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Atmospheric pressure microplasma for antibacterial silver nanoparticle/chitosan nanocomposites with tailored properties

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Abstract

Room temperature atmospheric pressure microplasma (APM) was deployed for the first time for the *in situ* synthesis of anti-bacterial silver nanoparticle/chitosan (AgNP/CS) nanocomposites. The plasma induced liquid chemistry plays a role in the *in situ* formation of AgNP, the size distribution of which depends on the silver salt precursor concentration. The microplasma process has also simultaneously tailored the physical properties of the composites, through molecular chain scission and formation of physically crosslinked polymer network. The formation of AgNP within the *in situ* modified chitosan has led to nanocomposites with overall improved mechanical properties and better stability in simulated body fluid. Our plasma synthesized AgNP/CS nanocomposites also demonstrate effective antibacterial properties against *E. coli* and *S. aureus* bacterial strains, showing their promise in potential antimicrobial applications.

Keywords: E. Atmospheric pressure microplasma; A. Silver nanoparticle; A. Chitosan; A. Nanocomposites; B. Antibacterial

1. Introduction

Antimicrobial resistance (AMR) presents a huge threat to the global health and economy and there is an urgent need to develop antibiotic alternatives to combat AMR [1]. Nanoparticles, in particular, silver nanoparticles (AgNPs) are promising for tackling this challenge due to their highly effective antibacterial properties against a wide range of gram-positive and gram-negative bacteria strains [2–4]. When interacting with bacterial cells, AgNPs can either lead to the formation of reactive oxygen species (ROS) or the release of silver ions (Ag^+), contributing to a wide spectrum of antibacterial activities via multiple reaction pathways [3]. Despite their promising applications, the health risk associated with the use of free NPs may be a concern for their impacts on healthy cells and host tissues [5]. To minimize the uncontrolled release of AgNPs and for localized treatment / enhanced treatment efficacy, polymer based nanocomposites incorporating AgNPs have been developed as an alternative solution for certain antimicrobial applications such as air filtration [4], tissue scaffolds [6], implant coating [7], and wound dressings [8].

The commonly used preparation methods for AgNP/polymer nanocomposites include physical mixing of pre-synthesized AgNPs with polymer solution and in situ chemical reduction of AgNO_3 in polymer solutions [8,9]. Other non-wet chemistry based synthesis approaches such as γ -irradiation [10], microwave [11], and thermal [12] irradiations have also been explored. Unfortunately, most of these methods would require long processing time (hours), complicated multi-step synthesis process, and/or the use of potentially hazardous chemicals /irradiation.

Non-thermal plasmas (NTP) such as dielectric barrier discharges (DBD) and corona discharges, can operate under near room temperature and is an alternative approach for the surface treatment and/or synthesis of a wide range of materials. Several researchers have deployed NTPs to produce antibacterial AgNP/polymer nanocomposites [13]. For instance, Vu et al. prepared AgNP/polyamide (PA) composites by immersing DBD activated PA into pre-formed AgNP colloid to achieve enhanced AgNP grafting on the polymer surface [14,15]. Other researchers deployed NTPs for in situ plasma polymerization (such as hydrocarbon based polymers [16–18], polyaniline [19], diethylglycol-dimethyl-ether (DEGME) [20], and polyethyleneoxide (PEO) [21], where AgNPs were sputtered simultaneously into the polymer matrices during their formation. Although the above mentioned NTP techniques benefit from reduced use of chemicals, faster fabrication, and easier application to various substrates, the technique was primarily constrained to surface treatment/*in situ* polymerization.

