Antimicrobial Hydrogels Formed by Crosslinking Polyallylamine with Aldaric Acid Derivatives

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ABSTRACT: Aqueous dispersions (0.1 wt %) of hydrogels **1** and **5**—formed by crosslinking polyallylamine hydrochloride (MW 60,000) with aldaric acid derivatives, diethyl L-tartrate and *N*,*N*′-bis(methoxycarbonylmethyl)-D-glucaramide, respectively—exhibited complete (log 5) kill within 4 h of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida albicans* suspended in culture medium. This antimicrobial activity was much higher than that of uncrosslinked polyallylamine (1 wt % killed only 75% of *E. coli* in 24 h). When dispersed at 10 and 100 ppm, hydrogel **5** displayed complete (log 5) kill of *E. coli* within 30–60 and 15 min, respectively. Hydrogels **1** and **5** were active against *S. aureus* and *Salmonella choleraesuis* dried on hard stainless steel surfaces and accelerated the deaths of *E. coli*, *P. aeruginosa*, *S. aureus*, and *C. albicans* in a model skin cream formulation. A 0.8% aqueous dispersion of hydrogel **5** was also effective as a hand sanitizer, killing 99.7% of *Serratia marcescens* on human hands within 5 min. Hydrogels **1** and **5** caused no dermal irritation or allergic contact sensitization under the conditions of a human repeat insult patch test. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 119: 3244–3252, 2011

Key words: antimicrobial; hydrogel; biocompatibility; crosslinking; polyamines

INTRODUCTION

Polymers and hydrogels that contain silver nanoparticles[1–7] or that release iodine,[8] proteins,[9,10] or antibacterial[11–17] or antifungal[18–20] drugs are well known. However, polymers[21–29] and hydrogels[30–35] that are inherently antimicrobial are relatively scarce, even though they have a number of advantages over small-molecule biocides and polymers that release them. Inherently antimicrobial polymers and hydrogels are nonvolatile and unlikely to contaminate water by dissolution or leaching. Because they do not penetrate the skin, they are unlikely to cause irritation or sensitization. Because their activity does not involve diffusion of a small molecule or protein, their activity should persist longer than that of a polymer that releases a diffusible agent. Finally, because inherently antimicrobial polymers and hydrogels are polycationic and kill on contact by disrupting the cell membrane,[36] they are unlikely to generate microbial resistance like diffusible agents that enter cells and inhibit a metabolic or anabolic pathway.

Potential applications of antimicrobial hydrogels include uses in bandages and tissue regeneration and in coatings for medical devices, such as catheters, to prevent nosocomial infections. Antimicrobial hydrogels may be useful as preservatives in personal care products or as the active ingredient in skin care products, such as acne medications. As surface disinfectants and hand sanitizers, antimicrobial hydrogels may offer activity that persists long after application.

Although hydrogels formed by crosslinked polyallylamine are known,[37–43] none has been shown to be antimicrobial. Here, we report on antimicrobial hydrogels formed by crosslinking polyallylamine with several aldaric acid derivatives. These hydrogels are bactericidal and fungicidal and are nonirritating and nonsensitizing to human skin.

EXPERIMENTAL

Materials

Polyallylamine hydrochloride (MW 60,000) and diethyl L-tartrate from Aldrich Chemical were used as received. Other chemicals were also used without further purification. D-Glucaro-1,4:6,3-dilactone (GDL) was synthesized as previously reported.[44]

Determining the swell ratio of crosslinked hydrogels

About 0.5 g of polymer followed by about 100 mL of distilled water at about 22°C were added to a dry,

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tared, 150 mL, coarse-fritted glass funnel, the stem of which had been sealed with a rubber stopper. The slurry was stirred, if necessary, to disperse the polymer fully in the water. The dispersion was left undisturbed for 15 min, the rubber stopper was removed from the stem of the funnel, and suction was applied to the funnel for 5 min. The stem and underside of the funnel were rinsed with ethanol to remove any remaining water droplets, and suction was continued for an additional 5 min. Any remaining water droplets were wiped off the funnel with a paper towel. The funnel and contents were then weighed to determine the weight of water retained by the polymer. Swell ratio was calculated according to eq. (1)

$$\text{Swell ratio} = \frac{W_{\text{wet}} - W_{\text{dry}}}{W_{\text{dry}}} \quad (1)$$

wherein W_{wet} and W_{dry} and are the weights of the water-swollen and dry hydrogel polymers, respectively.

