlet oxygen generation from TMPyP functionalised PAA/PVA nanofibre was slower than TMPyP in solution. They were still able to completely kill gram (-ve) *E. coli*. Reusing the same nanofibre, was carried out and the same efficiency of photoinactivation was observed (Figure 4) and (Figure 5).

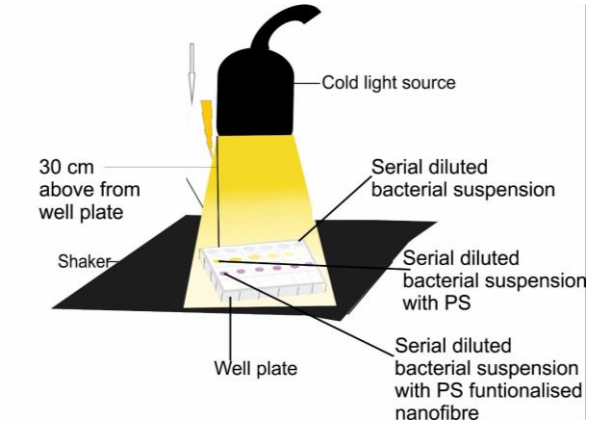

Figure 4: Schematic diagram of experimental set up of photodynamic inactivation (PDI) by cationic Photosensitiser (PS) functionalised PAA/PVA electrospun nanofibre

(A) No treatment, no Illumination

(B) Illumination but no PS-ENF

(C) After photo reatment with PS-ENF

Figure 5: Photo killing of antibiotic resistant bacteria (C) resulted from photodynamic inactivation by cationic TMPyP functionalised PAA/PVA ENF, (A) And (B) plates are control at dark and without PS functionalised nanofibres respectively. All experimental conditions were examined and repeated 3 times.

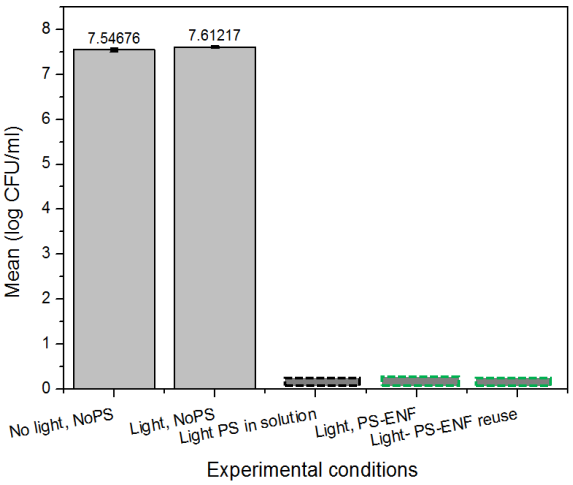

Figure 6: Log reduction of antibiotic resistant bacteria resulted from photodynamic inactivation by cationic TMPyP functionalised PAA/PVA ENF, demonstration PDI of 1st reuse of same nanofibre (right green dotted thick lines at far end). Black and green dotted bars and thick lines are representing numbers of CFU after PDT treatment control and test samples respectively. Where three samples last shows no CFU. The error bars are average ± SEM, where n=3.

4 CONCLUSION

The electrospun poly (acrylic acid)/ poly (vinyl alcohol) - glutaraldehyde double crosslinked nanofibre, neutralised by NaOH was developed as an ideal substrates for the adsorption of tetra cationic TMPyP. These nanofibres can effectively generate singlet oxygen. The electrospinning technique and electrostatic fabrication offer the highly efficient surface area, high porosity, lightweight, heat and water resistant platform. These nanofibres is chemically tailored for applications, including the potential capability of rapid and selective adsorption of cationic species, such as heavy metal cation contaminants or organic pollutants, and the killing of antibiotic resistant bacteria by singlet oxygen. This platform is capable of performing multiple tasks, e.g., photo disinfection, decontamination, and separation. In addition of being reusable and capable of generating singlet oxygen at low intensity of light these nanofibres will offer low energy consuming water treatment process.

5 ACKNOWLEDGMENTS

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