Antibacterial properties of electrospun Ti$_3$C$_2$T$_x$ (MXene)/chitosan nanofibers†

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Electrospun natural polymeric bandages are highly desirable due to their low-cost, biodegradability, nontoxicity and antimicrobial properties. Functionalization of these nanofibrous mats with two-dimensional nanomaterials is an attractive strategy to enhance the antibacterial effects. Herein, we demonstrate an electrospinning process to produce encapsulated delaminated Ti$_3$C$_2$T$_x$ (MXene) flakes within chitosan nanofibers for passive antibacterial wound dressing applications. In vitro antibacterial studies were performed on crosslinked Ti$_3$C$_2$T$_x$/chitosan composite fibers against Gram-negative Escherichia coli (E. coli) and Gram-positive Staphylococcus aureus (S. aureus) – demonstrating a 95% and 62% reduction in colony forming units, respectively, following 4 h of treatment with the 0.75 wt% Ti$_3$C$_2$T$_x$ – loaded nanofibers. Cytotoxicity studies to determine biocompatibility of the nanofibers indicated the antibacterial MXene/chitosan nanofibers are non-toxic. The incorporation of Ti$_3$C$_2$T$_x$ single flakes on fiber morphology was analyzed by scanning electron microscopy (SEM) and transmission electron microscopy equipped with an energy-dispersive detector (TEM-EDS). Our results suggest that the electrospun Ti$_3$C$_2$T$_x$/chitosan nanofibers are a promising candidate material in wound healing applications.

1. Introduction

With the discovery of graphene, a two-dimensional (2D) material comprised of a single atomic layer of sp$^2$ bonded carbons, interest in 2D materials suitable for biomedical applications has grown due to their inherent antibacterial activity, impressive mechanical properties, and thermal stability.\textsuperscript{1–4} MXenes—a family of 2D transition metal layered hexagonal ternary carbides and/or nitrides—have attracted a great deal of attention for applications including supercapacitors,\textsuperscript{5,6} batteries,\textsuperscript{7–9} water purification,\textsuperscript{10–12} catalysis,\textsuperscript{13–15} and desalination.\textsuperscript{16} These materials have a $M_nX_nT_x$ composition, where $M$ is an early transition metal, $X$ is carbon or nitrogen, $n = 1, 2, \text{ or } 3$, and $T$ is a variable surface termination (i.e. OH, F, O, Cl).\textsuperscript{5,7–37} Currently there are only a few studies on the antibacterial behavior of MXene, predominantly for use as antibiofouling agents in wastewater treatment.\textsuperscript{18,39} However, the antibacterial properties of MXene for their potential use as medical bandaging materials have yet to be studied.

As MXenes are nanoscale materials, usually manufactured in colloidal suspension, a carrier polymer is required for accurate dosage and immobilization for use as a medical bandage. Electrospun nanofibers are an ideal carrier medium, as they exhibit desirable characteristics such as high surface area and porosity, flexibility in chemical functionalization, excellent permeability and high absorption capabilities.\textsuperscript{29} Chitosan (CS), a polysaccharide derivative of chitin, was chosen as a carrier polymer due to its biocompatibility, biodegradability, and low-cost. CS is a natural polymer that has been used in numerous studies for biomedical applications including drug-delivery systems, tissue engineering, wound healing, and antibacterial effects derived from its cationic nature.\textsuperscript{21–27} While electrospinning and crosslinking of chitosan is possible,\textsuperscript{28,29} most studies of electrospun chitosan for antibacterial applications use a copolymer to increase stability and electrospinnability.\textsuperscript{30–32} In this study, Ti$_3$C$_2$T$_x$ (MXene) delaminated single and few layer flakes was electrospun within chitosan nanofibers for the first time. Only recently has MXene been successfully incorporated within electrospun polymers such as poly(acrylic acid) (PAA), poly(ethylene oxide) (PEO), poly(vinyl alcohol) (PVA), and alginate/PEO.\textsuperscript{31}