Synthesis of polyallylamine 30% crosslinked with diethyl L-tartrate (1)

A solution of 13.90 g (67.40 mmol) of diethyl L-tartrate (DET) in 12 mL of water was added to a solution of 42.04 g (0.449 mol equivalents of amine) of polyallylamine hydrochloride, MW 60,000, and 5.39 g (0.135 mol) of sodium hydroxide in 172 mL of water, and the resulting solution was stirred at ambient temperature for 4 days. The resulting gel was washed with 250 mL of methanol, separated from the supernatant liquid, and then washed in a blender with eight successive 250-mL portions of methanol. The resulting solid was ground and dried under vacuum to give 38.47g (86% yield) of hydrogel that exhibited a swell ratio of 224.

Synthesis of polyallylamine 50% crosslinked with diethyl L-tartrate (2)

A solution of 0.412 mL (2.41 mmol) of diethyl L-tartrate was added to a solution of 0.900 g (9.61 mmol equivalents of amine) of polyallylamine hydrochloride, MW 60,000, and 0.192 g (4.80 mmol) of sodium hydroxide according to the method used to synthesize **1**, and the resulting solution was stirred at ambient temperature for 88 h before washing with methanol.

Synthesis of hexadecylated polyallylamine crosslinked with GDL (3)

In a 2000-mL three-necked flask equipped with a heating mantle, reflux condenser, nitrogen inlet, and overhead stirrer, 17.08 g (55.94 mmol) of 1-bromohexadecane was added to a solution of 70 g (0.75 mol equivalent of amine) of polyallylamine hydrochloride and 2.24 g (56.0 mmol) of sodium hydroxide in 525 mL of water. The mixture was heated at reflux for 5 h, cooled to room temperature and stirred overnight. Additional sodium hydroxide (5.60 g, 140 mmol) was added, followed by a solution of 12.18 g (69.96 mmol) of GDL in 175 mL of water. Almost immediately, a gel formed. The gelled mixture was heated at 50°C for 7 h and filtered, and the residue was washed three times with methanol and then three times with THF. The gel was dried in a vacuum oven set at 80°C for 24 h. The pale yellow polymer (58.85 g, 60.5%) exhibited a swell ratio of 7.9.

Synthesis of *N*,*N*′-bis(methoxycarbonylmethyl)-D-glucaramide (4)

To a solution of 30.28 g (241 mmol) of methyl glycinate hydrochloride in 500 mL of methanol was added 50 mL (0.36 mol) of triethylamine. After the resulting solution had stirred at ambient temperature for 30 min, a solution of 20.99 g (120.6 mmol) of GDL in a total of 160 mL of methanol was added, and the resulting solution was stirred overnight at ambient temperature. The resulting white precipitate was collected by filtration, washed with 5 × 100 mL of methanol, and dried under vacuum to give 32.73 g (77% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.19 (t, J = 5.8 Hz, 1H), 7.96 (t, J = 5.8 Hz, 1H), 5.69 (d, J = 5.8 Hz, 1H), 5.40 (d, J = 4.5 Hz, 1H), 4.65 (d, J = 4.2 Hz, 1H), 4.48 (d, J = 6.0 Hz, 1H), 4.06 (br t, 1H), 4.01 (t, J = 5.8 Hz, 1H), 3.95–3.76 (m, 6H), 3.622 (s, 3H), 3.618 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 173.82, 172.97, 170.46, 170.35, 73.25, 72.80, 71.93, 70.64, 51.90, 51.86, 40.68, 40.66.

Synthesis of polyallylamine 2% crosslinked with *N*,*N*′-bis(methoxycarbonylmethyl)-D-glucaramide (5)

A slurry of 0.88 g (2.5 mmol) of *N*,*N*′-bis(methoxycarbonylmethyl)-D-glucaramide in 65 mL of methanol was added to a solution of 23.03 g (246.2 mmol equivalents of amine) of polyallylamine hydrochloride, MW 60,000, and 41.2 mL (296 mmol) of triethylamine in 950 mL of methanol. The resulting solution was stirred at ambient temperature for 5 days and then concentrated under reduced pressure to about 150 mL. The resulting solid was separated from the methanol, washed repeatedly with methanol and then dried under vacuum to give 12.92 g (88% yield) of hydrogel that exhibited a swell ratio of 125.

Antimicrobial activity of crosslinked hydrogels

Antimicrobial activity was determined by a standard micro-shake flask test. Bacterial cultures were

inoculated into TSB (trypticase soy broth) and incubated at 37°C overnight for 20 ± 2 h. The following day, the concentration of bacteria was adjusted to $\sim 1.0 \times 10^5$ CFU mL^{-1} (CFU = colony forming unit) by dilution with 0.6 m*M* phosphate buffer. Diluted bacterial culture (2.5 mL) was transferred into culture plate wells containing 2.5 mL of hydrogel (50, 25, 12.5 or 5.0 mg of solid dispersed in 2.5 mL of 0.6 m*M* phosphate buffer) or just 2.5 mL of 0.6 m*M* phosphate buffer (control). The culture plates were incubated at room temperature on a platform shaker with constant shaking motion. Three 100-μL aliquots were periodically removed from each well and serially diluted with 0.6 m*M* phosphate buffer. Undiluted and diluted samples from each well were plated onto duplicate TSA (trypticase soy agar) plates, and incubated at 37°C for 20 ± 2 h. After incubation, the number of bacterial colonies on each plate was counted using a Q-count instrument or equivalent counting method. The colony count was averaged, corrected for the dilution factor, and reported as the number of colony forming units (CFU) per mL. Log kill was calculated according to eq. (2)

