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Gallium-loaded dissolvable microfilm constructs that provide sustained release of Ga^{3+} for management of biofilms

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Abstract

The persistence of bacterial biofilms in chronic wounds delays wound healing. Although Ga^{3+} can inhibit or kill biofilms, precipitation as $Ga(OH)_3$ has prevented its use as a topical wound treatment. We report the design of a microfilm construct comprising a polyelectrolyte film that releases non-cytotoxic concentrations of Ga^{3+} over 20 days and a dissolvable micrometer-thick film of polyvinylalcohol that enables facile transfer onto biomedically important surfaces. By using infrared spectroscopy, we show that the density of free carboxylate/carboxylic acid and amine groups within the polyelectrolyte film regulates the capacity of the construct to be loaded with Ga^{3+}, and that the density of covalent cross-links introduced into the polyelectrolyte film (amide-bonds) controls the release rate of Ga^{3+}. Following transfer onto the wound-contact surface of a biologic wound dressing, an optimized construct is demonstrated to release of ~0.7 μg cm^{-2}

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day^{-1} of Ga^{3+} over 3 weeks, thus continuously replacing Ga^{3+} lost to precipitation. The optimized construct inhibited formation of *P. aeruginosa* (two strains; ATCC 27853 and PA01) biofilms for up to 4 days and caused pre-existing biofilms to disperse. Overall, this study provides designs of polymeric constructs that permit facile modification of the wound-contacting surfaces of dressings and biomaterials to manage biofilms.

Keywords

microfilms; gallium nitrate; biofilm; polymer; wound dressing

1. Introduction

Bacteria in chronic wounds are thought to grow predominantly within biofilms, structured communities of bacteria encased by polymeric matrices.[1,2] Once established, biofilms are highly tolerant of antimicrobial treatments[3–5] due to restricted penetration of agents, the presence of slow-growing (persister) cells, and other mechanisms.[6] These factors make management of biofilms, in general, more challenging than planktonic (free floating) bacterial cells. Biofilms formed by *Pseudomonas aeruginosa* (*P. aeruginosa*), in particular, are notorious for causing persistent infections in cystic fibrosis patients,[7] on burns[8] and on chronic wounds,[9] and they have been suggested to play a role in the delayed healing of wounds.[10]

Recently, gallium (i.e. Ga^{3+}; 1–10 μM) has been shown to be effective both in killing planktonic cells[11] and in preventing biofilm formation by *P. aeruginosa*.[12,13] It has been proposed that Ga^{3+} interferes with Fe^{3+} metabolism, which is important for bacterial growth and biofilm formation.[12] Due to its chemical similarity with Fe^{3+}, Ga^{3+} is taken up by bacteria (instead of Fe^{3+}) through their binding to iron siderophores that are part of the bacterial iron uptake system. Unlike Fe^{3+}, however, Ga^{3+} cannot be reduced under physiological conditions to Ga^{2+}. The binding of Ga^{3+} to the Fe^{3+} protein binding sites results in blockage of redox-driven biological processes that are important for bacterial growth and biofilm formation.[12] Ga^{3+} (100–1000 μM) has also been shown to kill established biofilm cells with bacteria within the inner layer of the biofilms being more susceptible due to the limited Fe^{3+} availability in this region.[12,14]

Due to its ability to inhibit biofilm formation and kill established biofilm cells, Ga^{3+} has been proposed as a topical wound treatment.[15] However, the direct application of Ga^{3+} [in the form of a salt, such as gallium nitrate, $Ga(NO_3)_3$] is not effective for management of biofilms in a wound environment because of two key issues: (i) Ga^{3+}-associated cytotoxicity (>36 μM) to mammalian cells, such as Balb/c 3T3 fibroblasts[16,17] and (ii) the tendency of Ga^{3+} to form insoluble precipitates [$Ga(OH)_3$] under physiological conditions, which reduces its bioavailability[18] and therefore its effectiveness in inhibiting biofilm formation (Figure S1). To address the issue of bioavailability, gallium solutions are usually prepared with complexing agents, such as citrate and maltolate[19,20] that can chelate Ga^{3+} to prevent precipitation. Although Ga^{3+} stability is improved by using complexing agents, toxicity associated with Ga^{3+} (as a therapeutic agent) remains an unresolved problem.[21] Here we

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propose an alternative strategy to manage biofilm formation on wounds by functionalizing the surfaces of biologic wound dressings with polymeric constructs that provide sustained release of non-cytotoxic concentrations of Ga^{3+}. The continuous release of Ga^{3+} replenishes the Ga^{3+} lost due to precipitation, thereby maintaining antibiofilm activity for a sustained duration (>24h). Because the management of established biofilms is particularly challenging, in this study, we also evaluated the effectiveness of our surface functionalization strategy for dispersion of pre-existing biofilms.

The approach described in this paper is based on the fabrication of a microfilm construct that comprises a nanoscopic polyelectrolyte layer containing Ga^{3+} that is integrated with a micrometer-thick (mechanically robust) sacrificial film comprised of polyvinylalcohol. Specifically, as shown in Figure 1, we demonstrate that the construct can be used to functionalize biological wound dressings (Biobrane®) with polymeric nanoporous films containing $Ga(NO_3)_3$ to confer antibiofilm activity on the wound dressings. The polyelectrolyte nanofilms used in our study are comprised of multilayers of poly(allylamine hydrochloride) (PAH) and poly(acrylic acid) (PAA). We hypothesized that the polyeletrolyte multilayers (PEMs) could serve as nanometer-thick reservoirs for spatial confinement and localized delivery of Ga^{3+}. We note that previously we reported the use of PEMs loaded with silver nanoparticles to release non-cytotoxic loadings of Ag^{+} ions that killed planktonic bacteria in *in vitro*[22,23] and *in vivo* studies[24,25]. In this study, we move to explore ways to manage bacterial biofilms based on the localized delivery of Ga^{3+} from PEMs. The fabrication of PEMs that release Ga^{3+} required that several materials-related challenges be addressed. In particular, we sought to understand how the chemical functionality of the polyelectrolytes could be exploited to tune the loading of Ga^{3+} achievable within the nanofilms, and how subsequent cross-linking of the PEMs could be used to tune the rate of release of the Ga^{3+}. Specifically, we predicted that a sustained local release of Ga^{3+} would exert antibiofilm activity without inducing local cytotoxicity or undesired gallium oxide precipitates.

As noted above, we transferred the cross-linked nanoporous Ga^{3+}-loaded PEMs onto the surfaces of biologic wound dressings using a sacrificial, water-soluble poly(vinyl alcohol) PVA support layer.[24] The PEMs were not synthesized directly on the Biobrane because the harsh conditions required to fabricate the PEMs (e.g. low pH (pH 2.5) for $Ga(NO_3)_3$ solution and T ~ 215°C for cross-linking the Ga^{3+}-loaded PEMs) will cause damage to the biological components (e.g., collagen) of the dressings.

2. Results

2.1. Fabrication of PEMs containing $Ga(NO_3)_3$

Gallium ions have not previously been incorporated into polyelecrotrolyte films, and thus the first challenge we addressed was to establish design rules that would permit use of the chemical functionality of the polyelectrolytes to control the loading of Ga^{3+}. Past studies have reported that Ga^{3+} interacts with ligands containing amine[26,27] and/or carboxylate/carboxylic functional groups[28,29] to form chelate complexes with the oxygen and/or nitrogen atoms of the ligands.[30] Thus, we hypothesized that Ga^{3+} might be incorporated into PEMs by coordination of Ga^{3+} to unbound COOH groups and/or NH_3^{+} groups. In an

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acidic environment, PEMs of PAA and PAH are known to reorganize to form microporous[31] or nanoporous[32] films and to generate 'free' or unbound NH_3^+ and COOH groups.[33] Thus we focused on this class of PEMs. We note, in addition, that when Ga^{3+} salts such as gallium nitrate ($Ga(NO_3)_3$) are dissolved in water, the solution becomes acidic due to hydrolysis (i.e., the Ga^{3+} ions coordinate hydroxide ions, thus producing hydronium ions as one of the products).[18] Thus we used the acid nature of Ga^{3+} solutions to generate unbound NH_3^+ and COOH groups within the above-mentioned PEMs.

To test the above design, we first measured the extent of incorporation of Ga^{3+} within PAH and PAA multilayers: $(PAH/PAA)_n$, where n is the number of bilayers. The PEMs were deposited layer-by-layer on poly(dimethylsiloxane) (PDMS) sheets from solutions of PAH (adjusted to pH 7.5) and PAA (adjusted to pH 5.5). The growth of the PEMs was confirmed by measurements of ellipsometric thicknesses. Post-assembly, the PEMs were incubated against solutions of gallium salts ($Ga(NO_3)_3$) at pH ~ 2.5.[18] The PEMs were subsequently rinsed 3× with distilled water (pH ~5–6; 15 s each) and dried under a stream of N_2. The incorporation of Ga^{3+} within the PEMs was determined by ICP-OES (see Experimental Section). Figure 2a shows that the Ga^{3+} loading within the PEMs (solid line) increased linearly with the number of bilayers (n), with concomitant increase in ellipsometric thickness of the PEMs (dashed line). Thus, a key result that emerges from this first experiment is that Ga^{3+} can be incorporated into the PEMs, and that the loading of Ga^{3+} within PEMs of PAH and PAA can be tuned by adjusting the number of bilayers within the PEMs.