Electrospun polymers functionalized with two-dimensional materials (i.e. graphene oxide (GO),\textsuperscript{14–37} exfoliated nanosheets of metal-organic frameworks (MOFs),\textsuperscript{38} transition-metal dichalcogenides (TMDs),\textsuperscript{39} and layered zeolites\textsuperscript{40}) and their antibacterial activities have been extensively studied. Chitosan/ poly(lactide-co-glycolide) (PLGA) functionalized with GO decorated with silver nanoparticles via a two-step process displayed excellent antibacterial activity to Gram-negative (E. coli and P. aeruginosa) (inactivation rate of 99%) and Gram-positive (S.}
aureus) bacteria (inactivation rate of 76%). Antibacterial properties of electrospun GO sheets that were blended directly with PVA/chitosan displayed clear zones of inhibition, whose mean diameter increased with increasing GO loading. Previous studies attribute the antibacterial mechanism of GO to oxidative and physical stresses, including production of reactive oxygen species (ROS) and penetration of the bacterial membrane by the two-dimensional sharp edges. Similarly, the antibacterial effects of Ti$_3$C$_2$T flakes and membranes have been suggested to be due to their sharp edges, high surface hydrophilicity and action as a reducing agent.

In this study, we report on the fabrication of glutaraldehyde-crosslinked and basified Ti$_3$C$_2$T/CS nanofibers produced by electrospinning a blend of the natural polymer CS and delaminated Ti$_3$C$_2$T single and few-layer flakes colloidal suspensions. To realize the potential bandage applications of Ti$_3$C$_2$T/CS, the antibacterial effects of the nanofibers on the Gram-negative Escherichia coli (E. coli) and the Gram-positive Staphylococcus aureus (S. aureus) were studied. This work shows that even with a low Ti$_3$C$_2$T concentration in the composite (less than 1.0 wt%), substantial antibacterial activity is achieved.

2. Results and discussion

2.1 Synthesis and characterization of Ti$_3$C$_2$T

Ti$_3$C$_2$T is synthesized via the selective etching of Al layers from the starting material, Ti$_3$AlC$_2$, using a LiF/HCl solution as an etchant as illustrated in (Fig. 1a). The resultant multilayered (ML) Ti$_3$C$_2$T flakes are then washed with distilled water, centrifuged, and decanted to remove any remaining reaction by-products until a certain degree of spontaneous delamination occurs. A typical SEM image of the ML structure is shown in (Fig. 1b). The ML Ti$_3$C$_2$T is sonicated in water under a flow of a protective argon gas, to produce delaminated Ti$_3$C$_2$T, or a colloidal suspension of single and few-layer thick flakes as seen in (Fig. 1c and d), using SEM and TEM, respectively. The obtained Ti$_3$C$_2$T flake sizes agreed with other studies following similar etching and delamination procedures.

A typical XRD pattern of a vacuum-assisted film of Ti$_3$C$_2$T flakes displays a sharp, intense (002) peak at 6.9°. This peak corresponds to (002) basal-plane reflections with a c lattice parameter of 25 Å, indicative of the presence of water and Li cations between the negatively charged sheets. For this study, the antibacterial properties of delaminated Ti$_3$C$_2$T composites were investigated due to the previously demonstrated antibacterial effects of the flakes.

2.2 Morphology and structure of Ti$_3$C$_2$T/CS nanofiber mats

2.2.1 Ti$_3$C$_2$T/CS fiber morphology. SEM micrographs (Fig. 2a) show the as-spun CS mats with uniform, cylindrical fibers with an average fiber diameter of 211 ± 40 nm. Fine-tuning of electrospinning parameters led to consistent formation of uniform CS nanofibers with Ti$_3$C$_2$T loads of 0, 0.05, 0.10, 0.25, 0.50, and 0.75 wt%. These Ti$_3$C$_2$T concentrations were chosen because they consistently formed uniform, bead-free nanofibers. SEM micrographs of the Ti$_3$C$_2$T/CS nanofibers revealed the presence of dense networks of non-woven fibers of relatively uniform diameters (Fig. 2b–f). No branching of the fibers was observed, which suggests that the electrical forces and surface tension of the Ti$_3$C$_2$T/CS solution during electrospinning were properly balanced. Morphology of the Ti$_3$C$_2$T/CS nanofibers were visually comparable to those of the neat CS nanofibers. Average fiber diameters of the 0.05, 0.10, 0.25, 0.50, and 0.75 wt% Ti$_3$C$_2$T/CS were 236 ± 59, 170 ± 54, 311 ± 144, 279 ± 80, and 298 ± 76 nm respectively (Fig. S1†). The data failed Levene’s test for homogeneity at a significance level of
0.05, so a Kruskal–Wallis non-parametric analysis of variance (ANOVA) was performed, finding significant differences between fiber diameters at $p \leq 0.05$. Dunn’s test was used for post hoc multiple comparison and indicated significant difference between the CS and the composite fibers containing 0.25, 0.50 and 0.75 wt% Ti$_3$C$_2$T$_x$. This difference in diameter may be due to the increasing water concentration in the electrospinning solutions from the increasing addition of Ti$_3$C$_2$T$_x$ aqueous suspensions.

