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Biocidal Nanofibers via Electrospinning

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ABSTRACT: To achieve biocidal properties, a cyclic *N*-halamine precursor, 7,7,9,9-tetramethyl-1,3,8-triazaspiro[4.5]-decane-2,4-dione (TTDD), was synthesized and introduced into nanosized polyacrylonitrile fibrous mat by an electrospinning technique. It was rendered antimicrobial by exposure to dilute hypochlorite solution. Synthesis routes and characterization data are presented. Scanning electron microscopy (SEM) demonstrated that the ultrafine fiber possessed average diameter 414 nm (from 240 to 650 nm). The chlorinated nanofibrous composites provided about 4.9 log reductions of both Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538) and Gram-negative bacteria *Escherichia coli* O157:H7 (ATCC 43895) within 5 min of contact time. This is indicative of promising possible applications in the filtration of water and air. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: nanofiber; composites; biomaterials; *N*-halamine; biocidal

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INTRODUCTION

Electrospinning is a straightforward method for forming fibers with diameters from micrometers to nanometers through the action of electrostatic forces. In the electrospinning process, a high voltage is applied to overcome the surface tension of a polymer solution at the end of a capillary. A charged polymer jet is ejected due to the increase of electrostatic forces to a grounded electrode. As the solvent evaporates, the polymer is deposited as a random micro- or nanofiber mat on a metal grid (counter or grounded electrode). Because of unique properties such as small diameters of fibers and the large surface area to volume ratio, nanosized fibers from electrospinning have been of great interest in a variety of applications such as for filtration,[1–5] tissue engineering,[6,7] drug release systems,[8,9] enzyme stabilization,[10,11] and protective clothes.[12]

The electrospun nanofibrous structured filters can effectively remove tiny particles with diameters between 1 and 5 μm. Polyacrylonitrile (PAN) is an important polymer to produce nanofibrous membranes adopted in filtration due to its great mechanical properties, resistance to chemicals, and thermal stability. Electrospun PAN nanofibrous mats have been reported to be used as filter media for nanoparticle filtration[5] and water filtration[4,13] due to concerns about the qualities of water and air. However, microorganisms can be easily absorbed by the filters, and they reproduce rapidly forming biofilms. The contaminated filters and biofilms are difficult to remove in a cleaning process. Electrospun nanofibers with antimicrobial functionality having potentials for filter media are summarized in Table I.

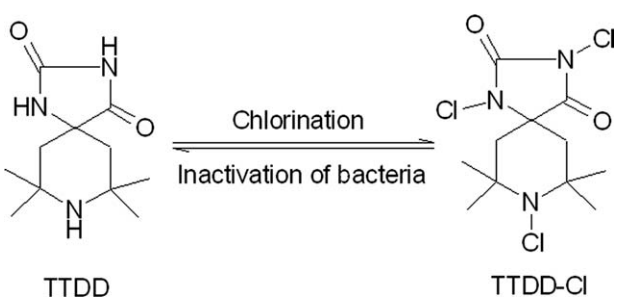
The antimicrobial activity introduced into fibers and polymers has attracted increasing attention for applications including protection of hospital personnel and first responders, for reducing odor, and for antimicrobial water and air filters. Antimicrobial agents such as quaternary ammonium salts,[27–29] metals,[30,31] phosphonium compounds,[32] and *N*-halamine compounds have been used to impart biocidal functions onto fibers and polymers for protection against infectious disease pathogens by using techniques such as grafting,[33,34] coating,[35–39] and blending.[40] Among these, the *N*-halamine compounds are the most desirable antimicrobial agents for producing antimicrobial materials due to their durable and rechargeable properties in killing of Gram-positive and Gram-negative microorganisms.[18,41–48] *N*-halamine compounds have been extensively studied as antimicrobial agents. These compounds have demonstrated excellent biocidal functions against a broad spectrum of microorganisms such as fungi, bacteria, and viruses.[49–51] In addition, *N*-halamines have demonstrated efficacy in total