Next, we sought to provide insight into the physical processes that determined the degree of loading of Ga^{3+} within the PEMs. We first investigated the possible influence of the pH-induced reorganization of the PEMs[33] on the loading of Ga^{3+}. Specifically, we characterized the uptake of Ga^{3+} into PEMs of PAH and PAA that were prevented from undergoing reorganization by cross-linking prior to loading of Ga^{3+}. The cross-linking was achieved by heating the as-assembled films at 215°C for 2 h under nitrogen. The cross-linking arises from amide (-NHCO-) bond formation between PAA and PAH,[34] a reaction that we confirmed to take place by using PM-IRRAS (Figure S2). The as-assembled PEMs and the cross-linked PEMs with 40.5 bilayers were then exposed to aqueous $Ga(NO_3)_3$ as described previously. As shown in the first two bars of Figure 2b, the loading in the as-assembled PEMs was 15.6 ± 0.3 μg cm^{-2} whereas less than 0.1 μg cm^{-2} was loaded into the PEMs that had been cross-linked prior to immersion in $Ga(NO_3)_3$ solution. As noted above, the reorganization involves the formation of nano- or micro-pores[31,32] and the generation of unbound NH_3^+ and COOH groups.[33] Therefore, the low loading of Ga^{3+} in the cross-linked PEMs may arise from either the absence of the porous morphology or the absence of chemical functionality (i.e. unbound NH_3^+ and COOH groups) that can bind to Ga^{3+}.

As shown in Figure S3, by using electron microscopy, we observed nanoporous structures (pore sizes of 50–200 nm) within Ga^{3+}-loaded PEMs. Hence, we explored further the hypothesis that the morphology (i.e. nanoporosity) of the PEMs, as generated by exposure to acidic conditions influenced the amount of Ga^{3+} incorporated into the PEMs. Specifically, we determined if Ga^{3+} salts were incorporated by trapping of the Ga^{3+} solution within the pores of the PEMs. Two sets of porous PEMs (40.5 bilayers) were made prior to immersion

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in $Ga(NO_3)_3$ solutions. The first set was pre-treated with HCl solution (pH = 2.5) for 1 min followed by 15 s water rinse.[31] The second set was pre-treated at pH 2.5/rinsed (similar to first set) but then cross-linked to stabilize the porous structure against additional rearrangement during subsequent immersion in the $Ga(NO_3)_3$ solution. Cross-linking of the acid-treated (porous) PEMs was confirmed by PM-IRRAS (Figure S2). Inspection of the last two bars in Figure 2b reveals that the loading of Ga^{3+} in the porous (but not subsequently cross-linked) PEM was 13.8 ± 0.3 μg cm^{-2} whereas less than 0.1 μg cm^{-2} was loaded into the porous PEM that was cross-linked prior to immersion into the $Ga(NO_3)_3$ solution. From this experiment, we conclude that morphology (porosity) is not the key factor determining the Ga^{3+} uptake into the PEMs, but rather the chemical functionality of the PEMs (available COOH and NH_3^+ groups) plays a central role.

The results above suggest that the availability of Ga^{3+} binding groups in the PEMs plays a key role in influencing the uptake of Ga^{3+} into the PEMs. Our next set of experiments was designed to identify these binding sites. To this end, PEMs containing 11 bilayers of PAA and PAH were fabricated on gold-coated silicon wafers. Ga^{3+} was loaded into the PEMs by immersing the PEMs into $Ga(NO_3)_3$ solutions (pH ~2.5). PEMs treated with HCl solution (pH~2.5) were used as a control. Following immersion in the Ga^{3+} solutions, the films were air dried (not rinsed) and characterized by PM-IRRAS. Figure 2c shows PM-IRRAS spectra of the PEMs, as assembled (purple), acid-treated (black), and Ga^{3+}-loaded (red).

Inspection of Figure 2c leads to four conclusions. First, treatment of as-assembled PEMs with acid converted the COO^- groups (that were electrostatically linked to NH_3^+ of PAH in as-assembled PEMs) to COOH, as evidenced by the diminished asymmetric and symmetric COO^- bands (at 1575 cm^{-1} and 1404 cm^{-1},[35] respectively) and enhanced COOH bands at 1715 cm^{-1}[36] (in comparison to the spectrum of the as-assembled PEMs). The formation of COOH groups in acid-treated PEMs is also accompanied by generation of unbound NH_3^+ groups at 1563 cm^{-1},[37] consistent with prior reports.[33,38] Second, for Ga^{3+}-loaded PEMs, we observed an interaction between Ga^{3+} and unbound carboxylic acid/carboxylate groups as seen by the shifts of COOH and symmetric COO^- bands to higher wavenumbers (~15 cm^{-1}) as compared to the bands of acid-treated PEMs. A similar spectroscopic shift has been reported in past studies using Ga^{3+} chelating agents (e.g., citrate).[29] Third, nitrate (NO_3^-) is also incorporated into Ga^{3+}-loaded PEMs, as seen by the appearance of a NO_3^- peak at 1349 cm^{-1}.[39] Consistent with this conclusion, XPS results obtained using Ga^{3+}-loaded PEMs (Figure 2d) also show a N1s peak (407 eV) consistent with NO_3^- ions.[40] Fourth, we observed an interaction between Ga^{3+} and unbound amine/ ammonium groups as evident from the appearance of an NH_3^+ band at 1528 cm^{-1} and an NH_2 band at 1621 cm^{-1} for the Ga^{3+}-loaded PEMs (these peaks are shifted to a lower wavenumbers with respect to the peaks in acid-treated PEMs). Additional observations of (i) a N-H stretching peak at 3210 cm^{-1} (attributed to coordinated amines)[41] (Figure S4) and (ii) a NH_2 peak at 399 eV in our XPS measurements (for Ga^{3+}-loaded PEMs, but not for as-assembled PEMs or acid-treated PEMs), also support our conclusion that coordination interactions occur between Ga^{3+} and NH_2 groups in the PEMs.[26,27]

Whereas the PEMs characterized above were not rinsed (to maximize the signal arising from the interactions of the Ga^{3+} ions with the polyelectrolytes), following incubation with

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$Ga(NO_3)_3$, the PEMs used in our biofilm experiments (see below) were rinsed with water to remove excess $Ga(NO_3)_3$. Examination of PM-IRRAS spectra of rinsed samples (Figure S4 and S5) revealed that some COOH groups in gallium nitrate-loaded PEMs are deprotonated to COO^- groups by the rinsing process (and re-form ionic bonds with NH_3^+). Nevertheless, evidence of interaction of the remaining Ga^{3+} with COO^-/COOH[28] and NH_2[26,41] groups within the PEMs can still be seen in the spectra (as detailed above). Finally, we comment that by decreasing the pH of the $Ga(NO_3)_3$ solution to 2.0, the number of available Ga^{3+} binding ligands (unbound COO^-/COOH and NH_2/NH_3^+) could be increased, which we observed to lead to an increase in the loading of Ga^{3+} within the PEMs (see Figure S5). This result provides further support for our conclusion regarding the role of the chemical functional groups of the polyelectrolytes in determining the loading of Ga^{3+}. More broadly, these results establish design rules for controlling the loading of Ga^{3+} into thin organic films.

2.2. $Ga(NO_3)_3$ release kinetics and antibiofilm activity of Ga^{3+}-loaded PEMs

Because past studies have demonstrated that Ga^{3+} is toxic to mammalian cells (e.g. fibroblast and keratinocytes) at high concentrations (36–100 μM),[16,18] we first determined the minimum concentration of Ga^{3+} (in solution) that inhibited biofilm formation by *P. aeruginosa* ATCC 27853. Figure S6 shows that addition of 6.3 μM of $Ga(NO_3)_3$ to solution is effective in inhibiting bacterial biofilm formation within 24 h, and the nitrate counter ions do not contribute to the inhibition of biofilm formation by $Ga(NO_3)_3$ These result suggest that PEMs releasing ~6.3 μM of $Ga(NO_3)_3$ into solution should exert antibiofilm activity without inducing potential cytotoxicity *in vitro*.

Next, we measured the release of Ga^{3+} from PEMs of $(PAH/PAA)_n$, with n = 6.5, 8.5, 10.5 (assembled on PDMS sheets with loadings of 0.9 ± 0.1, 1.9 ± 0.1, and 2.8 ± 0.2 μg cm^{-2}, respectively) into PBS (pH ~ 7.4). In these initial experiments, the PEMs were not cross-linked (see Section 2.3 below for studies at cross-linked PEMs). Figure 3a shows that a significant fraction (0.4 ± 0.01, 1.4 ± 0.1, 1.9 ± 0.1 μg cm^{-2} or 46 ± 1, 71 ± 2, 69 ± 2%) of the Ga^{3+} within the PEMs was released quickly (within 2 h) and that almost all (0.8 ± 0.03, 1.7 ± 0.04, 2.5 ± 0.1 μg cm^{-2} or 83 ± 4, 88 ± 2, 88 ± 2 %) of the Ga^{3+} was released in 24 h (see below for comments regarding the concentrations of Ga^{3+} generated by these PEMs).