2.2.2. Ti$_3$C$_2$T$_x$/CS chemical functionalities. The chemical functionalities of as-spun CS and Ti$_3$C$_2$T$_x$/CS fiber mats was investigated by FT-IR spectroscopy (Fig. 3a) to ensure that the addition of Ti$_3$C$_2$T$_x$ did not interfere in the crosslinking of the chitosan nanoﬁbers. Typical CS bands at 3418 (hydroxyl group), 1203 (bridge ether oxygen), 1653 (amide I group), and 1532 cm$^{-1}$ (amide II) appeared for all the nanofibrous mats (Table 1). The amine deformation displayed in the FT-IR spectra for both the neat and composite fibers indicates successful crosslinking, where glutaraldehyde undergoes a Schiff base reaction with the chitosan for the formation of an amine functionality and/or Michael-type adducts with terminal aldehydes to form carbonyl groups. As shown by the negligible differences between the spectra of the neat and composite nanofibers, the crosslinking of the chitosan was unaffected by the addition of Ti$_3$C$_2$T$_x$.

2.2.3. Ti$_3$C$_2$T$_x$/CS crystal structure. The effect of Ti$_3$C$_2$T$_x$ on the overall crystal structure of the CS nanofibers was investigated by X-ray diffraction (Fig. 3b). The neat CS and Ti$_3$C$_2$T$_x$/CS nanofibers displayed similar XRD patterns with the typical amorphous broad reflection at 20.1°, signaling the retardation of the crystallization process of the chitosan blend. However, the introduction of Ti$_3$C$_2$T$_x$ into chitosan nanofibers produced a somewhat broadened peak of decreased intensity, suggesting some form of interaction between the Ti$_3$C$_2$T$_x$ and CS. More specifically, Ti$_3$C$_2$T$_x$
single flakes may physically interfere with the semi-crystalline behavior of CS via interfacial interactions and steric hindrance.\textsuperscript{46}

2.3 Characteristics of Ti$_3$C$_2$T$_2$ immobilized within CS nanofibers

Two different interactions are likely to occur at the organic–inorganic interface between the negatively charged surface terminations (O, F, OH, Cl) of the Ti$_3$C$_2$T$_2$ and the positively charged nitrogen-containing groups of chitosan. The first being electrostatic interactions and the second being the less dominant hydrogen bonding.\textsuperscript{47,48} Fig. 4a illustrates the orientations of Ti$_3$C$_2$T$_2$ to the CS nanofibers based on these interactions.

Due to the encapsulation of the Ti$_3$C$_2$T$_2$ flakes within the chitosan nanofibers, they could not be visualized from the SEM micrographs. To identify the presence of Ti$_3$C$_2$T$_2$ flakes, Ti$_3$C$_2$T$_2$/CS fiber mats were analyzed by TEM (Fig. 4b). The TEM micrographs enabled the simultaneous visualization of the chitosan nanofibers and the Ti$_3$C$_2$T$_2$ flakes. Two orientations were observed: full embodiment of the Ti$_3$C$_2$T$_2$ flake within the fiber and protrusion of the Ti$_3$C$_2$T$_2$ flake, as suggested in Fig. 4. The TEM-EDS micrograph and results on the composite fibers shows the presence of Ti$_3$C$_2$T$_2$ within the fiber (Fig. 4c). The chitosan was not doped and so the peaks for C and O are combined.