$$\log \text{ kill} = \log_{10} C - \log_{10} T \quad (2)$$

wherein *C* is the mean CFU density of microbes in untreated (control) flasks and *T* is the mean CFU density of microbes in (test) flasks containing hydrogel. Microbes tested were *Escherichia coli* (*E. coli*, ATCC 25922), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*, ATCC 29213), and *Candida albicans* (*C. albicans*, ATCC 10231). The three hydrogel samples tested for antimicrobial activity were **1**, **3**, and **5**.

Speed of kill of crosslinked hydrogels

For each hydrogel, an exposure of *E. coli* to a 100 ppm loading was effected by dispersing 5 mg of hydrogel in 25 mL of 0.6 m*M* phosphate buffer, stirring overnight, and then adding 25 mL of a culture broth ($\sim 1.0 \times 10^5$ CFU mL^{-1}) of *E. coli* (ATCC 25922). The untreated (control) sample was created by adding 25 mL of the culture broth of *E. coli* ($\sim 1.0 \times 10^5$ CFU mL^{-1}) to 25 mL of 0.6 m*M* phosphate buffer alone. After 15, 30, 60, 120, 180, and 240 min, aliquots of each test mixture were removed and serially diluted 1 : 10 with Letheen broth in a 96-well microtiter plate. After incubating overnight at 37°C, each plate was scored for microbial growth using a Most Probable Number (MPN) protocol, and log kill was calculated according to eq. (2) wherein *C* is the mean density of *E. coli* in untreated (control) samples and *T* is the mean MPN density of *E. coli* in the hydrogel containing (treated) samples. Exposure of *E. coli* to a 10-ppm loading of each hydrogel was effected similarly except using 1 mg of hydrogel in 50 mL of buffer and adding 50 mL of a culture broth ($\sim 1.0 \times 10^5$ CFU mL^{-1}) of *E. coli* (ATCC 25922). The three hydrogel samples tested were **1, 2,** and **5**.

Hard surface disinfection by crosslinked hydrogels

Tests were performed by Consumer Product Testing Company, Fairfield, NJ following Association of Official Analytical Chemists (AOAC) Use Dilution test methods 955.14 and 955.15. Hydrogels **1** and **5** were dispersed in deionized water at 0.5 wt % (w/v) and 1 wt % (w/v), respectively. Type 304 stainless steel penicylinders (8 mm OD, 6 mm ID, 10 mm L) were soaked overnight in 1*N* sodium hydroxide, washed with water until the rinse water was neutral to phenolphthalein, and autoclaved in 0.1% w/v aqueous asparagine solution. The sterile penicylinders were drained and transferred aseptically into a 48-h culture broth (1 mL per cylinder) of *S. aureus* (ATCC 6538) or *Salmonella choleraesuis* (*S. choleraesuis*, ATCC 10708). After being immersed in culture broth for 15 min, the penicylinders were drained and transferred by sterile hook into a sterile glass petri dish lined with sterile filter paper so that the cylinders stood on end without touching one another. The penicylinders were dried at 37°C for 40 min.

For each hydrogel tested, 10 penicylinders inoculated with a given test organism were immersed individually for 10 min at 20°C in 10 mL of aqueous hydrogel dispersion. Each penicylinder was then removed from the hydrogel dispersion, drained, and deposited into a primary culture tube containing 10 mL of Letheen broth and incubated at 37°C. After 30 min, each penicylinder was transferred into secondary culture tube containing 10 mL of Letheen broth, and both primary and secondary culture tubes were incubated at 37°C for 48 h, after which they were examined for microbial growth as evidenced by turbidity. Neutralization of each antimicrobial hydrogel by double serial subculture was shown to be effective by inoculating tubes showing no growth with low levels of test organism. Viability of test organisms was demonstrated by incubating inoculated penicylinders in deionized water instead of a hydrogel suspension.