To determine if the Ga^{3+} released from the above-described PEMs into solution was active against biofilm formation, PEM-coated PDMS sheets (d=6 mm, Ga^{3+} loadings = 0.9 ± 0.1, 1.9 ± 0.1, and 2.8 ± 0.2 μg cm^{-2}) were incubated against 0.5 mL suspensions of *P. aeruginosa*. Biofilms were stained using crystal violet (CV) dye and quantified using a plate reader (see Supplementary Information for details). Specifically, we exposed PEM-coated PDMS disks containing Ga^{3+} to fresh cultures of bacteria over a two day period: 1st incubation (over 0–24 h) and 2nd incubation (over 24–48 h), with the disks washed 3× using sterile PBS between each incubation period. When released into a volume of 0.5 mL, we note that the concentrations of Ga^{3+} generated by these PEMs within the first and second incubations periods are 6 ± 0.3 – 20 ± 0.5 μM and 0.7 ± 0.2 – 1.1 ± 0.1 μM, respectively, all of which are below the cytotoxic thresholds for mammalian cells (36–100 μM). Inspection of Figure 3b reveals that Ga^{3+}-loaded PEMs (loadings of 0.9 ± 0.1 – 2.8 ± 0.2 μg cm^{-2}) are

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effective at inhibiting biofilm formation during the first 24-h incubation, but are unable to inhibit biofilm formation during the second 24-h incubation (see also Figure S7 and S8). Although this result demonstrates that the Ga^{3+} released from the polyelectrolyte film is an active antibiofilm agent, additional results support prior conclusions that its activity is transient due to precipitation of $Ga(OH)_3$. Specifically, we measured a decreased antibiofilm efficiency of precipitated $Ga(NO_3)_3$ solution, when compared to the non-precipitated solution (Figure S1). While the burst release of the Ga^{3+}, as described above leads only to transient activity (insufficient in duration for a biological wound dressing), we note that in some clinical contexts such transient activity of a surface may be useful.

We also comment that the results obtained above were based on the use of a CV stain. In addition to the measurement of biofilm biomass using CV stain, we also characterized bacterial colonization by (i) quantifying the colony forming units (CFU) of surface attached-bacteria and (ii) by imaging of fluorescent (Syto®9)-stained surface-attached bacteria following a 48h-incubation of PDMS in a bacterial suspension (see Supplementary Information for details). Figure S7 and S9 confirm that Ga^{3+} released from the PEM prevented formation of bacterial biofilms (note that it also shows that Ga^{3+} does not prevent planktonic cells from replicating and attaching on surfaces). Significantly, however, by comparing the results from the CV and CFU methods, we have validated that CV permits quantification of the amount of bacterial biofilms. For example, CV data shows that after 48 h, PEMs containing 0.9 ± 0.1 μg cm^{-2} Ga^{3+} have 87 ± 2 % less biofilm biomass than PEMs containing no Ga^{3+}. Similarly, CFU data shows that the Ga^{3+}-loaded PEMs have 87 ± 4 % (1 log_{10}) less attached viable bacteria than PEMs containing no Ga^{3+}. Guided by this result, we used the CV stain in the subsequent experiments presented in this paper.

2.3. Biofilm inhibition using Ga^{3+} released from cross-linked PEMs

Figure 3 led us to hypothesize that the release profile of Ga^{3+} from the PEMs needed to be extended to durations longer than 24 h (i.e. for multiple days) in order to sustain antibiofilm activity. Our approach to achieve this goal was guided by past studies that have shown that small hydrophobic drugs can be released from cross-linked nanoporous multilayer films with zero-order release kinetics.[42] Therefore, we hypothesized that the burst release of Ga^{3+} shown in Figure 3a might be slowed by cross-linking of the PEMs. To test this hypothesis, we thermally cross-linked the Ga^{3+}-loaded PEMs by heating the PEMs at 215 °C for 2 h. Spectroscopic measurements confirmed the formation of amide bonds between the PAA and PAH within Ga^{3+}-loaded PEMs (Figure S2). To determine if the high temperature associated with the cross-linking process impacted the amount of Ga^{3+} that could be recovered from the PEMs, we characterized the loadings of Ga^{3+} before and after the cross-linking step. Figure S10 shows that the Ga^{3+} recovered from the PEMs was not changed by cross-linking. Next, we characterized the Ga^{3+} release profile from PEMs of $(PAH/PAA)_{60.5}$ loaded with 24.5 ± 0.4 μg cm^{-2} of Ga^{3+}, with and without cross-linking. Here we note that the higher loading of Ga^{3+} (as compared to that reported earlier in this paper) was used to prolong the release of Ga^{3+} over two weeks (the time it takes for *in vivo* model wound beds, reported previously,[24] to close completely). As seen in Figure 4, the amount of Ga^{3+} released from the PEMs without cross-linking and PEMs with cross-linking after 24 h were 22.7 ± 0.4 and 2.5 ± 0.1 μg cm^{-2}, respectively. In other words, within 24 h,

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94 ± 1% of Ga^{3+} was released from PEMs without cross-linking whereas only 10 ± 1 % of Ga^{3+} was released from the cross-linked PEMs. Figure 4 also shows that the amounts of Ga^{3+} released from the cross-linked PEMs over 3 days following the initial 24 h incubation period were 0.9 ± 0.1, ~0.7 ± 0.1, and ~0.7 ± 0.2 μg cm^{-2} day^{-1}, respectively. Over the subsequent 19 days, the cross-linked Ga^{3+}-loaded PEMs provided a sustained zero-order release of ~0.7 μg cm^{-2} day^{-1}. We note that PM-IRRAS revealed that the amide peak of the cross-linked Ga^{3+}-loaded PEMs did not change during a 4-day incubation in water (shown as Figure S11), indicating the absence of measurable hydrolysis of the amide bonds during this period.

In clinical settings, biofilms on wounds are typically removed by physical methods (i.e. wound debridement to surgically remove the biofilms along with necrotic host tissue) so that wounds can progress beyond the inflammatory stage toward healing.[43] However, debridement of topographically complex wound surfaces can be a challenge and fragments of biofilms may persist. It has also been shown that even following debridement, residual bacteria-embedded biofilms can re-attach on wounds creating bacterial microstructure within 24 hours and forming mature biofilms within 3 days.[44] Motivated by the need for protection against recurring biofilm formation and to provide a tool to augment surgical removal of biofilms, here we assess the potential utility of the cross-linked PEMs to inhibit biofilm formation over an extended period. Specifically, we successively inserted and removed PEMs (with or without cross-linking) into four fresh cultures of bacteria over a four day period (see Experimental Section for details) to evaluate impacts on biofilms. Figure 5 shows that PEMs with loadings of Ga^{3+} of 24.5 ± 0.4μg cm^{-2} (without cross-linking) inhibited biofilm formation by *P. aeruginosa* ATCC 27853 on adjacent uncoated surfaces for two successive 24-h inoculations, but failed to inhibit biofilm formation for the next two 24-h inoculations. In contrast, Ga^{3+}-loaded PEMs with cross-linking inhibited biofilm formation against four inoculations over four days. Similar long term antibiofilm activities of cross-linked Ga^{3+}-loaded PEMs against another strain of *P. aeruginosa* (PA01) can be seen in Figure S12. When released into a volume of 0.5 mL, we note that the concentrations of Ga^{3+} generated by the above-described cross-linked PEMs (d = 6mm) over the four incubations are 19.8 ± 1.0, 7.0 ± 0.6, 6.0 ± 0.9, and 5.6 ± 1.4 μM. These concentrations are consistent with a result reported earlier in this paper – namely that 6.3 μM was effective in inhibiting bacterial biofilm formation of *P. aeruginosa* over 24 h (Figure S6a). Importantly, these Ga^{3+} concentrations are below the concentration reported to be toxic to Balb/c 3T3 fibroblasts (36 –100 μM).[16,17] Finally, we note that between each inoculation of bacteria in the experiments shown in Figure 5, the surfaces were rinsed with PBS. As shown in Figure 5, the biofilm biomass on the surfaces of wells in a control group (samples without Ga^{3+}) was measured to steadily increase even when we performed the PBS rinse between each inoculation. For this reason, we do not expect the PBS rinsing to interfere with biofilm formation.

2.4 Surface functionalization of a biologic wound dressing using gallium-loaded dissolvable microfilm constructs

Motivated by the challenges of managing biofilms on burn and chronic wounds,[45] we next modified the surface of a biosynthetic dressing (Biobrane®) using Ga^{3+}-loaded PEMs.

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Biobrane® is a biosynthetic dressing composed of a silicone film impregnated with nylon that has been coated with porcine collagen. It is widely used in hospitals to treat burn wounds and promote tissue growth.[46] Because biologic dressings are particularly susceptible to infection,[47] and in turn, biofilm development, we used Biobrane as a model wound-contacting surface at which we aimed to demonstrate the efficacy of Ga^{3+}-loaded PEMs for control of biofilms.

We integrated the cross-linked Ga^{3+}-loaded PEMs (shown to be effective in inhibiting biofilm formation) onto the wound-contact surface of Biobrane using a dissolvable microfilm, reported previously for Ag-nanoparticle-containing PEMs.[24] Briefly, to assemble the microfilms, aqueous solutions of PVA were spin-coated over Ga^{3+}-loaded PEMs supported on PDMS sheets and then the microfilm construct was dried at 70°C for 5 min. After drying, the composite PEM/PVA microfilm was peeled from the PDMS surface and placed on the surface of pre-moistened Biobrane®, with the PEMs facing the surface of Biobrane (see Experimental Section for details). The moisture on the Biobrane dissolved the PVA component of the microfilm leaving the nanoscopically-thick PEM adhered to the surface of Biobrane. We confirmed transfer of the PEM onto the Biobrane using ICP-OES to quantify the loading of Ga^{3+} on the Biobrane. Figure 6a shows the loadings of Ga^{3+} (i) within the PEM/PVA microfilm on the PDMS sheet, (ii) on the PDMS substrate after peeling of the PEM/PVA microfilm from the PDMS sheet, (iii) within the peeled microfilm, and (iv) on the Biobrane after transfer of the microfilm. These results indicate that most (98 ± 3%) of the Ga^{3+} within the PEMs on the PDMS support was retained in the free-standing PEM/PVA microfilms, and most of the Ga^{3+} in the microfilm (98 ± 4%) was transferred onto Biobrane.