### Table 1 FTIR peak annotations of the CS and Ti$_3$C$_2$T$_2$/CS nanofibers

| Peak frequency, cm$^{-1}$ | CS          | Ti$_3$C$_2$T$_2$/CS | Peak assignment   | References |
|--------------------------|-------------|----------------------|------------------|------------|
| 3418                     | 492, 3418   | —                    | –OH stretching   | 28, 29, 46 and 62 |
| —                        | 957         | Ti-O stretching      | 46 and 47        |
| 2887                     | 2887        | C-H stretching       | 28, 29, 46 and 62 |
| 1653                     | 1653        | Amide I              | 28, 29, 46 and 62 |
| 1532                     | 1532        | Amide II             | 28, 29, 46 and 62 |
| 1430                     | 1430        | C-N stretching       | 28, 29, 46 and 62 |
| 1203                     | 1203        | Bridge ether oxygen  | 28, 29, 46 and 62 |
| 1078, 1027               | 1078, 1027  | C-O stretching       | 46 and 47        |

2.4 Antibacterial activity of electrospun Ti$_3$C$_2$T$_2$/CS mats

#### 2.4.1 GA crosslinked CS and Ti$_3$C$_2$T$_2$/CS

The antibacterial activity of the glutaraldehyde-crosslinked 0.75 wt% Ti$_3$C$_2$T$_2$/CS composite nanofibers after 4 h of contact with *E. coli* and *S. aureus* is shown in (Fig. 5a and b). The glutaraldehyde-crosslinked Ti$_3$C$_2$T$_2$/CS fibers exhibited a bacterial cell reduction of over 95% for *E. coli*, relative to the unmodified control CS fibers. A lower cell reduction of 62% was achieved against *S. aureus* due to the thicker peptidoglycan cell wall of Gram-positive bacteria which may provide protection against the antibacterial effects of the Ti$_3$C$_2$T$_2$/CS composite nanofibers. Our results are in agreement with a previous study on PLGA/CS mats functionalized with silver nanoparticles, which reported higher cell reduction for *E. coli* than *S. aureus*.\textsuperscript{44} The *E. coli* data passes Levene’s test for homogeneity of variance (significance level of 0.05), so a one-way ANOVA with post hoc Tukey test was performed. The *S. aureus* data also does not pass Levene’s test for homogeneity of variance (significance level of 0.05), so a Kruskal–Wallis one-way ANOVA with post hoc Dunn’s test at significance level 0.05 was performed.

#### 2.4.2 Basified CS and Ti$_3$C$_2$T$_2$/CS

Two methods of crosslinking, glutaraldehyde vapor deposition and basification, were used to compare the effect on the nanofiber antibacterial efficacy as a function of their crosslinking stability. Basification
remains a common method for creating aqueous insoluble CS nanofiber mats via removal of the acetate salt. The application of NaOH neutralizes the amines on the chitosan, which prevents the mat from immediately dissolving into solution, but does not prevent the mat from beginning to degrade and smoothing into a film.59

The basified composite proved to be effective against E. coli but showed less antibacterial activity against S. aureus, indicating that the charged NH3 group may be required for S. aureus activity. While the mat did not disintegrate appreciably over the 4 h time period (measured via OD600, data not shown), this smoothing effect may also have covered surface MXene flakes, reducing their efficacy against the S. aureus cell wall. Both basified CS and basified MXene composite nanofiber mats were effective against more susceptible E. coli, which may be due to the inherent antibacterial activity of CS. The mechanism is proposed to be direct adsorption of dissolved water-insoluble CS onto the surface of the bacterial cell, forming an imperious layer that blocks membrane transport channels and leads to cell death.59 This is likely the same effect found in other studies of electrospun CS, as the combination of CS with a copolymer would not prevent bacteria from contacting regions of CS on the surface of the nanofibers.36–32

2.4.3. Antibacterial mechanism. Following exposure to the Ti3C2Tz/CS composite nanofibers, SEM micrographs of the attached cells were examined as shown in Fig. 5c and d. Morphological integrity was lost upon contact with the Ti3C2Tz/CS mats as seen by the flattened and burst features of the bacteria. These SEM micrographs are consistent with previous images obtained for E. coli following treatment with AgNPs.31