Preservation of a skin cream by crosslinked hydrogels

A model skin cream was formulated by adding a mixture of 99.0 g of octamethylcyclotetrasiloxane, 10.0 g of Abil® EM-90 cetyl dimethicone copolyol, and 15.0 g of Stepan TAB-2®, heated to 77°C, to a vigorously stirred mixture of 5.0 g of **1** in 346.5 g of deionized water, also heated to 77°C. After 15 min

of vigorous agitation at 77°C, 12.0 g of triethanolamine was added to the mixture. After an additional 15–25 min of vigorous agitation at 77°C, external heating was discontinued, and the vigorously agitated mixture was allowed to cool. When the temperature of the mixture reached 37–38°C, 12.5 g of Dow Corning 200® fluid dimethicone was added, the speed of agitation was reduced, and the mixture was allowed to cool to room temperature. A control skin cream was made by an identical procedure, except using 350.5 g of deionized water without **1** and 100.0 g of octamethylcyclotetrasiloxane. A second test skin cream was formulated by the same procedure, using 59.4 g of octamethylcyclotetrasiloxane, 6.0 g of Abil® EM-90 cetyl dimethicone copolyol and 9.0 g of Stepan TAB-2®, 3.0 g of **5** in 207.9 g of deionized water, 7.2 g of triethanolamine, and 7.5 g of Dow Corning 200® fluid dimethicone.

Microbiological tests were performed by Consumer Product Testing Company, Fairfield, NJ, according to the *United States Pharmacopoeia* (USP), 24th Edition, <51> Antimicrobial Effectiveness Testing. Twenty-gram portions of each skin cream formulation were aseptically transferred into sterile glass containers and inoculated with 100 μL of a 1×10^8 CFU mL^{-1} culture of *S. aureus* (ATCC 6538), *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC 9027), *C. albicans* (ATCC 10231), or *Aspergillus niger* (*A. niger*, ATCC 16404), yielding microbial loads between 1×10^5 and 1×10^6 CFU g^{-1}. Inoculated samples were incubated at 20–25°C protected from light. Periodically, samples of each inoculated skin cream were serially diluted 10-fold, and microbial counts were determined by the pour plate method, using trypticase soy agar (TSA) plates incubated at 20–25°C for 3 days for bacteria and Sabouraud dextrose agar (SDA) plates incubated at 20–25°C for 5 days for the fungi.

Hand sanitization by crosslinked hydrogels

Hand sanitization efficacy tests were performed by Consumer Product Testing Company, Fairfield, NJ. The 11 subjects completing the test had no cuts, scrapes, or compromised skin on their hands and had been instructed to refrain from using topical antimicrobial products, topical steroids, and topical or systemic antibiotics for 7 days prior to beginning the study. The subjects washed their hands and forearms with a 1.0% solution of Triton-X 100, patted them dry with paper towels, and then applied three sequential 1.5-mL portions of a 24-h culture broth (TSB) of *Serratia marcescens* (ATCC 14756) containing 1×10^8 CFU mL^{-1} to their hands, up to the base of the palm, and allowed them to dry. Subjects' hands were each placed in an antimicrobial-free, low bioburden plastic bag containing 75 mL of sterile 75 m*M* phosphate buffer, pH 7.8, containing 0.1% Triton-X 100, and each hand was massaged in the bag for 1 min to recover bacteria. Recovery solutions were diluted 100, 1000, and 10,000-fold with diluent/neutralizer (0.1% peptone and 0.3% lecithin in 1.0% Tween 80, pH 7), plated onto TSA, incubated at 25°C ± 2°C for 48 ± 4 h, and counted by standard plate counting technique.

After baseline recovery, the subjects' hands were rinsed with running tap water. Their hands and forearms were then washed with a 1.0% solution of Triton-X 100, rinsed in running tap water, rinsed with 70% aqueous isopropanol, dried, rinsed again with 70% aqueous isopropanol, and dried with paper towels.

Subjects' hands were contaminated with *Serratia marcescens* as before. Each subject then distributed a 1-mL portion of a 0.8% dispersion of hydrogel **5** in deionized water over the entire surface of both hands, extending two finger widths below each palm, and rubbing for 30 s. Five minutes after application of the 0.8% dispersion of hydrogel, hands were sampled for bacterial recovery as before. The bacterial counts recovered with and without treatment with hydrogel **5** were compared.

In vitro control experiments verified the ability of the diluent/neutralizer to neutralize the antibacterial activity of the 0.8% dispersion of hydrogel under the conditions of the test, allowing the growth of 1.0×10^2 CFU mL^{-1} of *Serratia marcescens*.

Human repeat insult patch test of crosslinked hydrogels

Hydrogels **1** and **5** were dispersed in deionized water at 0.5% (w/v) and 0.8% (w/v), respectively. The human repeat insult patch test was performed by Consumer Product Testing Company, Fairfield, NJ. The 52 subjects completing this test included 12 men, age 32–68 years, and 40 women, age 22–79 years. Subjects had no visible skin disease, were in good health, were not pregnant or nursing, were not under a doctor's care or taking medication that would influence the outcome of the study, and had not used a topical or systemic steroid or antihistamine for at least 7 days prior to beginning the study.