Following validation of transfer of the Ga^{3+}-loaded PEMs onto Biobrane, we tested the functionalized Biobrane for its ability to inhibit biofilm formation. In brief, we sequentially exposed the functionalized Biobrane to four fresh cultures of bacteria over a four day period (see Experimental Section for details). As shown in Figure 6b, we measured the modified Biobrane to be effective in inhibiting biofilm formation for all four successive 24-h inoculations. These results demonstrate that the Ga^{3+}-loaded PEMs provided a sustained release of Ga^{3+} that protected the biologic dressing against biofilm formation.

2.5. Dispersal of pre-formed biofilms from biologic dressings

Once established, biofilms are known to be difficult to eradicate.[6,45] Specifically, a past study reported that biofilm communities become increasingly resistant to antibiotics as the biofilm matures.[48] Hence, strategies for biofilm management not only need to be developed for prevention of biofilm formation, but also for eradication of mature biofilms. To determine the concentrations of Ga^{3+} needed to disperse pre-existing biofilms in our system, we performed a biofilm dispersion assay using a microtiter plate. Following maturation of biofilms for 48 h, fresh media containing different concentrations of Ga^{3+} (in citrate buffer to maintain Ga^{3+} stability) were added for an additional 24, 48, or 72 h (see Supplementary Information for details). Figure 7a shows that treatment with Ga^{3+} (12.5 – 100 μM) for 24 h does not disperse the pre-existing biofilms, while 12.5 μM and 50 μM Ga^{3+} treatment for 72 h is able to disperse biofilms with an efficiency of 52 ± 11 % and 79 ± 9 %, respectively.

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The results reported above indicate that Ga^{3+} treatment needs to be sustained for more than 24 h to achieve significant dispersion of pre-existing biofilms. Thus, we investigated if Ga^{3+} that was released from cross-linked PEMs loaded with 24.5 ± 0.4 μg cm^{-2} of Ga^{3+} (with a sustained release profile characterized in Figure 4 and shown to be effective in inhibiting biofilm formation as seen in Figure 5 **and** 6) was able to disperse pre-existing biofilms that were formed on the wound-contact surface of biologic dressings (Biobrane). Briefly, the biofilms were first allowed to form on the biological dressing for 48 h. Next, PEMs with or without Ga^{3+} (supported on PDMS sheets) were placed on top of the Biobrane with the PEM surface in contact with the Biobrane. Following treatment, the biofilm remaining on the surface of the Biobrane samples was quantified (see Experimental Section for details) (Figure 7b). After a 24-h incubation period, we measured no significant dispersal of pre-existing biofilms on the Biobrane. However, after a 48- or 72-h incubation, cross-linked Ga^{3+}-loaded PEMs caused dispersal of 82 ± 4 % and 87 ± 4 % of the pre-existing biofilms, respectively. In contrast, Ga^{3+}-loaded PEMs (without cross-linking) were not able to cause significant dispersal after 48 h and permitted additional biofilm growth. These results demonstrate that sustained release of Ga^{3+} from cross-linked PEMs (but not burst release) is able to disperse biofilms pre-existing on biologic dressings *in vitro*.

3. Discussion

This study presents a materials-oriented approach for management of biofilms that uses a microfilm construct to enable the functionalization of surfaces with cross-linked PEMs containing Ga^{3+}. Our approach results in the delivery of Ga^{3+} in a sustained manner (~0.7 μg cm^{-2}/day) over a prolonged duration (20 days). The overall significance of the sustained release of the Ga^{3+} is that it allows replenishment of Ga^{3+} that is lost from solution due to precipitation as $Ga(OH)_3$ in neutral pH solutions.

We show that Ga^{3+}-loaded microfilms that provide a sustained release of Ga^{3+} can (i) inhibit biofilm formation for up to 4 days and (ii) disperse pre-existing biofilms present on a biologic wound dressing. Past studies have described the use of chelating agents as the basis of an approach aimed at achieving the sustained presence of Ga^{3+} (i.e., minimizing loss of Ga^{3+} via formation of a precipitates of $Ga(OH)_3$), such as the use of citrate,[19] deferoxamine,[14] or maltolate.[20] In those studies, however, it was reported that a Ga^{3+} concentration of more than 1000 μM was required to kill bacteria in pre-existing biofilms.[14] This concentration exceeds the reported *in vitro* cytotoxicity limit (36 μM) for fibroblasts.[16,17] In contrast, by releasing the Ga^{3+} continuously from the cross-linked PEMs, we show that it is possible to both inhibit biofilm formation and disperse existing biofilms using concentrations of Ga^{3+} (~6 μM was released into 0.5 mL solution from PEMs with 6 mm diameter) that are below the cytotoxicity limits. We note that the Ga^{3+} concentration that is effective at dispersing existing biofilms (6 μM, continuously released from PEMs), as reported in this study, is approximately 100× less than the Ga^{3+} concentration needed to kill existing biofilms reported by Banin et al. (1000 μM, in solution).[14] Banin et al did not, however, report the concentration of Ga^{3+} required in their system for dispersal of biofilms. In addition, we note that the use of Ga^{3+}-loaded microfilms to manage *P. aeruginosa* biofilms (as presented in this study) is potentially advantageous relative to commercially available antibiotics since bacterial biofilms formed by *P.*

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aeruginosa do not respond to many antibiotic treatments (i.e. enrofloxacin, gentamicin, ampicillin, oxytetracycline, and trimethoprim/sulfadoxine).[49] In addition, whereas bacterial resistance can develop against conventional antibiotics, the effectiveness of Ga^{3+} (which binds iron-scavenging siderophores that are secreted and shared among bacteria) is retained over time.[50] Another key outcome of our study is the demonstration of a process that permits the integration of Ga^{3+}-loaded PEMs onto the wound-contacting surface of the biologic wound dressing (Biobrane®). This was achieved by prefabricating the PEMs on PDMS sheets and subsequently transferring the PEMs to Biobrane using water-dissolvable poly(vinyl alcohol) (PVA) microfilms.[24] The successful transfer of the Ga^{3+}-loaded PEMs using PVA microfilms is significant because PVA microfilms permit the transfer of PEMs onto a wide range of biomedically relevant interfaces including wound dressings and tissues. The PVA microfilms are easily handled and thus potentially offer the basis of an easy and facile procedure by which clinicians can place Ga^{3+}-loaded PEMs onto biofilm contaminated wounds to treat persistent infections. A key merit of this approach to management of biofilms is that the sustained release of Ga^{3+} from the functionalized wounds potentially circumvents the need for clinicians to apply daily Ga^{3+} solutions to treat biofilms on wounds, thereby preventing further disturbance of wounds (including wounds that have been sealed with biologic dressings). In addition, the ability of the functionalized surface to continuously exert antibiofilm activity when the surface was challenged repeatedly with fresh bacteria inoculum (up to four times) suggests that this approach can be potentially used to manage recurring biofilm formation that is often reported on wounds.[44] The successful fabrication of PEMs that released Ga^{3+} for 20 days at concentrations that inhibited and dispersed biofilms, as described above, required the development of methods that permitted the loading of Ga^{3+} into PEMs. Past studies have reported the loading of small hydrophobic drugs into PEMs via trapping of the drugs within the pores of the PEMs.[42] In contrast, our results indicate that following immersion of PEMs under acidic conditions, free COOH and NH_3^+ groups were generated within the PEMs and that these free chemical functional groups of the PEMs plays a central role in binding Ga^{3+}. Furthermore, we also note that there are key differences in the methods and mechanism of incorporation of Ga^{3+} into PEMs versus the incorporation of other metal ions/metals, such as Ag^+/AgNPs, as has been reported in a previous study.[24] In particular, Ag^+ is incorporated into PEMs (of PAA and PAH) by incubation in an $AgNO_3$ solution at pH 5–6. During incubation, Ag^+ ions exchange with protons of the COOH groups of the PAA in the PEMs resulting in carboxylate-bound Ag^+ ions. In contrast, Ga^{3+} is incorporated into the PEMs by incubation in an acidic (pH ~2.5) $Ga(NO_3)_3$ solution, whereby Ga^{3+} binds with the available COOH and NH_3^+ groups that are generated in acidic conditions. As a result, the loading of Ga^{3+} within PEMs can be increased by decreasing the pH of the incubating solution (e.g., from pH 2.5 to 2.0, see Figure S5).