While there are many proposed mechanisms for the antibacterial nature of Ti3C2Tz flakes,18–19 the most likely mechanisms involve direct contact of the bacteria with the MXene flakes. Direct mechanical destruction by MXene penetration through bacterial membranes has been observed by TEM,18 and is also considered to be the main cause of the antibacterial activity of two-dimensional GO.1–4,19 Additionally, the hydrophilic, negatively-charged MXene flakes (due to the reactive –OH, –F, and –O surface terminations) may promote agglomeration of the bacteria, a significant indicator of antimicrobial activity in GO.25 Despite being incorporated into the nanofibers, the Ti3C2Tz flakes retain a high degree of antibacterial effectiveness. Our findings compare well with other studies that tested 200 µg mL−1 Ti3C2Tz colloidal suspensions against E. coli – resulting in a 99% bacterial inhibition.18

2.5 Biocompatibility of electrospun Ti3C2Tz/CS mats

To determine the biocompatibility of Ti3C2Tz/CS nanofibers, an in vitro cytotoxicity test was performed using HeLa cells, with results evaluated using an alamarBlue® assay. As shown in Fig. 6, the average cell viabilities relative to the control were over 85% at all test concentrations. The data passed Levene’s test for homogeneity (at a significance level of 0.05), so a one-way analysis of variance (ANOVA) was performed, and no difference between samples was found (p ≤ 0.05). This indicates that the mats are not cytotoxic to HeLa cells over 72 h of exposure.
2.6 Material comparison

A comprehensive literature review of electrospun materials with incorporated antibacterial two-dimensional and metal oxide nanoparticles (e.g., zinc oxide, titanium dioxide, and carbon nanotubes) clearly indicates that Ti$_3$C$_2$T$_x$/CS nanofibers are the most effective in reducing *E. coli* and *S. aureus* at incredibly low doses (e.g., 0.75 wt% Ti$_3$C$_2$T$_x$/CS). Given that the most common application method for these nanoparticles is spraycoating or dipcoating onto the surface of electrospun fibers, their effectiveness may be lowered as the particles may be washed away in aqueous environments. Electrospinning a composite nanofiber, as detailed in this paper, allows the nanoparticles to be fully or partially encapsulated, limiting surface erosion.

Though 2D materials and metal oxides display excellent antibacterial properties, it is important to consider their biocompatibility for use in wound healing dressings. For example, GO at higher doses was shown to exhibit stark cytotoxicity reflected in the protrusion into mitochondrion and cell nuclei thus leading to apoptosis. Carbon nanotubes have been found to promote inflammation and granuloma formation due to the formation of free radicals, release and accumulation of peroxidative products, depletion of antioxidant and oxidation of protein sulphydryl groups. TiO$_2$ was revealed to be toxic to the cells of major organs such as the brain and liver, causing acute necrosis. It was also demonstrated that TiO$_2$ was capable of travelling through the placental barrier of pregnant mice and relaying neurotoxicity to their offspring. Despite these properties displayed by commonly studied 2D materials and metal oxides, Ti$_3$C$_2$T$_x$ encapsulated by the chitosan nanofibers proved to be non-toxic. This is supported by previous studies demonstrating the high biocompatibilities of MnO$_x$/Ti$_3$C$_2$ composite materials and functionalized Ti$_3$C$_2$ nanosheets with GdW$_{10}$-based polyoxometalates (POMs) (Fig. 7).