In recent years, the use of room temperature atmospheric pressure microplasma (APM), in particular APM/ liquid interaction has emerged as a new technique for the in situ synthesis of metal NPs or nanocomposites containing metal NPs [22]. The plasma induced liquid chemistry (PiLC) can produce various energetic/reactive species such as solvated electrons, radicals (e.g. OH•, H•, and O•), and H₂O₂ etc. at the plasma-liquid interface or inside the bulk liquid. These species could contribute to the reduction of metal cations, leading to formation of various metal NPs such as AuNPs and AgNPs in aqueous solutions [23,24]. With the use of PiLC, we have successfully synthesized a wide range of nanocomposites containing metal based NPs, such as AuNP/CNT [25], AuNP/GO [26], Fe₃O₄/PINIAM [27], AuNP/PEDOT:PSS [28], and PVA hydrogel composites containing AuNP, AgNP or AuAg alloyed NPs [29]. One of the unique features of the PiLC synthetic approach is its ability to create highly charged NP surfaces within minutes, resulting in highly dispersed / stable NPs without the need for reductants, surfactants or ligand chemistry. The plasma synthesized composites can be further processed into different forms such as gels, sponges, coatings and films for further applications.

Chitosan (CS) is a biocompatible and biodegradable natural polymer, commonly used in the biomedical field [30–33]. By incorporating AgNPs into CS, one can obtain anti-microbial nanocomposites that can be used for a wide range of applications such as wound dressings, tissue engineering, drug delivery and water treatment, etc [34–36]. In the present work, a direct current (DC) atmospheric pressure microplasma has been deployed for the in situ synthesis of AgNP/CS nanocomposites. The resulting AgNP/CS nanocomposites were further processed into films, and their antibacterial activities were tested against *E. coli* and *S. aureus* bacterial strains.

2. Methods

2.1 Materials

Chitosan (CS, deacetylation > 92.6%) was purchased from Heppe Medical Chitosan GmbH company. Acetic acid (100%, AnalaR NORMAPUR® ACS, Reag. Ph. Eur. Analytical Reagent) was supplied by VWR. Silver nitrate (99.9999% trace metals basis) was purchased from Sigma-Aldrich. *Escherichia coli* (*E. coli*, ATCC 11303) and *Staphylococcus aureus* (*S. aureus*, ATCC 6538) used for antibacterial investigation were received from LGC Standards, Middlesex, UK. *E. coli* and *S. aureus* were grown in Mueller-Hinton broth (MHB), obtained from Oxoid Ltd, Hampshire, UK. Phosphate buffered saline (PBS), nutrient agar (NA) plates and Mueller-Hinton agar (MHA) plates were obtained from Oxoid Ltd, Hampshire, UK.

2.2 APM experiment

2 wt% CS solution was prepared by dissolving appropriate amount of as-purchased CS powder in 2% (v/v) acetic acid aqueous solution under room temperature for 24 h. Appropriate amount of AgNO_3 solution (10 mM) was introduced into the CS/acetic acid aqueous solutions to obtain AgNO_3 /CS mixtures with 1 % CS and different AgNO_3 concentrations (1, 2, and 4 mM, respectively). After 30 min stirring, the AgNO_3 /CS mixtures were subjected to plasma treatment.

Scheme 1 shows the plasma set-up in the present work. The anode (carbon rod) was immersed into the AgNO_3 /CS aqueous mixtures, while the cathode (stainless steel capillary with inner diameter of 250 μm) was positioned approximately 2 mm above the mixture surface. Helium (He) gas (flow rate = 25 sccm) was supplied through the capillary and the plasma can be ignited at the gas / liquid interface at a voltage of ~ 4 kV. Once the plasma was ignited, the voltage dropped to ~ 2.8 kV. The current was then adjusted and maintained at 5 mA to sustain the plasma and the AgNO_3 /CS solutions were gently stirred using magnetic stirrer throughout the treatment (10 min). The plasma treated samples were named as 1, 2, and 4 mM AgNP/CS, respectively, according to the initial concentration of the AgNO_3 used. For comparison, pure CS (1%) solution was also treated by plasma under the same condition.

2.3 Characterization

Ultraviolet-visible (UV-vis) spectra of all samples were recorded by a Cary 60 UV-Vis Spectrometer (Agilent Technologies). Diluted (10x) liquid AgNP/CS samples were drop-casted / dried in dark on TEM sample grids (Agar Scientific), to study the size and morphology of AgNPs within the nanocomposites using transmission electron microscope (TEM, Tecnai G2 F20 S-TWIN, FEI, USA). Based on the TEM images, AgNPs size distributions were analysed using “FUJI” software, where over 300 NPs were selected for each sample. The particle size was evaluated automatically using the “Particle Size” function within the software. X-ray photoelectron spectroscopy (XPS) of AgNP/CS nanocomposites were conducted using

Scheme 1. Diagram of plasma set-up used in this work.

a Kratos Axis Ultra XPS system and the results were analysed by an open source software CasaXPS. Samples for XPS were prepared by drop casting liquid AgNP/CS solutions onto intrinsic silicon wafer, followed by drying under ambient condition.