4. Conclusion

A key conclusion of the study reported in this paper is that nanometer-thick polymeric films (PEMs) containing controlled loadings of Ga^{3+} can be fabricated and transferred using PVA microfilms onto biomedically-relevant surfaces to provide sustained release (over 20 days) of concentrations of Ga^{3+} that are effective at inhibiting formation of biofilms as well as

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dispersing existing biofilms. The study focused on biofilms formed by two strains of *P. aeruginosa* because they are notorious for causing persistent infections in burns and in chronic wounds (see Introduction for details). Two key materials-related challenges were addressed in the context of fabrication of the PEMs used in the above-described microfilm construct (i) the development of methods that permitted the loading of the PEMs with Ga^{3+}, and (ii) the post-processing of the Ga^{3+}-loaded PEMs to achieve sustained release of the Ga^{3+}. Specifically, we used infrared spectroscopy to establish that the Ga^{3+} was taken into the nanofilms by coordination interactions between Ga^{3+} and free COO^{-}/COOH and NH_2/NH_3^{+} groups within the PEMs that were generated upon exposure of the PEMs in acidic solutions of $Ga(NO_3)_3$. We also determined that the release rates of Ga^{3+} from the PEMs could be tailored by thermal cross-linking the PEMs to provide a sustained, zero-order release over almost 3 weeks. This latter accomplishment is particularly significant because we observed the sustained release of Ga^{3+} from the PEMs to be necessary for both long-term prevention of biofilm growth and for dispersing of pre-existing biofilms formed on biological dressings. Because the cross-linking of the PEMs involved the use of high temperatures, we used PVA microfilms to transfer pre-fabricated Ga^{3+}-loaded cross-linked PEMs onto the wound-contact surfaces of biologic wound dressings. The transfer of pre-fabricated PEMs using PVA microfilms has the advantage that the biologic wound dressings are not exposed to conditions (temperature or pH, for example) that would potentially degrade its functional properties (note that biologic wound dressings contain peptide and protein fragments that are thermally and chemically labile). More broadly, however, the use of PVA microfilms potentially allows facile transfer of the prefabricated Ga^{3+}-loaded PEMs onto a range of biomedically relevant surfaces, including wounds and biomedical devices. An additional merit of the approach described in this paper is that the approach is modular and PEMs containing Ga^{3+} can potentially be loaded with other bioactive factors to confer on the surfaces of biological materials a spectrum of desired functionalities.

5. Experimental Section

5.1 Materials

PAH (Mw = 65 kDa) and gallium(III) nitrate hydrate [$Ga(NO_3)_3 \cdot \times H_2O$] were obtained from Sigma Aldrich (St. Louis, MO) and PAA (Mw = 50 kDa) from Polysciences (Warrington, PA). Wound dressing Biobrane® was purchased from UDL Labs, Rockford, IL. Reagent grade solvents, standard salts and media were purchased from Sigma Aldrich (Milwaukee, WI), Fisher Scientific (Pittsburgh, PA), or other commercial sources and used without further purification unless otherwise noted. A 6 mm biopsy punch was purchased from Miltex, Inc., York, PA. *P. aeruginosa* (ATCC 27853) used in biofilm assays was obtained from ATCC (Manassas, VA). Tryptic soy broth yeast extract (TSBYE), and 48-well tissue culture plates (#353078) were purchased from Becton, Dickinson, and Company (Franklin Lakes, NJ). Crystal violet (#65092A-85) was purchased from EMD Chemicals (Gibbstown, NJ). Trypticase soy agar with 5% sheep blood (#221261) was obtained from BD (Sparks, MD). M63 medium (comprised of 2.0 g $(NH_4)SO_4$, 13.6 g KH_2PO_4, 0.5 mg $FeSO_4 \cdot 7H_2O$, 10 mL 20% glycerol, and 1 mL 1M $MgSO_4$ in 1.0 L of distilled H_2O) was adjusted to pH ~ 7 before use in biofilm assays.

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5.2 Preparation of PEMs

Poly(dimethylsiloxane) (PDMS) sheets were fabricated by curing Sylgard 184 (Dow Chemical, Midland, MI) on OTS-functionalized silicon wafers or glass slides at 60°C for 24 h (see details elsewhere).[24] Aqueous solutions of polyelectrolytes were prepared at 0.01 M (with respect to the molecular weight of the polymer repeat unit) and adjusted to the desired pH. To assemble the PEMs, the PDMS sheets were first immersed into a PAH solution for 10 min followed by three 1 min rinses with deionized water (Millipore, 18.2 MΩ). Next, the substrates were immersed in PAA solution for 10 min followed by the rinsing steps described above. The adsorption and rinsing steps were repeated until the desired number of multilayers was deposited, as described elsewhere.[23] Post fabrication, the PEMs were stored at ambient conditions.

5.3 Loading of Ga^{3+} into PEMs and quantification of Ga^{3+} released from PEMs

Incorporation of Ga^{3+} into the PEMs was initiated by incubation of the pre-assembled PEMs on PDMS in an aqueous solution of $Ga(NO_3)_3$ (15 mM) at pH ~2.5 for at least 2 h, followed by rinsing in distilled water (pH~5–6) three times (15 s each) and air drying. The amount of Ga^{3+} loaded into the PEMs was determined by extracting the Ga^{3+} from the PEMs (which were punched into circles of 6 mm diameter using a biopsy punch) into 2% nitric acid (5 mL) by incubation for 24 h. The concentration of Ga^{3+} extracted from the PEMs was measured by elemental analysis using inductively coupled plasma optical emission spectrometry (ICP-OES, Vista-MPX CCD Simultaneous ICP-OES, Varian Inc., CA, US) at a wavelength of 417.2 nm. Similarly, the release of Ga^{3+} from the PEMs into aqueous solution was quantified by ICP-OES. For the latter measurements, sample substrates were individually placed into 15 mL centrifuge tubes, immersed under PBS buffer (3 mL) and incubated without agitation. Substrates were removed daily and placed into new vials containing fresh PBS, with at least 3 samples prepared per experimental group. For ICP-OES analysis, 1 mL of solution from each vial was combined with 2 mL of 3% nitric acid.

5.4 Preparation of the microfilm and transfer of the microfilm to Biobrane

PVA (25–50 μL, 5 wt.%) was deposited over the PEMs (with or without Ga^{3+}) supported on PDMS sheets (6 mm diameter) by spin coating at 1500 rpm (ac = 27) for 10s (WS-400A-6NPP/Lite, Laurell Technologies, North Wales, PA). The PEM/PVA microfilm construct was then dried in an oven for 5 min at 70°C. After drying, the microfilm was peeled from the PDMS surface using tweezers starting from the edge of microfilm. The PEM/PVA microfilm was then placed on the wound-contact surface of a pre-moistened piece of Biobrane® (with the PEMs facing the surface of Biobrane®). The surface of Biobrane was pre-moistened by pipetting water onto the Biobrane. The moistened Biobrane dissolved the PVA microfilm, leaving the PEMs adhered to the surface of Biobrane.

5.5 In vitro antibiofilm activity over extended periods of time

P. aeruginosa (ATCC 27853 or PA01) was incubated in tryptic soy broth yeast extract (TSBYE) overnight at 37°C with rotation until a cell density of approximately ~10^9 CFU/mL was achieved. The ability of PEM-coated PDMS or modified Biobrane to inhibit biofilm formation over extended periods was examined in the following manner. We placed

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6 mm-diameter PDMS/ Biobrane disks at the bottom of the wells of 48-well tissue culture plates and exposed the disks to four fresh 500 μL cultures of ~10^5 CFU *P. aeruginosa* over a four day period: 1st incubation (over 0–24 h), 2nd (24–48 h), 3rd (48–72 h), and 4th (72–96 h), with the disks washed 3× using sterile PBS and transferred to new plates every 24 h (i.e. between each incubation period). The incubation was done at 30°C (temperature relevant to outer skin temperature) without shaking. After incubation, the medium was removed, the wells rinsed 3× with distilled H_2O, and the plates allowed to dry for at least 1 h. Next, biofilms that formed on adjacent surfaces uncoated by the disks were stained by adding 600 μL of 0.35% filtered crystal violet to each well for 15 minutes at room temperature. After the dye was removed, the wells were rinsed 5× with tap water, and air dried for at least 1 h. We then added 30% glacial acetic acid to solubilize the dye and quantified absorbance at 595 nm using a Beckman Coulter DTX880 spectrophotometer.

5.6 Dispersal of pre-existing biofilms on biological dressings using Ga^{3+}-loaded PEMs

Biofilms on the biological dressing (Biobrane) were prepared by cutting the dressing into disks using a 6 mm biopsy punch, placing the disks into the bottom of the wells of 48-well plates, and then incubating with M63 medium containing ~10^5 CFU *P. aeruginosa* for 48 h at 30°C. Biobrane samples were then washed 3× with sterile PBS and air dried. One set of samples was processed to determine the amount of biofilm present before treatment (pre-existing biofilm). Ga^{3+}-containing PEMs (supported on PDMS sheets) were put on top of Biobrane disks with the PEM surface in contact with the Biobrane. Fresh M63 media was added and the plate incubated at 30°C, with the media being replaced every 24 h. Following treatment, the Biobrane samples were washed, stained, and the biofilms remaining on the Biobrane surface quantified by crystal violet staining.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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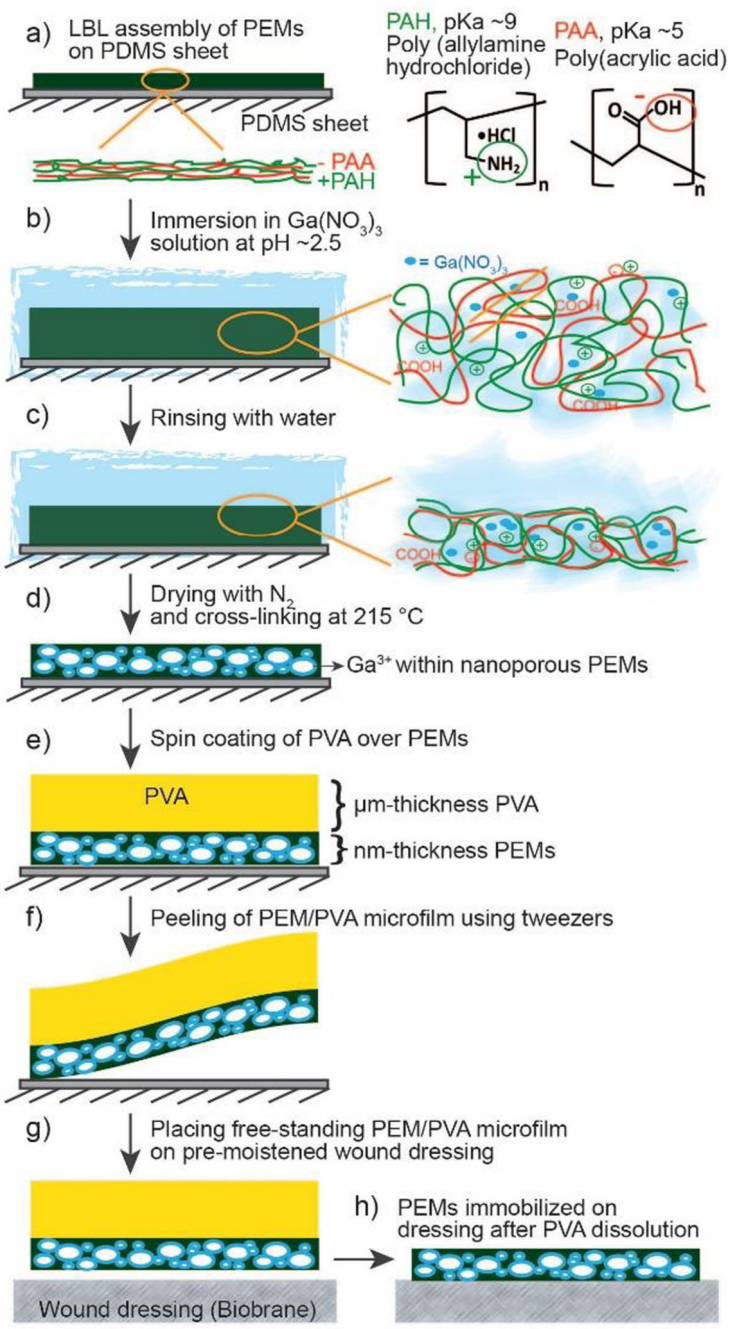