3. Conclusions

We present the functionalization of electrospun CS nanofiber mats with Ti$_3$C$_2$T$_x$ flakes to develop flexible bandage materials with antimicrobial properties. Ti$_3$C$_2$T$_x$ flakes were successfully incorporated into chitosan nanofibers without alteration to the nanofiber integrity as confirmed by SEM and TEM. Contact with the Ti$_3$C$_2$T$_x$/CS nanofiber surface imparted remarkable antibacterial activity, leading to a cell reduction rate of 95% against Gram-negative (*E. coli*) and 62% against Gram-positive (*S. aureus*) bacteria. Our findings strongly suggest the application of Ti$_3$C$_2$T$_x$/CS nanofiber mats as excellent wound dressing materials.
4. Experimental

4.1 Materials

Medium molecular weight chitosan with 77% deacetylation ($M_w = 190$–$310$ kDa), glutaraldehyde solution (50%), ethanol (EtOH, 99.5%), sodium hydroxide (NaOH), phosphate buffered saline (PBS), glycine and hydrochloric acid (HCl, 37.0%) were obtained from Sigma Aldrich. Lithium fluoride (LiF, 99% trace metals basis) and trifluoroacetic acid (TFA, 99%) were obtained from Alfa Aesar. Sodium chloride (NaCl), BD Bacto™ Agar and yeast extract were purchased from Fisher Scientific and Tryp- tone from Oxoid. NEB® 5-alpha competent Escherichia coli was purchased from New England Biolabs and Staphylococcus aureus subsp. aureus Rosenbach was purchased from American Type Culture Collection (ATCC).

4.2 Synthesis and characterization of MXene ($\text{Ti}_3\text{C}_2\text{Tx}$)

A colloidal suspension of $\text{Ti}_3\text{C}_2\text{Tx}$ flakes was obtained via sonication of multilayer $\text{Ti}_3\text{C}_2\text{Tx}$, after etching the MAX phase $\text{Ti}_2\text{AlC}_2$ in a LiF/HCl solution as described previously. Briefly, $\text{Ti}_2\text{AlC}_2$ (1.0 g) was gradually added to 12 M HCl solution (10 mL) containing LiF (1.0 g) and left to stir for 24 h at 35 °C. The solution was then washed five to eight times with deionized (DI) water via a procedure of agitation, centrifugation (3500 rpm/2301 $\times$ g for 2 min), and decantation, until the supernatant became dark in color. The solution was then sonicated in ice water for 1 h under flowing argon gas and centrifuged (5000 rpm/4696 $\times$ g for 1 h). Information regarding the morphological properties of $\text{Ti}_3\text{C}_2\text{Tx}$ flakes was obtained through scanning electron microscopy (SEM) (Zeiss Supra 50VP SEM) and transmission electron microscopy (TEM) (JEOL JEM2100) operating at an accelerating voltage of 200 kV. Samples for the TEM, started as $\text{Ti}_3\text{C}_2\text{Tx}$ flakes in a colloidal suspension, were diluted 1 : 20 in DI water, sonicated for 10 min in a water bath, and drop-cast onto copper aperture grids. Samples were allowed to fully dry before imaging. X-ray diffraction (XRD) patterns of the free-standing membrane comprised of $\text{Ti}_3\text{C}_2\text{Tx}$ flakes were acquired with a Rigaku Smartlab with Cu Kα radiation with step size of 0.04 degrees and dwell time of 0.7 s per step.

4.3 Fabrication of $\text{Ti}_3\text{C}_2\text{Tx}/\text{CS}$ nanofibers

Chitosan solutions 2.7% (w/v) were prepared in trifluoroacetic acid (TFA), and then loaded with MXene at 0, 0.05, 0.10, 0.25, 0.30 or 0.75 wt% based on the solid CS. The solution was rotated overnight or until uniform. The electrospinning setup was comprised of a high voltage power supply (20–25 kV), a syringe and metal needle (5 mL; 21 gauge), with a tip-to-copper collector distance of 10–14 cm. All solutions were pumped at 1 mL h$^{-1}$ at room temperature (23.0 ± 0.5 °C) and under 9–15% relative humidity. The glutaraldehyde-crosslinked mats were prepared by exposing the electrospun fibers to glutaraldehyde vapor for 24 h in an airtight chamber. The basified samples were prepared by soaking in 1 M NaOH in EtOH for 0.5 h, then washed in changes of ethanol until the pH returned to neutral as measured via litmus paper.