Approximately 0.2 mL of each hydrogel dispersion, or an amount sufficient to cover the contact surface, was applied to the 3/4″ × 3/4″ absorbent pad of an adhesive dressing. The dressing was then applied to a marked spot between the scapulae of each subject, thus forming an occlusive patch. Patches were applied to the same site three times a week (typically, Monday, Wednesday, and Friday) for three consecutive weeks (total of nine applications). Each patch was removed after 24 h of contact.

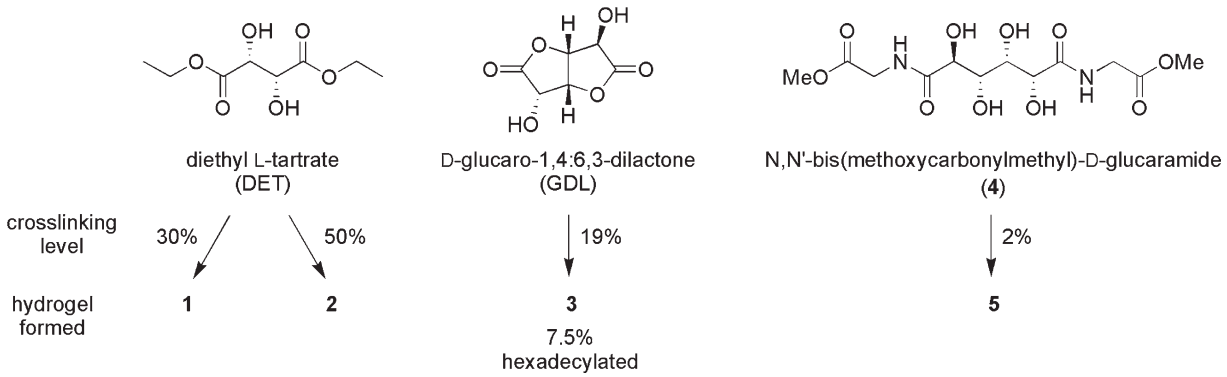

Figure 1 Compounds used to crosslink polyallylamine and the hydrogels formed.

The site of application was examined and scored upon removal of the first patch and again 24 h after removal of the first patch. Thereafter, the site of application was examined and scored 24 or 48 h after the removal of each patch, usually just before application of the subsequent patch. Thus, the application site on each subject was examined 10 times during the Induction Phase. Approximately 2 weeks after application of the final induction patch, a challenge patch was applied to a virgin site adjacent to the original site, following the same procedure as described above. The patch was removed 24 h after application, and the site was examined and scored. The challenge site was examined and scored again 48 h after removal of the challenge patch.

Each time an induction or challenge site was examined, it was scored according to the following scale: 0 = no visible skin reaction, + = barely perceptible or spotty erythema, 1 = mild erythema covering most of the test site, 2 = moderate erythema with possible presence of mild edema, 3 = marked erythema with possible edema, and 4 = severe erythema with possible edema, vesiculation, bullae, or ulceration. For both materials tested, all scores (10 induction and 2 challenge for each of 52 subjects) were 0. In addition, five subjects who began the study but discontinued for various reasons not related to the test materials generated scores of only 0 as well.

RESULTS AND DISCUSSION

Synthesis of crosslinked hydrogels

Commercially available polyallylamine hydrochloride (MW 60,000) was crosslinked using several diester and dilactone derivatives of polyhydroxylated dicarboxylic acids (aldaric acids). The crosslinkers used (Fig. 1) were commercially available diethyl L-tartrate (DET), D-Glucaro-1,4:6,3-dilactone (GDL),[44] and *N,N′*-bis(methoxycarbonylmethyl)-D-glucaramide (**4**). Compound **4** was synthesized by treating GDL in methanol with two equivalents of methyl glycinate (Fig. 2) and collecting the precipitate. Crosslinking was generally performed by dissolving polyallylamine hydrochloride in water or methanol, adding enough base (sodium hydroxide or triethylamine, respectively) to deprotonate the proportion of amine groups intended to be crosslinked, and then adding the crosslinker. The resulting gel was washed and dehydrated by rinsing with methanol and was then dried under vacuum. Swell ratios of the resulting hydrogels ranged from 23 to 224.

Hydrogels **1** and **2** were formed by crosslinking nominally 30 and 50%, respectively, of the amine groups of polyallylamine with DET. Hydrogel **3** was formed by partially (7.5%) alkylating polyallylamine with 1-bromohexadecane and then crosslinking 19% of the amine groups with GDL. Hydrogel **5** was much less tightly crosslinked than **1**, **2**, or **3**, having only 2% of its amine groups crosslinked with **4** (Fig. 1).