Figure 1.

Schematic illustration of the procedure used to fabricate PEM/PVA microfilms containing Ga^{3+} and apply them to wound-dressings. a) Layer-by-layer (LBL) assembly of PEMs of $(PAH_{7.5}/PAA_{5.5})_n$ on PDMS sheets. b) Loading of PEMs with Ga^{3+} by immersion in 15 mM $Ga(NO_3)_3$ solution at pH 2.5 for 2h. c) Rinsing of PEMs with deionized water three times (15 s each). d) Drying of PEMs under a stream of N_2 and subsequent cross-linking by heating to 215 °C for 2 h under N_2. e) Spin-coating of PVA solution on cross-linked Ga^{3+}-loaded PEMs with subsequent drying of PEM/PVA microfilm for 5 min at 70 °C. f) Peeling

of microfilm from the PDMS sheet. g) Placement of PEM/PVA microfilm onto pre-moistened wound dressing (Biobrane®). h) Dissolution of PVA leaving PEM immobilized on Biobrane.

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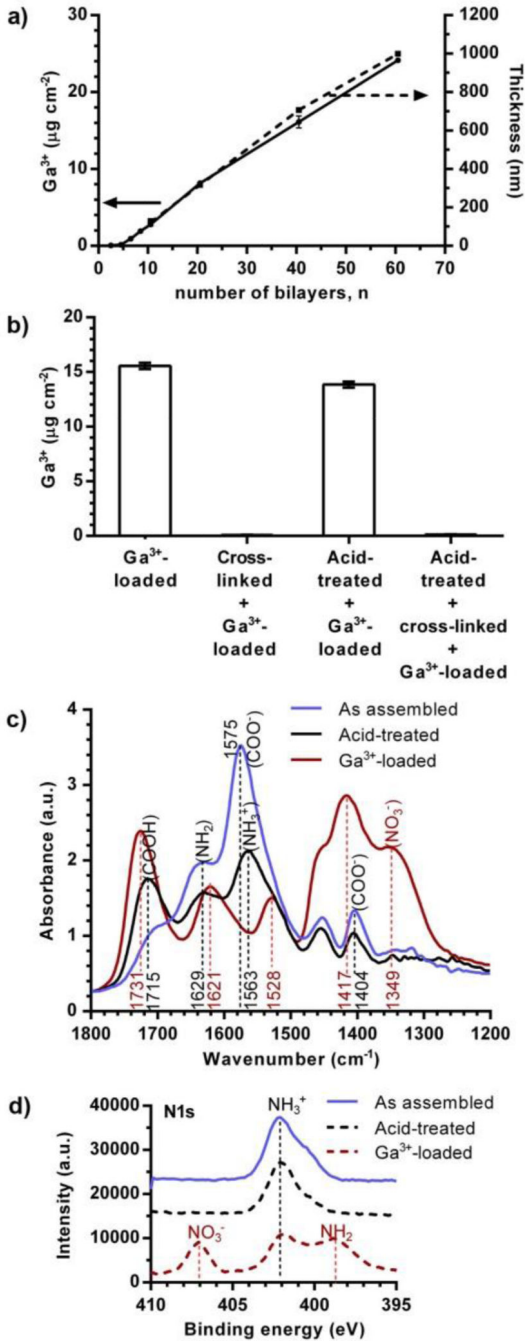

Figure 2.

a) Ga^{3+} loading (μg cm^{-2}) within PEMs $(PAH_{7.5}/PAA_{5.5})_n$ as a function of the number of bilayers (n). The ellipsometric thickness of the PEMs, before Ga^{3+} loading, is also presented. Data presented as mean ± standard deviation (n > 4). b) Uptake of Ga^{3+} from $Ga(NO_3)_3$ solution at pH 2.5 into (i) as-assembled PEMs, (ii) cross-linked PEMs, (iii) acid-treated PEMs, and (iv) acid-treated and cross-linked PEMs. c) PM-IRRAS spectra of PEMs, as assembled (blue), acid-treated PEMs (black), and Ga^{3+}-loaded PEMs (red) without

subsequent water rinsing. d) XPS spectra (N1s region) of PEMs as assembled, acid-treated PEMs, and Ga^{3+}-loaded PEMs after rinsing.

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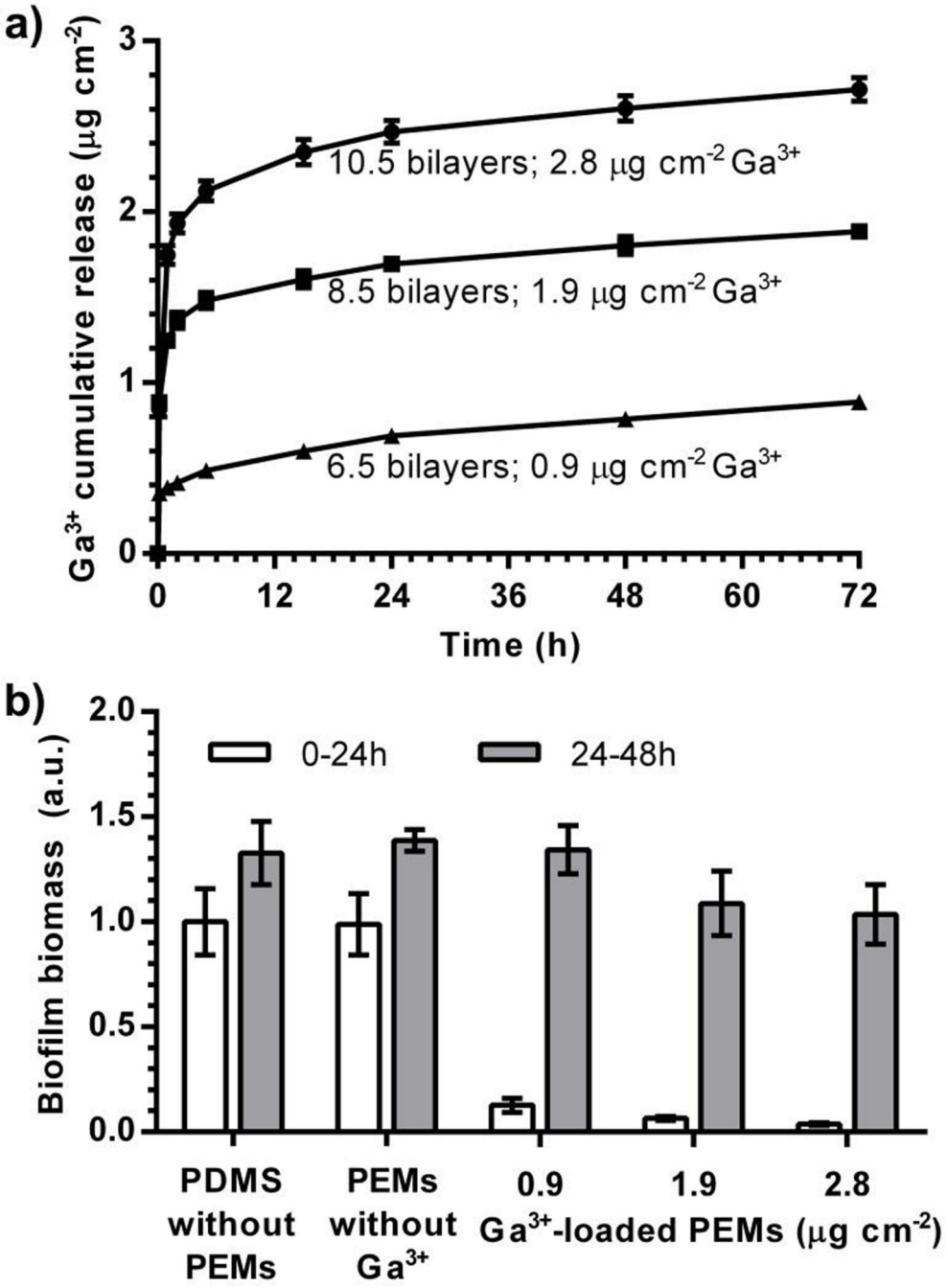