2.4 Preparation and characterization of AgNP/CS composite films

The 1, 2, 4 mM AgNP/CS composite films were prepared by casting 9 ml of each plasma treated sample onto a 5 cm × 9 cm glass slide. The samples were then oven dried at 60 °C for 6 h (no further weight loss). Control film samples (pure CS and plasma treated CS) were also prepared for comparison. The thickness of the all film samples was 0.02 ± 0.002 mm.

Fourier transform infrared (FTIR) of all films were conducted under ambient condition using Perkin Elmer Spectrum 100 FT-IR Spectrometer (equipped with a Universal Attenuated Total Reflectance (ATR) sampling accessory). Differential Scanning Calorimetry (DSC) analysis was carried in a N₂ atmosphere at a heating rate of 10 °C min⁻¹ using a Perkin-Elmer DSC 6 instrument to examine the thermodynamic characteristics of the films. Thermogravimetric analysis (TGA) was carried out using a TA Instruments SDT-Q600 in a N₂ environment with scan range of 50 to 800 °C at a heating rate of 10 °C min⁻¹.

2.5 Swelling ratio

The swelling ratio of the samples were tested using the gravimetric method following established procedure [37]. The film of each sample was cut into triplicate pieces using pin-punch set. After initial weighing of the dried film (W_d), all film samples were immersed in simulated body fluid (10 ml PBS, PH=7.4) at 37 °C. After 24 h, the films were taken out and the excess water were removed using filter paper. The swelling ratio of each film sample can be calculated according to the following equation:

$$\text{Swelling ratio (\%)} = [(W_s - W_d) / W_d] \times 100$$

where the W_s and W_d are the weight of swollen film and dry film, respectively.

2.6 Mechanical tests

The tensile tests of all film samples were measured using a Lloyds LRX series materials testing machine (50 N) at a speed of 2 mm min⁻¹ following ASTM D882-01 [38]. Films were cut into dumb-bell coupons with gauge length of 25 mm and gauge width of 4 mm using a Ray-Ran test machine cutter. Stress-strain curves of each sample, and the corresponding tensile strength (MPa) and Young modulus (MPa) were all recorded by the machine. Five measurements were repeated for each group and the mean value was taken for further analysis.

2.7 Antibacterial tests

The antibacterial activities of all the films were evaluated against gram-negative *E. coli* and gram-positive *S. aureus* strains using the CLSI method [39]. The bacterial strains were cultured

in MHB for 24 h in a 37 °C orbital incubator to obtain bacterial pellets. The as-obtained pellets were re-suspended in PBS to an optical density (OD₅₅₀) value of 0.3 at 550 nm, corresponding to an approximately 1x10⁸ cfu mL⁻¹ inoculum. The bacteria suspensions were spread evenly onto NA plates for *E. coli* and MHA plates for *S. aureus* using cotton swabs, after which 4 mm circular film samples were directly placed on the inoculated agar plates. After 24 h incubation under 37 °C, the diameter of the incubation zones were measured to determine their antibacterial activity. Pure CS and plasma treated CS films were used as references, and five replicates were tested for each group.