Antimicrobial activity of crosslinked hydrogels

In an initial screening, the antimicrobial activities of hydrogels **1, 3,** and **5** were determined by a standard micro-shake flask test using *E. coli* (Gram negative fermentative), *P. aeruginosa* (Gram negative nonfermentative), *S. aureus* (Gram positive), and *C. albicans* (fungus) as test organisms. Although unmodified (soluble) polyallylamine hydrochloride at 1 wt % killed only 75% of *E. coli* in 24 h, hydrogels **1** and **5** displayed complete (log 5) kill of all four test organisms within 4 h at a loading of only 0.1 wt % (Table I).

Figure 2 Synthesis of *N,N′*-bis(methoxycarbonylmethyl)-D-glucaramide (**4**).

TABLE I
Antimicrobial Activity of Crosslinked Hydrogels

Microbe	log CFU (control)	Hydrogel loading (wt %)	Hydrogel 1	Hydrogel 3	Hydrogel 5
			log kill[a]		
E. coli	4.86	1.0	4.86	3.49	4.86
		0.50	4.86	3.13	4.86
		0.25	4.86	3.16	4.86
		0.10	4.86	2.64[b]	4.86
P. aeruginosa	4.86	1.0	4.86	4.86	4.86
		0.50	4.86	4.86	4.86
		0.25	4.86	4.86	4.86
		0.10	4.86	4.86	4.86
S. aureus	4.66	1.0	4.66	0.72	4.66
		0.50	4.66	0.74	4.66
		0.25	4.66	1.15	4.66
		0.10	4.66	1.19[c]	4.66
C. albicans	5.09	1.0	5.09	5.09	5.09
		0.50	5.09	5.09	5.09
		0.25	5.09	5.09	5.09
		0.10	5.09	3.17	5.09

[a] After an exposure time of 4 h.
[b] After 24-h exposure, **3** at 1.0, 0.5, 0.25, and 0.10 wt % displayed a log kill of 4.86.
[c] After 24-h exposure, **3** at 1.0, 0.5, 0.25, and 0.10 wt % displayed log kill of 2.26, 3.29, 3.49, and 2.99, respectively.

The activities of **1** and **5** were equal to or greater than those of recently reported poly(*N*-isopropylacrylamide)/polyurethane[45] and cationic phosphonium terpolymer[31,32] hydrogels. In comparison to **1** and **5**, the partially long-chain alkylated hydrogel **3** was less active, particularly against the Gram positive bacterium, *S. aureus*. While the antibacterial activity of phosphonium terpolymer hydrogels has been shown to increase with increasing alkyl chain length,[31,32] that of amphiphilic tryptophanamide hydrogelators reached a maximum and then decreased as alkyl chain length increased.[46]

Speed of kill of crosslinked hydrogels

To further characterize the antibacterial activity of crosslinked polyallylamine hydrogels, the two most potent antimicrobial hydrogels, **1** (30% crosslinked with DET) and **5** (2% crosslinked with **4**), were tested for speed of kill using *E. coli* as the test organism. To explore the effect of crosslinking density, a more highly crosslinked analog of **1** was also tested. Hydrogel **2** had nominally 50% of the amine groups of polyallylamine crosslinked with DET. Of the three hydrogels, **5** proved to be the most potent, 10 ppm of which achieved complete (log 5) kill of *E. coli* within 30–60 min (Fig. 3). In addition, 100 ppm of **5** achieved complete (log 5) kill of *E. coli* within 15 min (data not shown). The bactericidal activity of hydrogel **5** is thus comparable to that of soluble polycationic poly(acrylamide-*co*-*N*-methyl-4-vinylpyridinium sulfate).[26] While crosslinking is required to impart significant antimicrobial activity to native polyallylamine, a lower degree of crosslinking and, perhaps, longer, more flexible crosslinks seem to maximize the antibacterial performance.

End-use application testing of crosslinked hydrogels

Finally, we examined the antimicrobial performance of crosslinked polyallylamine hydrogels **1** and **5** in a number of end-use application tests. These tests were quite demanding and highlighted the differences between shaker-flask assays in the laboratory and performance expectations in real-world applications.

Hard surface disinfection by crosslinked hydrogels

The ability of hydrogels **1** and **5** to disinfect hard surfaces was assessed using AOAC Use Dilution test methods 955.14 and 955.15. Briefly, stainless steel tubes that were 10-mm long, 1-mm thick and 8 mm in outer diameter were soaked for 15 min in a 48-h culture broth of *Staphylococcus aureus* or *Salmonella choleraesuis*, drained, dried, and then exposed for 10 min to an aqueous suspension of one of the hydrogel materials. The tubes were then treated so as to deactivate the antimicrobial hydrogel, and the presence of any remaining viable bacteria on the stainless steel tubes was determined by culturing experiments. The presence of residual bacteria was graded in a pass/fail manner.