Table I. Electrospun Nanofibrous Materials with Antimicrobial Functionality

Polymer	Biocide	Bacteria inactivated	Reference
Polyacrylonitrile	Silver nanoparticles	*E. coli, B. cereus*	14
Polyacrylonitrile	Silver nanoparticles	*S. aureus, E. coli*	13, 15
Polyacrylonitrile and β-cyclodextrin	Silver nanoparticles	*E. coli, S. epidermidis*	16
Polyacrylonitrile, poly(vinylchloride), cellulose acetate	Silver nanoparticles	*E. coli, P. aeruginosa*	17
Polyacrylonitrile	*N*-halamine	*S. aureus, E. coli*	18
Nylon	*N*-halamine	*S. aureus, E. coli*	19
Polycarbonate	Quaternary ammonium salt	*S. aureus, E. coli, K. pneumoniae*	20
Poly(methyl methacrylate)	Silver nanoparticles	*S. aureus, E. coli*	21
Cellulose acetate	Silver nanoparticles	*S. aureus, E. coli, K. pneumoniae, P. aeruginosa*	22
PU	Silver nanoparticles	*S. aureus, E. coli*	23
PU	Silver nanoparticles	*E. coli, S. typhimurium*	24
PU	Quaternary ammonium moieties	*S. aureus, E. coli*	25, 26

inactivation of microorganisms without causing the microorganisms to develop resistance to them.[52]

N-halamines can contain one or more of amine, amide, and imide halamine bonds. The rate of inactivation of bacteria is directly related to the *N*-halamine structures. The killing rate of bacteria follows the order of imide > amide > amine halamines, which is in the reverse order of their stabilities. In this study, an *N*-halamine precursor, 7,7,9,9-tetramethyl-1,3,8-triazaspiro[4.5]-decane-2,4-dione (TTDD), was synthesized and incorporated into electrospun PAN fiber. A molecule of TTDD has one amide, one amine, and one imide nitrogen atom, which allows its chlorinated derivative to be efficacious in inactivating bacteria (Scheme 1). TTDD can be rendered antimicrobial by exposure to dilute household bleach. TTDD-Cl (Scheme 1) with its cyclic structure is very stable due to the absence of a α-hydrogen, thus preventing a dehydrohalogenation process. TTDD-Cl is very stable in aqueous solutions because there is only a very low concentration of "free chlorine" released into water due to low N—Cl dissociation constants. The chlorinated nanofibrous samples will be shown to demonstrate excellent antimicrobial properties in inactivating *Staphylococcus aureus* and *Escherichia coli* O157:H7.



Scheme 1. The function of a biocidal *N*-halamine.

EXPERIMENTAL

Materials

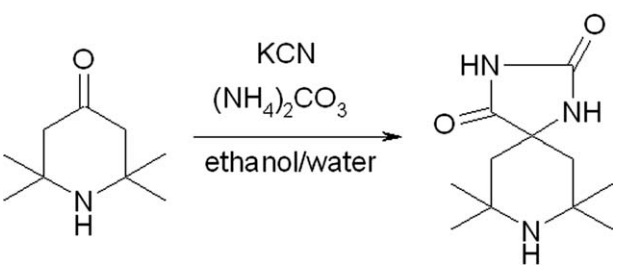
PAN was purchased from Aldrich Chemical Company (St. Louis, MO). All other chemicals used in this research were purchased from Fisher Scientific (Fair Lawn, NJ) or Aldrich Chemical Company and used as received, without further purification, unless otherwise noted.

Characterization

A JEOL JSM7000 Field Emission Scanning Electron Microscope was used to characterize the surface morphology of the nanosized fibers. Samples were coated with gold under an argon purge before analysis. The NMR spectra were recorded by a Bruker AV-400 (400 MHz) spectrometer. Powder X-ray diffraction patterns of polymers were collected with a Rigaku Miniflex powder X-ray diffractometer using Cu-Kα (λ = 1.54056 Å) radiation at ambient temperature over the angular range 5°–90° (2θ, Cu-Kα) with a step width of 0.05° and a fixed counting time of 1 s/step.

Synthesis of TTDD

TTDD was prepared by the reaction of 2,2,4,4-tetramethyl-4-piperidone, potassium cyanide, and ammonium carbonate in a mole ratio of 1 : 2 : 6, respectively, in 500 mL of water/ethanol (1 : 1 v/v) solution at ambient temperature for 48 h (Scheme 2).