Figure 3.

a) Cumulative release of Ga^{3+} into PBS solution from PEMs of $(PAH/PAA)_n$, with n = 6.5, 8.5, or 10.5 (corresponding to Ga^{3+} loadings of 0.9, 1.9, and 2.8 μg cm^{-2}, respectively). PEMs supported on PDMS sheets (6 mm diameter) were incubated in PBS and the concentration of eluted Ga^{3+} was measured by ICP-EOS. Data represents the mean ± standard deviation (n = 4). b) Biofilm biomass on the surfaces of tissue culture plates that were adjacent to PEM-coated PDMS sheets that were placed into the wells of the tissue culture plates (see Figure S7a for experimental set-up). Over a 2 day period, two fresh

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bacterial inoculums were added to the wells (first incubation over 0–24 h and second incubation over 24–48 h with the wells rinsed between the incubation periods). Error bars show standard error (n = 4)

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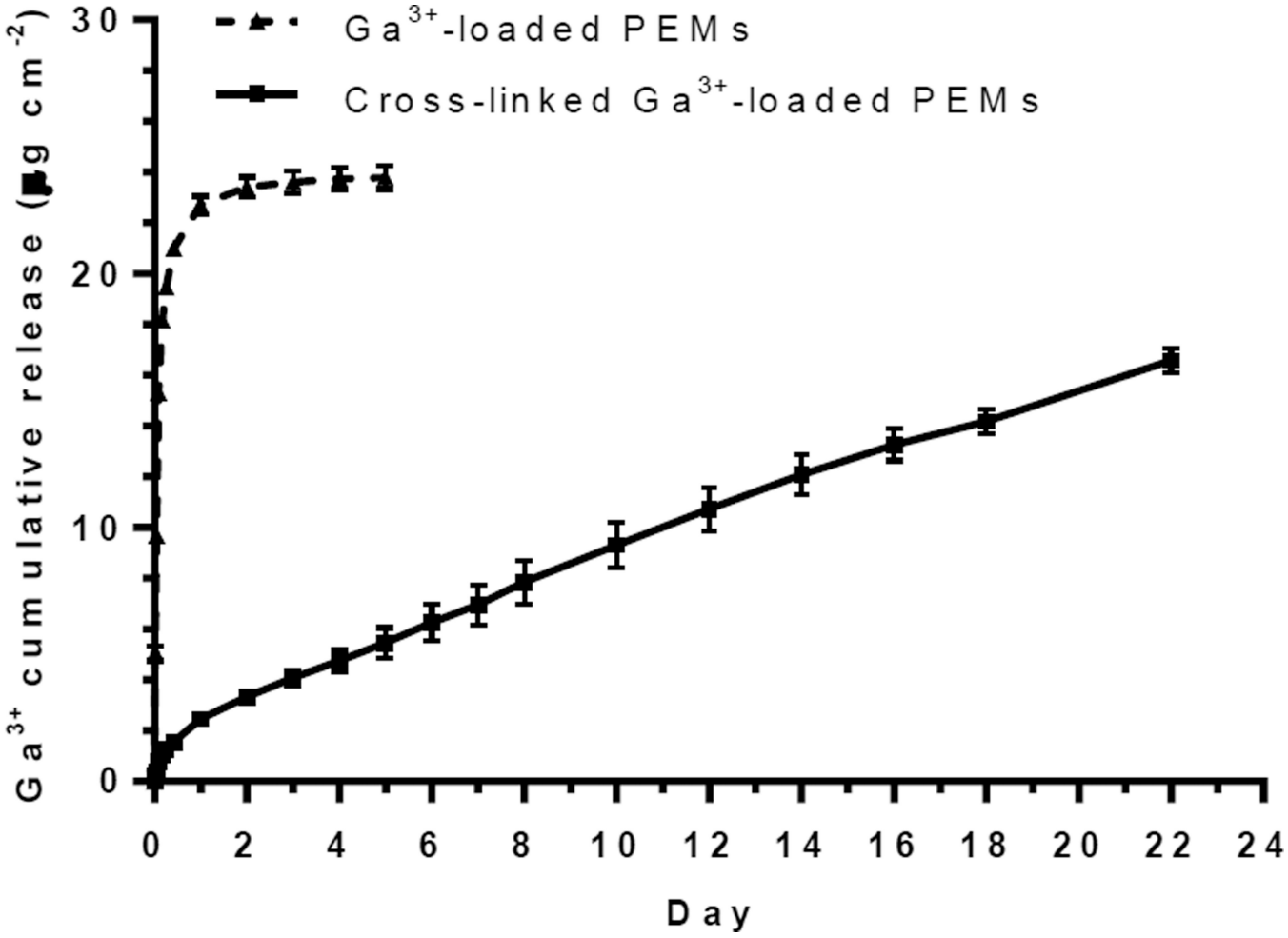

Figure 4.

Cumulative release of Ga^{3+} into PBS solution from either cross-linked or non-crosslinked PEMs of $(PAH/PAA)_{60.5}$ impregnated with a Ga^{3+} loadings of 24.5 ± 0.4 μg cm^{-2}. PEMs (6 mm diameter) were incubated in PBS and the concentration of eluted Ga^{3+} was measured by ICP-EOS. Error bars show standard deviation (n = 4).

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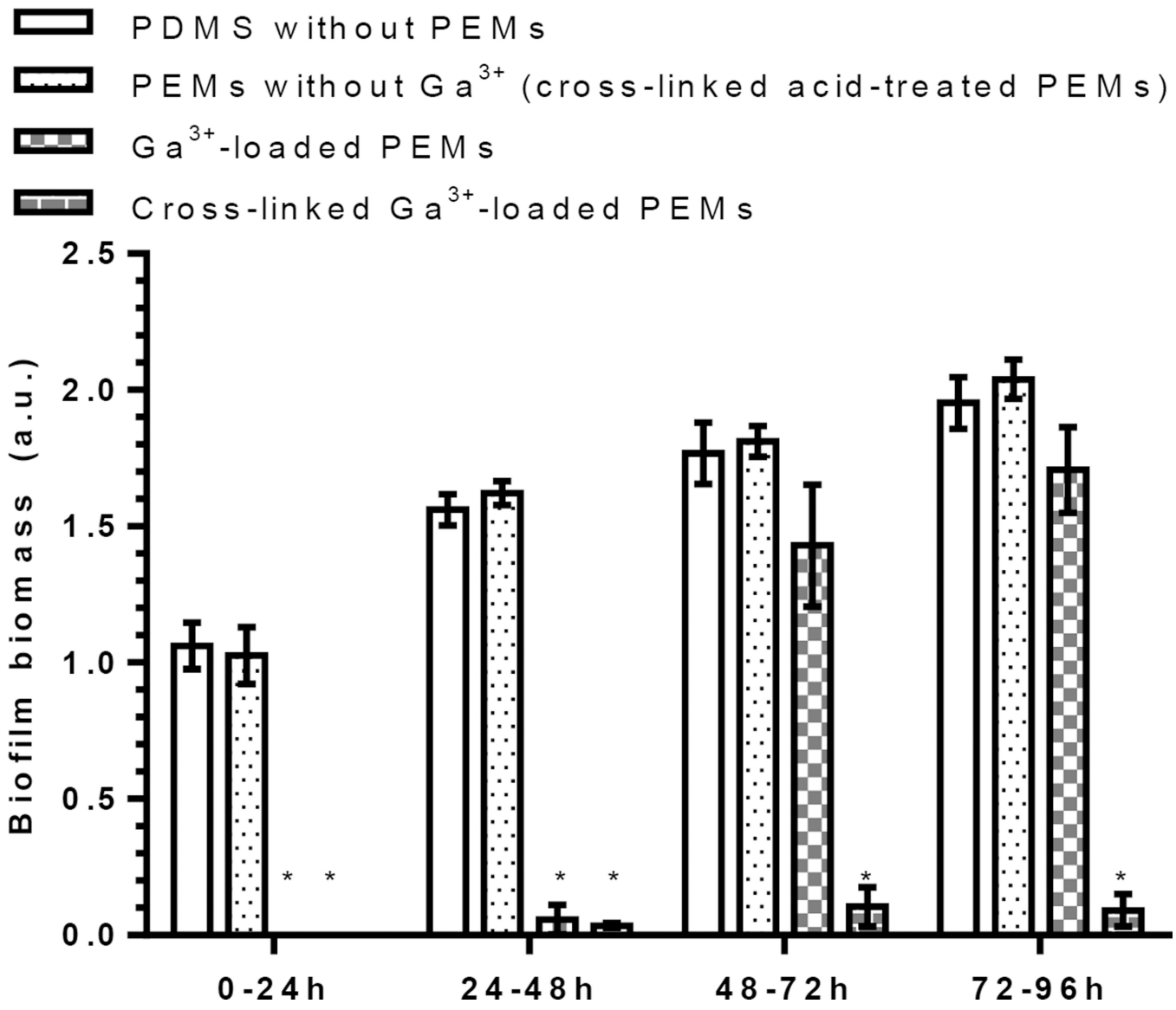

Figure 5.