Hydrogels **1** and **5** were tested as 0.5 and 1.0 wt % dispersions in deionized water, respectively. The two concentrations were chosen so as to give dispersions that had similar, nearly water-like viscosities, as would be desirable for a home or industrial hard surface disinfectant. Since a given mass of hydrogel **1** led to a more viscous dispersion than the same mass of hydrogel **5**, it was used at a lower loading.

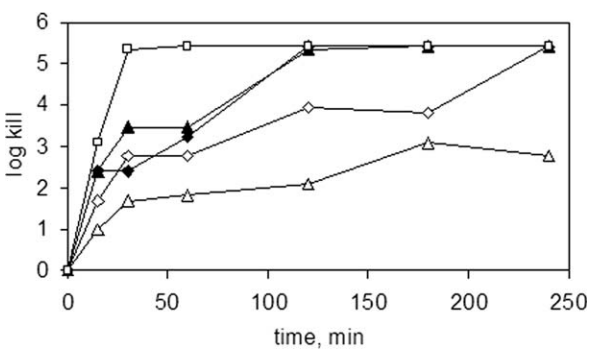

Figure 3 Speed of kill of *E. coli* when exposed to crosslinked polyallylamine hydrogels: 10 ppm **1** (◇), 100 ppm **1** (◆), 10 ppm **2** (△), 100 ppm **2** (▲), or 10 ppm **5** (□).

TABLE II
Use Dilution Test: Number of Penicylinders (Out of 10) Showing Residual Microbial Activity After a 10-Minute Exposure to an Aqueous Hydrogel Dispersion

		Test organism			
		Staphylococcus aureus		*Salmonella choleraesuis*	
		Culture experiment			
Hydrogel	wt %[a]	Primary	Secondary	Primary	Secondary
1	0.5	10	10	10	10
5	1.0	0	0	3	3

[a] Loading of hydrogel (w/v) in aqueous dispersion.

As can be seen in Table II, hydrogel **5** was bactericidal against *S. aureus*, giving 10 kills out of 10 at 1.0 wt % loading. While **5** was also active against *S. choleraesuis*, it did not completely eradicate viable *S. choleraesuis* under the conditions employed. Hydrogel **1** at 0.5% loading displayed no antibacterial activity in this rigorous assay. While a higher concentration of **1** might have been active, the resulting aqueous dispersion would have been too viscous for customary use as a hard surface disinfectant.

Preservation of a model skin cream formulation by crosslinked hydrogels

The ability of hydrogel **1** or **5** to preserve a personal care product against microbial contamination was assessed by formulating 1 wt % of **1** or **5** into a representative skin cream, inoculating the skin cream with one of five microorganisms, and assaying the resulting test samples over a 4-week period for the presence of viable microbes by standard culturing techniques. The microorganisms tested were *E. coli* and *P. aeruginosa* (Gram negative), *S. aureus* (Gram positive), *C. albicans* (yeast), and *Aspergillus niger* (mold). The ability of **1** or **5** to kill microorganisms in this test was complicated by the presence of the various skin cream ingredients, most of which are lipophilic or surfactant in nature, and the relatively low activity of water in the system.

As can be seen in Table III, the skin cream formulation itself (control with no hydrogel additive) was hostile to all of the microbes tested, resulting in a reduction or elimination of each of the microbial populations over the course of 4 weeks. Nevertheless, even at 1 wt % loading, **1** and **5** each accelerated the eradication of each of the microorganisms except for the mold, *A. niger*.

Hand sanitization by crosslinked hydrogel 5

Since hydrogel **5** showed the most rapid bactericidal activity (Fig. 3), it was examined as a potential hand sanitizer. The test required that an aqueous dispersion of **5** cause a $\geq$ 99% ($\geq$log 2) reduction of an originally 1×10^8 CFU mL^{-1} population of *Serratia marcescens* within a total of 5-min contact time on human skin. *Serratia marcescens* is a Gram-negative bacterium and human pathogen often found in bathrooms and involved in nosocomial infections, particularly catheter-associated bacteremia, urinary tract infections, and wound infections. A 0.8% dispersion of **5** in deionized water was tested since, when applied to human hands, it dried readily without feeling sticky or giving the sensation of leaving a residue.

As shown in Table IV, the 0.8% dispersion of **5** was an effective hand sanitizer. Despite several anomalously low baseline bacterial levels on hands before treatment, the aqueous dispersion of **5** caused a 99.7% (2.5 log) reduction in the numbers of *Serratia marcescens* present, thereby exceeding the expectation of 99% reduction for a hand sanitizer.