Scheme 2. Synthesis of TTDD.

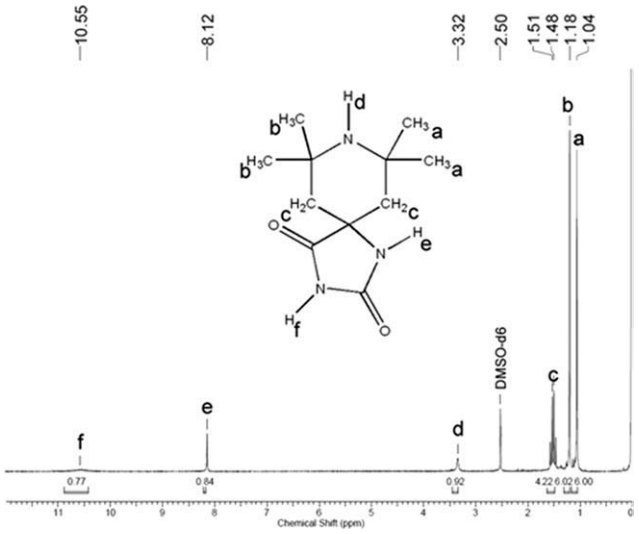


Figure 1. [1]H-NMR spectra of TTDD.

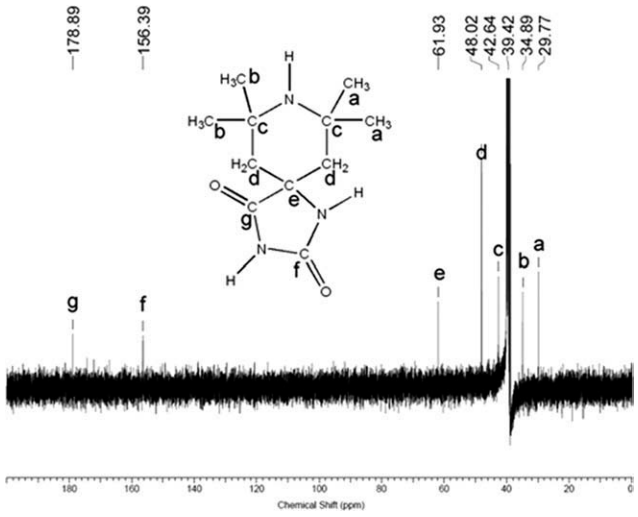


Figure 2. [13]C-NMR spectra of TTDD.

The reaction mixture was filtered and washed with three portions of 100 mL of distilled water (Yield 97%).

Electrospinning

The PAN spinning solution was prepared by adding 10% wt polymer into dimethyl formamide and stirring for 2 days at ambient temperature. Upon complete dissolution of PAN in dimethyl formamide, 5% wt TTDD was added into the above polymer solution and continuously stirred until a homogeneous solution was obtained. The PAN polymer solution loaded with TTDD was spun from an 18 Gauge needle. The high voltage applied to the needle of the syringe was 20 kV. The distance of the spinneret-to-metal grid was 20 cm. The flow rate of the polymer solution was 2 mL/h.

Chlorination and Analytical Titration

The fibrous composites were chlorinated with a 10% aqueous solution of commercial aqueous sodium hypochlorite (NaOCl) (6% sodium hypochlorite) at ambient temperature for 1 h to produce the antimicrobial material. After rinsing thoroughly with distilled water, the chlorinated samples were dried at 45°C for 1 h to remove any occluded chlorine from the surface of the fibers. The amount of chlorine loaded on the samples was determined by an iodometric/thiosulfate titration method. The weight percent Cl^+ in the samples was calculated by the following equation:

$$Cl^+(\%) = \frac{N \times V \times 35.45}{W \times 2} \times 100 \quad (1)$$

where Cl^+ (%) is the weight percent of oxidative chlorine on the samples, N and V are the normality (equiv/L) and volume (L) of the titrant sodium thiosulfate, respectively, and W is the weight of the sample in g.