3. Result and discussions

In contrast to the clear, colourless pure CS solution, plasma treatment led to immediate colour change in all samples, see Figure 1 inset. To further confirm the optical property of all samples, the test solutions were analysed using the UV-vis spectroscopy (see Figure 1). It can be seen that the plasma treatment had no significant influence on the absorption spectrum of CS, despite the slight colour change seen for the plasma treated CS sample. With increasing concentration of AgNO₃ added into CS, the plasma treated AgNO₃/CS mixtures exhibit more distinctive colour change. This is accompanied by new characteristic peaks emerged at 412, 420, 425 nm for plasma treated 1, 2, 4 mM AgNO₃/CS mixtures, respectively, which coincide with the typical surface plasmon resonance (SPR) band of AgNPs[40]. The red shift of SPR peaks in the UV-Vis spectra is indicative of growing AgNP size within the plasma treated samples [40]. The morphologies and sizes of AgNPs formed within the plasma treated AgNO₃/CS were further investigated by TEM. Figure 2a-c show some typical images, where the synthesized AgNPs (dark particles) are well-dispersed. Particle size distribution analysis (Figure 2d-f)

Figure 1. UV-vis spectra of pure CS and plasma treated CS, 1 mM, 2 mM, and 4 mM AgNO₃/CS mixtures. Insets: images of (i) CS and (ii) - (v) plasma treated CS, 1 mM, 2 mM, and 4 mM AgNO₃/CS mixtures, respectively.

Figure 2. TEM images of (a) 1 mM, (b) 2 mM, and (c) 4 mM AgNP/CS nanocomposites, respectively. Corresponding size distribution of AgNPs within (d) 1 mM, (e) 2 mM, and (f) 4 mM AgNP/CS nanocomposites, respectively.

suggests that the average AgNPs size increases with increasing initial AgNO₃ precursor concentration, consistent with the red shift of the SPR peak found in the UV-vis analysis.

XPS analysis of plasma treated AgNO₃/CS was carried out to confirm the atomic states of silver element. It is observed that the survey scans of the three Ag containing samples (Figure 3a) all present distinguished Ag peaks. The core Ag 3d peaks of each samples (Figure 3b-d) can be all well fitted into two doublet components, where the doublet centred at binding energy (BE) $\sim 367.9/374.0$ eV correlates to the atomic Ag⁰ state and the doublet centred at BE $\sim 368.9/375.0$ eV correlates to the ionic Ag^{δ+} state, respectively [41]. The detailed BE and atomic fraction of all fitted elements are listed in Table 1. The XPS results further confirm that the Ag⁺ ion within the AgNO₃/CS were mostly reduced into Ag⁰ state by plasma treatment.

As discussed earlier, the PiLC can generate various energetic/reactive species, which may participate in the multiphase interaction (that involves gas, AgNO₃, CS, and water) at the plasma/liquid interface. The AgNP formation triggered by PiLC has been investigated by several workers. Despite the various plasma sources used in different studies, it is commonly recognized that both the highly energetic solvated electrons (from the plasma phase) and H radicals (induced by plasma-liquid interaction) play a role in the AgNP reduction [24,42–44]. In addition, the ultraviolet (UV) irradiation can be produced in accompany of our plasma process [45,46], which is also likely to contribute to the AgNP formation [47].

The chemical structural properties of all samples were studied by FTIR, see Figure 4. The broad bands around 3200 to 3500 cm⁻¹ is attributed to the –OH and –NH₂ groups stretching vibrations;

Figure 3. (a) Survey XPS spectra of 1 mM, 2 mM, and 4 mM AgNP/CS nanocomposites; (b)-(d) Ag 3d core XPS spectrum of 1 mM, 2 mM, and 4 mM AgNP/CS nanocomposites, respectively.

the peaks at around 1560 cm^{-1} is the NH bending (amide II) (NH_2) peak; peaks at around 1647 cm^{-1} is due to the C-O stretching (amide I) of O-C-NHR; multiple peaks at around 2927, 2884, 1411, 1321 and 1260 cm^{-1} correspond to CH_2 bending (within pyranose rings), 1078 cm^{-1} is for saccharide structures and the band at 1380 cm^{-1} is due to CH_3 wagging [48]. It is noticed that, comparing to CS and plasma treated CS films, new peaks at 825 and 800 cm^{-1} emerged in the spectra of AgNP/CS films. This can be attributed to the plane vibration bands of N-H due to the interaction between AgNPs and CS [49,50].

The thermal properties of all film samples were analysed by DSC and TGA respectively and the results are shown in Figure 5. The DSC thermograms (Figure 5a) of all samples exhibit a broad endothermic peak ranging from 120 to $200\text{ }^\circ\text{C}$, which are due to the dehydration process

Table 1. Ag 3d peak analysis results for 1, 2, and 4 mM AgNP/CS nanocomposites.