4.4 Characterization of as-spun and crosslinked nanofibers

Fourier transform infrared spectroscopy (FTIR) spectra were recorded from 4000 to 400 cm$^{-1}$ using a Perkin Elmer Spectrum Two FTIR spectrometer. X-ray diffraction (XRD) patterns of the fiber mats were acquired with a Rigaku Smartlab with Cu K radiation with step size of 0.04 degrees and dwell time of 0.7 s per step. Nanofiber morphology and size was examined in a SEM (Zeiss Supra 50VP) using an accelerating voltage of 3.50 kV. Average fiber diameters were calculated from fifty measurements per sample using ImageJ software. A TEM (JEOL JEM2100) equipped with an ultrathin window energy-dispersive detector (TEM-EDS) was used to identify $\text{Ti}_3\text{C}_2\text{Tx}$ within the chitosan nanofibers using an accelerating voltage of 200 kV. Samples were prepared by placing them on a copper aperture grid. The hydrophilicity of the fiber surface was evaluated by measuring the water contact angle.

4.5 Antibacterial tests

To prepare for testing, the electrospun mats were weighed into 1.0 ± 0.1 mg samples and soaked in EtOH for 1 h to sterilize them ($n = 4$ per type). The EtOH was then removed, and the mats were allowed to dry completely under sterile conditions. The glutaraldehyde-crosslinked mats then had residual reactive aldehyde groups capped using a sterile saturated glycine solution. Finally, the mats were washed and conditioned in 3 changes of sterile 1 × PBS. The bacterial strains were inoculated and cultured in sterilized lysogeny broth (LB) overnight in a shaking incubator at 37 °C. The cultures were then diluted to $\sim 3 \times 10^8$ CFU mL$^{-1}$ in sterile PBS, corresponding to an OD$_{600}$ of 0.375 ± 0.02 for E. coli and OD$_{600}$ of 0.200 ± 0.02 for S. aureus. Both bacteria solutions were then diluted 1 : 1000 in sterile PBS to a concentration of $\sim 3 \times 10^6$ CFU mL$^{-1}$, and 1 mL of this bacteria solution was combined with each sterile electrospun mat. All samples were prepared using sterile techniques and cultured in a shaking incubator at 37 °C for 4 h. The same volume of bacteria, without electrospun mats, was also incubated as a control. The antibacterial activity of the electrospun samples was assessed using the spread plate method. At 4 h incubation time, a serial dilution of each sample was plated onto LB agar plates using sterile techniques and incubated in a still incubator at 37 °C overnight. Following incubation, the colonies were counted using a Bio-Rad ChemiDoc™ MP System and Quantity One® 1-D analysis software with manual verification of each colony count. The dilution used for the spread plate method was previously determined and found to be within the statistically relevant range. The antibacterial effect was calculated relative to the controls containing no electrospun mats. After contact with the electrospun mats, the morphology of the attached bacteria cells was observed using a SEM. The fibers were first rinsed with sterile PBS pH 6.8 and fixed using 2.5% paraformaldehyde/2.5% glutaraldehyde diluted in 0.2 M Sorensen’s buffer pH 7.2 for several hours. A series of water–ethanol treatments (50 : 50, 30 : 70, 20 : 80, 10 : 90, and 100 ethanol) for 10 min each were used to dehydrate the attached cells. The fibers were then dried at room temperature.
4.6 Cytotoxicity studies using mammalian cells

Cytotoxicity of the glutaraldehyde-crosslinked bandages was measured using HeLa cells cultured in complete Dulbecco's Modified Eagle Medium (DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin). Samples (1.0 ± 0.1 mg, n = 4 per type) had reactive aldehydes capped using a saturated glycine solution as detailed previously, and then were sterilized using EtOH and dried under UV light. Samples were added to a 24-well plate well containing 5 × 10^4 HeLa cells in 1.5 mL complete DMEM. The cells were then cultured for 72 h at 37 °C and 5% CO2. A negative control of the cells with no bandages present was also prepared. Cell viability was assessed using an alamarBlue® assay by first removing media and bandages from the well, adding 1.5 mL warmed complete media containing 10% alamarBlue® and incubating them for 4 h at 37 °C protected from light. The number of live cells was assessed by measuring absorbance at 570 nm, normalized to the 600 nm reading value. Cell viability was calculated relative to the control wells.

Conflicts of interest

There are no conflicts to declare.

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