Biocidal Efficacy Test

A "sandwich test" was used to evaluate the biocidal efficacies of the samples. Both unchlorinated and chlorinated nanofibrous samples were challenged with *S. aureus* (ATCC 6538) and *E. coli* O157:H7 (ATCC 43895). In this test, 25 μL of the bacterial suspensions (pH 7, 100 m*M* phosphate buffer) were added to the center of two layered 1 inch square swatches. A sterile weight was placed on the top of the sandwich to ensure sufficient contact of the swatches with the inocula. After contact times of 5, 10, and 30 min, the samples were quenched with 5.0 mL of

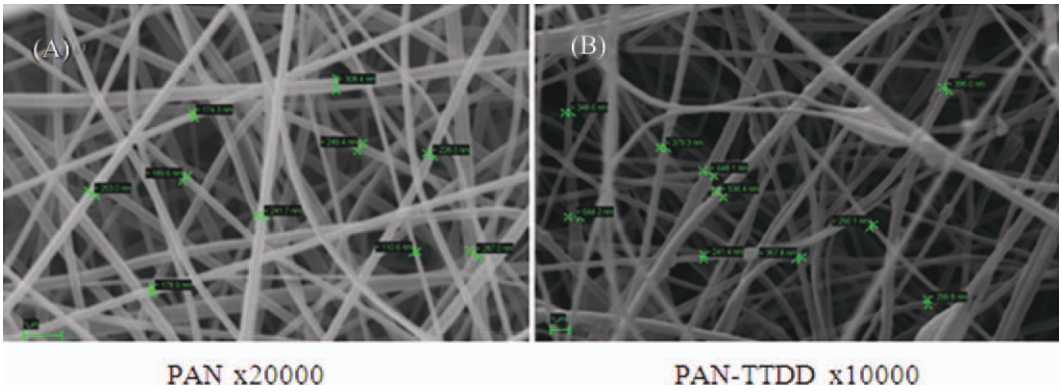


Figure 3. SEM micrographs of the electrospun PAN and PAN-TTDD fibers. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

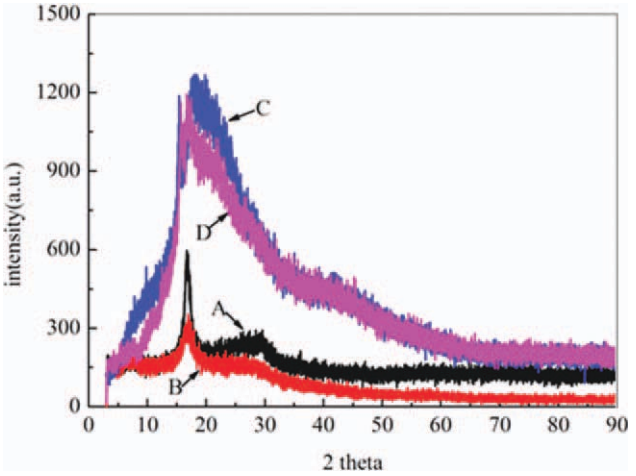


Figure 4. X-ray analysis of electrospun PAN and PAN-TTDD fibers. (a) PAN; (b) electrospun PAN; (c) unchlorinated electrospun PAN with TTDD; (d) chlorinated electrospun PAN with TTDD. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

sterile 0.02*N* sodium thiosulfate solution to remove all oxidative chlorine which could cause extended disinfection. Serial dilutions of the quenched samples were plated on Trypticase soy agar plates. The plates were incubated at 37°C for 24 h, and bacterial colonies were counted to determine the surviving bacterial populations.

RESULTS AND DISCUSSION

Characterization of TTDD

The NMR spectroscopic data for TTDD were as follows: ^{1}H-NMR TTDD (DMSO-d6, 400 MHz, Figure 1) δ 1.04 (6H), 1.18 (6H), 1.48 (4H), 3.32 (1H), 8.12 (1H), 10.55 (1H); ^{13}C-NMR (DMSO-d6, 400 MHz, Figure 2) δ 29.77, 34.89, 42.64, 48.02, 61.93, 156.39, 178.89. The above data confirmed that the synthesized TTDD possessed three types of N—H bonds, amine, amide, and imide, which can be halogenated by dilute aqueous sodium hypochlorite.