Samples		Ag species			
		Ag ⁰ 3d _{5/2}	Ag ^{δ+} 3d _{5/2}	Ag ⁰ 3d _{3/2}	Ag ^{δ+} 3d _{3/2}
1 mM AgNP/CS	BE (eV)	367.9	368.9	373.9	375.0
	Fraction (%)	54.04	4.04	37.87	3.25
2 mM AgNP/CS	BE (eV)	367.9	368.9	373.9	374.9
	Fraction (%)	50.61	8.84	35.08	5.46
4 mM AgNP/CS	BE (eV)	368.1	369.0	374.1	375.0
	Fraction (%)	54.99	4.14	38.12	2.75

Figure 4. FTIR spectra of CS, plasma treated CS, 1 mM, 2 mM, and 4 mM AgNP/CS films.

of water constrained in the sample via hydrogen bonds [51]. The exothermic peaks starting from 275 °C within the DSC thermograms correspond to the decomposition process of the CS polymer chains [52]. The TGA (Figure 5b) graphs suggest the decomposition of all tested samples involves two stages. Stage I (100 to 190 °C) can be associated to the weight loss of water, and stage II is due to the decomposition of polysaccharide structures, which closely matches the DSC profiles. When the temperature reached 800 °C, samples with higher initial AgNO₃ concentration show less weight loss, indicating greater remnant of AgNPs.

The water uptake ability of CS and CS based nanocomposites is key to their biomedical applications [53]. The swelling behaviours of pure CS, plasma treated CS and AgNP/CS films were evaluated and their swelling ratios are demonstrated in Figure 6. It was observed that pure CS films dissolved completely in PBS solution after 24 h (See Figure S1, supporting

Figure 5. The (a) DSC and (b) TGA curves of CS, plasma treated CS, 1 mM, 2 mM, and 4 mM AgNP/CS films.

Figure 6. Swelling ratio of (I) plasma treated CS, (II) 1 mM, (III) 2 mM, and (IV) 4 mM AgNP/CS films in PBS solution (PH=7.4), respectively.

information). In contrast, the plasma treated CS films remain stable in PBS and demonstrate a swelling ratio of 186.16 ± 9.27 %. This can be explained by the formation of crosslinked structures in plasma treated CS [54]. As discussed earlier, PiLC can generate various energetic/reactive radicals, such as $\text{OH}\cdot$, $\text{H}\cdot$, and $\text{O}\cdot$. It is widely accepted that $\text{OH}\cdot$ can induce cleavage of the β -1-4 glycosidic linkages and the oxygenation of d-glucopyranose rings within CS chains, leading to effective CS chain scission (see Step I in Scheme 2) [55,56]. At the same time, this process can also lead to the formation of aldehyde groups, which can effectively crosslink with amino groups of CS fragments via hydrogen bonds [55]. As a result, the PiLC has altered the physical structures of CS, leading to a more robust crosslinked network consisting of shorter CS molecular chains (Step II in scheme 2). This has in turn enhanced the stability of CS and prevented its dissolution in PBS. Compared to plasma treated CS film, the swelling ratio of AgNP/CS films (also stable in PBS, see Figure S1 in supporting information) decreases with increasing initial AgNO_3 concentration, see Figure 6. This may be due to the

Scheme 2. Simplified scheme illustrating formation of crosslinked CS structures with shorter molecular chains induced by plasma chemistry.

greater AgNO_3 initial concentration has resulted in greater AgNP contents in the polymer network (as indicated by the TGA results), hence there is less room for holding water [57].