Human repeat insult patch test of crosslinked hydrogels

Before hydrogel **5** was tested as a hand sanitizer, it and hydrogel **1** were tested for their potential to cause

TABLE III
Survival of Microorganisms in a Skin Cream Formulation Containing 1 wt % of Hydrogel 1 or 5

	Test microorganism														
	S. aureus			*E. coli*			*P. aeruginosa*			*C. albicans*			*A. niger*		
	hydrogel additive														
	none	**1**	**5**	none	**1**	**5**	none	**1**	**5**	none	**1**	**5**	none	**1**	**5**
Day	log (CFU g^{-1})														
0	5.91	5.91	5.91	6.04	6.04	6.04	5.99	5.99	5.99	5.96	5.96	5.96	5.79	5.79	5.79
7	3.34	<1	<1	3.88	2.57	2.46	3.70	2.15	2.60	3.56	2.28	2.08	5.08	5.20	5.04
14	2.70	<1	<1	2.15	<1	<1	1.00	<1	2.43	2.67	<1	<1	5.04	5.18	4.98
28	<1	<1	<1	<1	<1	<1	<1	<1	<1	2.66	<1	<1	3.45	4.15	4.58

TABLE IV
Hand Sanitization by a 0.8 wt % Aqueous Dispersion of Hydrogel 5

	Bacteria recovered from hands (log CFU)			
	Before treatment		After treatment	
Subject	Right hand	Left hand	Right hand	Left hand
1	7.9	6.2	5.4	5.3
2	6.8	6.9	4.4	4.3
3	7.2	6.3	3.6	3.5
4	5.4	5.6	2.9	2.7
5	6.2	6.2	4.1	4.0
6	6.2	6.1	2.1	2.5
7	4.6[a]	4.6[a]	3.2	3.7
8	4.7[a]	4.6[a]	2.1	2.6
9	3.7[a]	4.5[a]	2.6	3.0
10	6.2	6.1	3.3	3.2
11	6.0	5.8	2.1	1.9
Mean	5.8		3.3	

[a] Anomalously low baseline (before treatment) bacterial recovery.

dermal irritation or allergic contact sensitization. Fifty-two subjects completing the study (12 men and 40 women, ages 22–79 years) were exposed nine times over a period of 3 weeks to a 0.5 or 0.8% aqueous dispersion of **1** or **5**, respectively. Each time during this induction period, the hydrogel was applied to the same spot of skin and left in contact for 24 h. Approximately 2 weeks after the final application during the induction period, a challenge patch of hydrogel dispersion was applied adjacent to the original site and left in contact for 24 h. Ten times during the induction period and twice after application of the challenge patch, subjects' skin was examined for erythema (reddening), edema (fluid accumulation), blistering, and ulceration and scored from 0 (no visible skin reaction) to 4 (severe erythema with possible edema, vesiculation, bullae, or ulceration). For both **1** and **5**, all scores were 0. In addition, five subjects who began the study but discontinued for various reasons not related to the test materials generated scores of only 0 as well. Thus, hydrogels **1** and **5** caused no dermal irritation or allergic contact sensitization under the test conditions used.

CONCLUSIONS

Crosslinking as few as 2% of the amino groups of polyallylamine hydrochloride resulted in a greater than 10,000-fold increase in its antimicrobial activity. The resulting hydrogels displayed inherent antimicrobial potencies and speeds of kill comparable to or greater than those reported previously for other hydrogels. The most potent hydrogel, **5**, was formed by crosslinking polyallylamine hydrochloride with *N*,*N*′-bis(methoxycarbonylmethyl)-D-glucaramide (**4**) and was active as a hard surface disinfectant, as a preservative in skin cream and as a hand sanitizer. It and hydrogel **1**, formed by crosslinking polyallylamine hydrochloride with diethyl L-tartrate, were found to be safe for use on human skin, causing no dermal irritation or allergic contact sensitization under the conditions of a human repeat insult patch test.

Crosslinked polyallylamine appears to function as a polycationic biocide.[47,48] The fact that hydrogel **5** is more active than hydrogel **3** indicates that a cation exchange mechanism of cell membrane disruption[49,50] is probably more important than penetration of the cell membrane by hydrophobic alkyl chains.[51] The difference in activity between the crosslinked polyallylamine hydrogels and native polyallylamine indicates that crosslinking is significant in imparting antimicrobial activity. However, the cause for this effect remains uncertain. Crosslinking may simply preorganize the polycationic environment, reducing degrees of motional freedom, and thereby enhance multivalent interactions with microbial surfaces. Crosslinking may create a porous structure that entraps microorganisms. The greater activity of **1** compared to **2** indicates that pore size and swellability need to be above a certain threshold for maximal activity. Finally, a crosslinked hydrogel may be more surface-like in character than the corresponding soluble polycationic polymer, and this surface-like character may be important in generating the microbial response that ultimately leads to its death. Further experimentation will be required to distinguish between these mechanisms of action.

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