Table II. Biocidal Efficacy Against *S. aureus*[a]

Samples	Contact time (min)	Log bacterial reduction
PAN[b]	5	0.45 ± 0.06
	10	0.62 ± 0.06
	30	1.48 ± 0.08
PAN-TTDD[c]	5	0.70 ± 0.03
	10	0.73 ± 0.05
	30	0.94 ± 0.09
PAN-TTDD-Cl[d]	5	4.94 ± 0.00
	10	4.94 ± 0.00
	30	4.94 ± 0.00

[a]Each sample was inoculated with 25 μL of bacterial suspension at 8.67 × 10^4 cfu/sample, [b]PAN nanofibrous mats, [c]PAN-TTDD nanofibrous mats, [d]Chlorinated PAN-TTDD nanofibrous mats (0.03 wt % Cl^+).

Table III. Biocidal Efficacy Against *E. coli* O157 : H7[a]

Samples	Contact time (min)	Log bacterial reduction
PAN[b]	5	0.26 ± 0.03
	10	0.27 ± 0.03
	30	0.31 ± 0.02
PAN-TTDD[c]	5	0.21 ± 0.02
	10	0.23 ± 0.06
	30	0.27 ± 0.05
PAN-TTDD-Cl[d]	5	4.90 ± 0.00
	10	4.90 ± 0.00
	30	4.90 ± 0.00

[a]Each sample was inoculated with 25 μL of bacterial suspension at 8.00 × 10^4 cfu/sample, [b]PAN nanofibrous mats, [c]PAN-TTDD nanofibrous mats, [d]Chlorinated PAN-TTDD nanofibrous mats (0.03 wt % Cl^+).

Characterization of Nanofibers

The surface structures of the electrospun fibers were examined by scanning electron microscopy (SEM). Figure 3 shows SEM images of the electrospun PAN and electrospun PAN loaded with TTDD fibers. The fiber diameters of electrospun PAN were in the range of 110–310 nm [222 ± 59 nm; Figure 3(A)], whereas the fiber diameters of electrospun PAN loaded with TTDD were larger, in the range of 240–650 nm [414±145 nm; Figure 3(B)]. At a voltage such as 20 kV, the differences in fiber diameters were attributed to highly unstable streams dispersed in different directions. The addition of TTDD must be responsible for the diameter increase and high standard deviation of the fiber diameters.

In Figure 4, curves A and B show PAN powders and electrospun PAN as controls, which indicate peaks at intensities $2\theta = 16.7$. Curve C and D show the unchlorinated electrospun PAN fibers with TTDD and the chlorinated electrospun PAN with TTDD showing broader peaks at $2\theta = 17.7$, 17.5, respectively. The addition of TTDD in the PAN nanofibers causes the peak intensities to increase, and the peaks become broader and are also slightly shifted to the right.

Biocidal Efficacy Testing

The electrospun fibers were challenged with *S. aureus* (Gram-positive) and *E. coli* O157:H7 (Gram-negative) bacteria at 10^5 cfu (colony-forming units) per sample, and the biocidal test results are shown in Tables II and III, respectively. The oxidative chlorine loading of the electrospun fibers containing TTDD was ~ 0.03% (wt %) determined by titration. The chlorinated electrospun nanofibrous samples completely inactivated both *S. aureus* and *E. coli* O157:H7 within 5 min with complete log reductions of 4.9. The inactivation of the bacteria is attributed to the direct transfer of attached oxidative chlorine to the bacterial cells. The rate of inactivation of bacteria by *N*-halamine moieties always depends on the nature of the N—Cl moieties. The *N*-halamine studied possesses one imide, one amide, and one amine type N—Cl bond. The inactivation rates of the bacteria follow the order of imide N—Cl > amide N—Cl > amine N—Cl because the dissociation constant of an imide structure is