Figure 7 shows typical stress-strain curves of the composite film samples obtained from the tensile tests. Compared to pure CS, the tensile strength of plasma treated CS decreased, while the strain at break increased significantly. The tensile strength of polymers can be affected by both molecule weight and crosslinking [58]. It is widely accepted that reactive species generated by PiLC can lead to the scission of CS chains (see Scheme 2) and formation of crosslinked network between these shortened CS chains via hydrogen bonds [55,56]. It is worth noting that the hydrogen bonds based physical crosslinking is different from those covalent crosslinks seen in thermosetting materials. The hydrogen bonds can be mobile within the gel network under dynamic loading [59]. The mobility of hydrogen bonding and the presence of shorter CS molecular chains could be the reason for the greater elongation observed for the plasma treated CS films [60]. On the other hand, the shorter CS chains could be the reason for the decreased tensile strength of CS after the plasma treatment. Figure 8a and 8b shows that both tensile strength and Young's modulus of AgNP/CS are generally greater than those of plasma treated CS. The enhanced tensile strength can be attributed to the presence of AgNPs, which act as nanoreinforcement enhancing the mechanical properties (e.g., stiffness and tensile strength) of the composites through their interaction with the CS- NH_2 functions (see Figure 4 FTIR spectra) [37,61]. However, when AgNP content further increases, the nanoparticles may

Figure 7. (a) The stress - strain curves of CS, plasma treated CS, 1 mM, 2 mM, and 4 mM AgNP/CS composite films.

Figure 8. (a) Tensile strength–strain and (b) Young modulus of (I) CS, (II) plasma treated CS, (III) 1 mM AgNP/CS, (IV) 2 mM AgNP/CS, and (V) 4 mM AgNP/CS, respectively.

have the tendency to agglomerate. Such agglomeration may serve as stress concentration sites, facilitating the crack propagation during tensile testing hence reducing the samples strain at break, see Figure 7.

The antibacterial performance of pure CS, plasma treated CS, and AgNP/CS films towards *S. aureus* and *E. coli* bacterial strains are shown in Figure 9. Pure CS and plasma treated CS films displayed no antibacterial activity, which is consistent with previous reports [62]. In contrast, bacteria inhibition zones were clearly seen for all the AgNP/CS films against both bacterial strains. For *E.coli* particularly, the size of the inhibition zones increases significantly with increasing Ag content. The presence of AgNPs can contribute to the strong antimicrobial effect by generating reactive oxygen species (ROS) [63,64]. The ROS species formed within the AgNP/CS films can diffuse / migrate to the sample surface, during swelling of the nanocomposite films. When in contact with the bacterial strains, the ROS could destabilize their plasma membrane potential and deplete the levels of intracellular adenosine triphosphate, resulting in death of bacterial cells [65]. In addition, the small amount of residual Ag ions (as evidenced in the XPS results, Table 1) can also partly contribute to the antibacterial effects due to their ability to interact with the bacterial cell envelope and cellular molecules [29,66]. It is also noticed that the inhibition zone size for *E. coli* is larger than that of *S. aureus* for all AgNP/CS films. This is because *S. aureus* are surrounded by a thick peptidoglycan based cell wall [2,67], whereas the gram-negative *E. coli* cell wall comprises of a thin layer of negatively charged lipopolysaccharide, which is more prone to attack by antibacterial agents.

4. Conclusion

In conclusion, we have successfully demonstrated a facile approach for the synthesis of AgNPs loaded CS nanocomposites deploying atmospheric pressure microplasma liquid interaction.

Figure 9. Antibacterial analysis using *S. aureus* and *E. coli*; (a) and (b) antibacterial performances of (i) CS, (ii) plasma treated CS, (iii) 1 mM AgNP/CS, (iv) 2 mM AgNP/CS, and (v) 4 mM AgNP/CS against *S. aureus* and *E. coli* bacterial strain, respectively; (c) corresponding average diameter of each inhibition zone. Data were presented as mean $\pm$ S, E, and N=5. One way ANOVA followed by Dunnett's multiple comparisons test. **** $p < 0.0001$.

The unique process serves dual functions, i.e., *in situ* reduction of AgNP and tailoring the CS structure and properties, leading to nanocomposites with improved mechanical properties compared to pure CS. This shows the potential of the new approach for the rapid synthesis of functional nanocomposites with be-spoke properties. Our plasma synthesised AgNP/CS nanocomposites also demonstrates effective anti-microbial properties, which may enable them for a wide range of applications such as antibacterial coatings, wound dressings and membranes for water sanitation, etc.

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