Biofilm biomass on surfaces adjacent to PEM-coated PDMS sheets placed into the wells of the tissue culture plates. Fresh bacterial inoculums were added to the wells over a four-day period (first incubation between 0–24 h, second between 24–48 h, third between 48–72 h, and fourth between 72–96 h). The PEM-coated PDMS sheets were washed and transferred to new wells between each incubation period. Ga^{3+}-loaded PEMs contained 24.5 ± 0.4 μg cm^{-2} of Ga^{3+}. Error bars shows standard error (n > 4).

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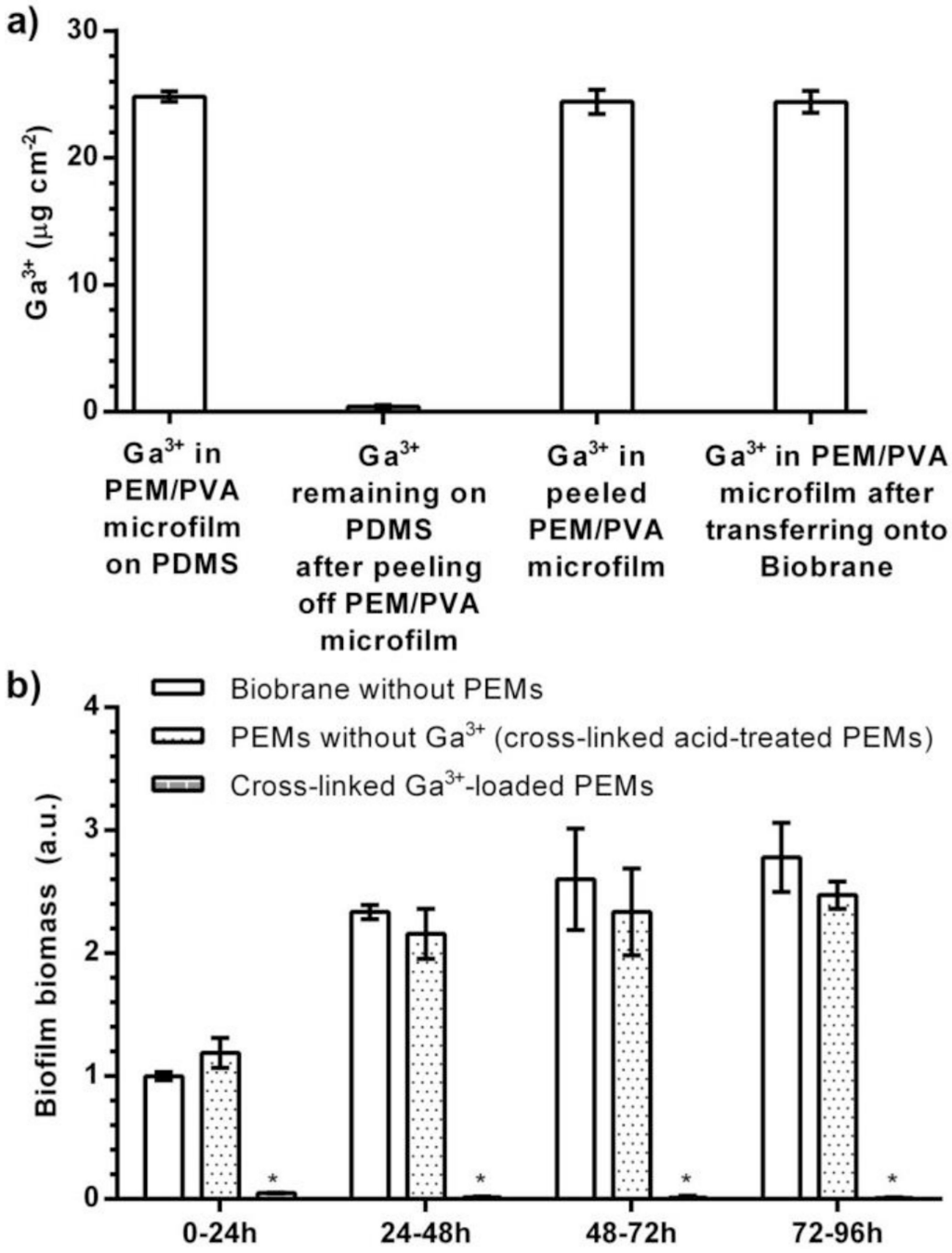

Figure 6.

Functionalization of Biobrane with Ga^{3+}-loaded PEMs. a) Ga^{3+} loadings within the PEM/PVA microfilms on the PDMS sheet, on the PDMS substrates after peeling the PEM/PVA microfilms from the PDMS sheets, within the peeled microfilms, and on the Biobrane after transfer of the microfilms. b) Biofilm biomass on surfaces of wells not coated by (functionalized) Biobrane following exposure of the Biobrane to four fresh bacterial inoculums over a four-day period (first incubation over 0–24 h, second over 24–48 h, third

over 48–72 h, and fourth over 72–96 h). The Biobrane was washed and transferred to new plates between each incubation period. Error bars shows standard error (n = 4).

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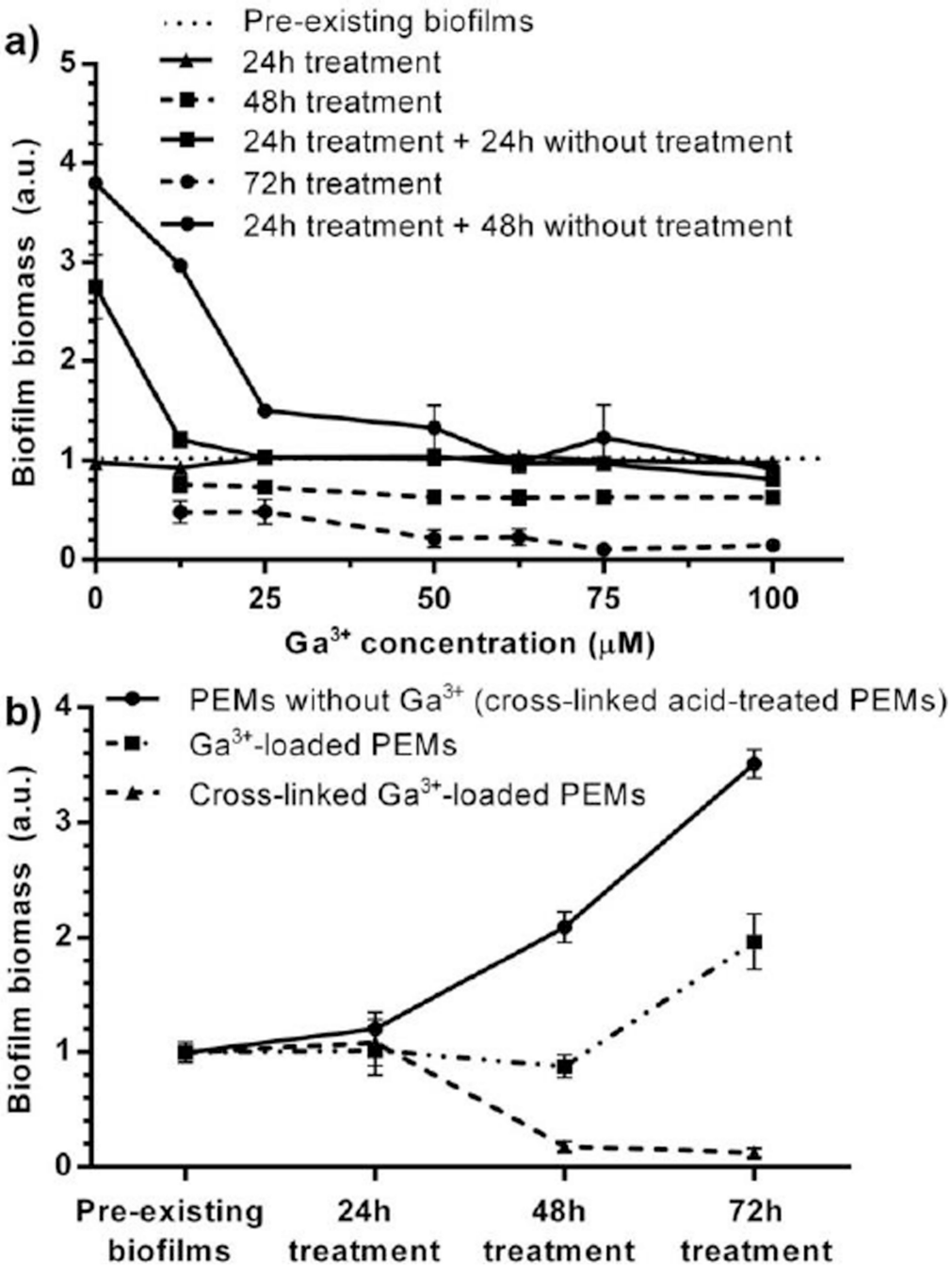

Figure 7.

Dispersal of pre-existing biofilms using Ga^{3+} solutions and cross-linked Ga^{3+}-loaded PEMs. a) Biofilm biomass of mature 48-h biofilms (dashed line) remaining on the surface of wells of 48-well plates following treatment with Ga^{3+} solutions (in citrate buffer to maintain Ga^{3+} stability) for 24, 48, or 72 h. Error bars show standard error ($n > 3$). b) Following maturation of biofilms on Biobrane for 48h (pre-existing biofilms), Ga^{3+}-loaded PEMs (either cross-linked or non-crosslinked) were placed on top of Biobrane with the PEMs in contact with the Biobrane. Fresh media was added and the plate incubated at 30 °C, with the media being

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replaced every 24 h. The plot shows the amount of mature biofilms on Biobrane (pre-existing biofilms) remaining after treatment with Ga^{3+}-loaded PEMs (with or without cross-linking). Error bars shows standard error ($n > 4$).