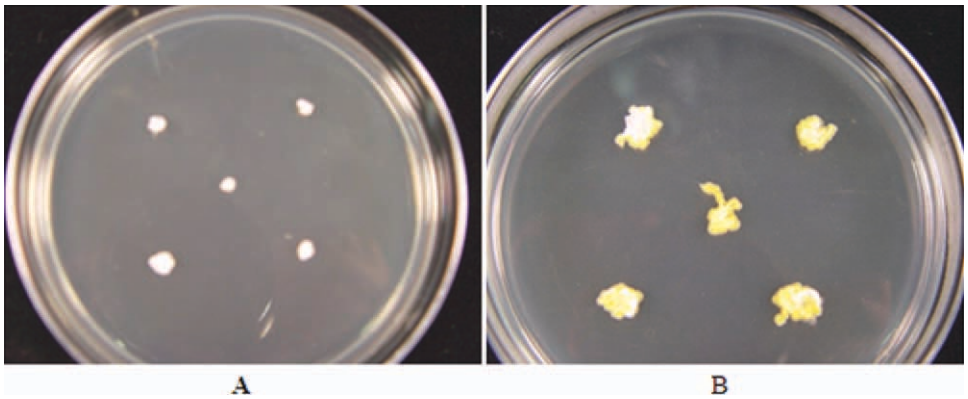


Figure 5. Plates with colonies for (A) PAN-TTDD-Cl (0.03%); (B) PAN-TTDD. The challenge time of *S. aureus* was 5 min.

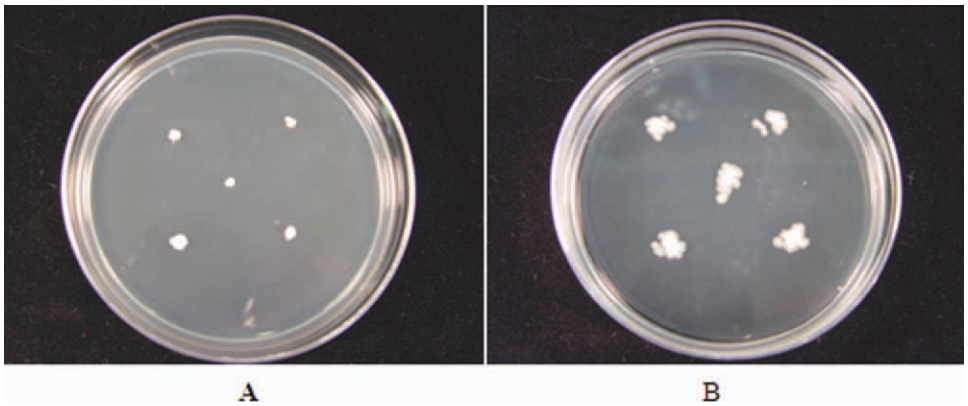


Figure 6. Plates with colonies for (A) PAN-TTDD-Cl (0.03%); (B) PAN-TTDD. The challenge time of *E. coli* O157:H7 was 5 min.

higher than for an amide structure and, in turn, an amine structure. The high surface areas of the nanosized fibers exposed to the bacteria could explain the reason why the nanofibrous mats studied in this work with relatively low chlorine loading inactivated the bacteria remarkably well.

For comparison, two control samples, electrospun PAN fibers and unchlorinated PAN fibers containing TTDD, were also evaluated against the bacteria. These samples provided ~0.45–0.70 log reductions of *S. aureus* and 0.21–0.26 log reductions of *E. coli* O157:H7 within 5 min of contact time, respectively. The reductions were due to the adhesion of the bacteria to the pores inside of nanofibrous mats, rather than to inactivation. The difference of log reductions between *S. aureus* and *E. coli* O157:H7 for the controls can be explained by the different shapes of the two species of bacteria.

To further demonstrate that all bacteria were inactivated by the chlorinated samples rather than just adhered to the fibers, the plates with small amounts of samples were incubated at 37°C for 24 h. The bacteria colonies on the plates are shown in Figures 5 and 6. Clearly, the chlorinated samples showed that there was no growth of the bacteria for both *S. aureus* and *E. coli* O157:H7 after 5 min of contact, while the unchlorinated control samples indicated significant propagation of the bacteria.

CONCLUSIONS

An *N*-halamine precursor, TTDD with one amine, one amide, and one imide nitrogen was synthesized and incorporated into nanosized PAN fiber by using an electrospinning technique. The fabricated nanosized fibers were characterized by electron scanning microscopy and X-ray diffraction. The chlorinated nanofibrous composites completely inactivated about 4.9-logs of both *S. aureus* and *E. coli* O157:H7 within 5 min of contact time. The biocidal nanofibrous composites provide potential disinfectant filters for the filtration of air and water.

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