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Research paper

Bioburden-responsive antimicrobial PLGA ultrafine fibers for wound healing

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ABSTRACT

Despite innovation in the design and functionalization of polymer nanofiber wound healing materials, information on their interaction with the biochemical wound environment is lacking. In an earlier study, we have reported the interaction of fusidic acid-loaded PLGA ultrafine fibers (UFs) with wound bacteria. Massive bacterial colonization and the formation of a dense biofilm throughout the mat were demonstrated. This was associated with a marked enhancement of initial drug release at concentrations allowing eradication of planktonic bacteria and considerable suppression of biofilm. The present study aimed at extending earlier findings to gain more mechanistic insights into the potential response of the fusidic acid-laden UFs under study to controlled microbial bioburden. Initial drug release enhancement was shown to involve surface erosion of the ultrafibrous mats likely mediated by microbial esterase activity determined in the study. Release data could be correlated with microbial bioburden over the inoculum size range 10^3–10^7 CFU/ml, suggesting a bioburden-triggered drug release enhancement mechanism. Moreover, the effectiveness of fusidic acid-laden UFs in the healing of either lightly contaminated or *Staphylococcus aureus* heavily infected wounds in a rat model suggested in-use relevant antimicrobial release patterns. Findings indicated active participation of polymer ultrafine wound dressings in a dynamic interaction with the wound milieu, which affects their structure–function relationship. Understanding such an interaction is fundamental to the characterization and performance assessment of wound materials under biorelevant conditions and the design of polymer-based infection-responsive biomaterials.

1. Introduction

Electrospun polymer nanofibers have demonstrated great potentials as wound dressing materials [1,2]. Compared to conventional dressings, nanofibrous mats possess intrinsic wound healing properties by virtue of their close structural resemblance to the native extracellular matrix, large surface to volume ratio and high porosity [3]. Nanofibers prevent exogenous infections via an efficient sieve effect [4] and rapidly enhance epidermal cell attachment and proliferation [5]. Further, polymer nanofibers with biodegradable chemistries allow bioresorption of the dressing material in synchronization with tissue regeneration and drug release functions [4]. This precludes wound repair disturbance and patient discomfort caused by dressing change, making wound care easier. Combined structural and functional characteristics of nanofibers allow for faster and more esthetic repair of wounds and burns [4–6].

Research efforts for further improvement of nanofibrous wound healing materials have been mainly targeting wound dressing design [7,8], material properties [9,10], manipulation of nanofibers characteristics [11,12], functionalization with antimicrobial agents, and growth factors [13–15] as well as drug release modulation [16,17]. The developed materials have been usually evaluated using in vitro testing and less frequently in vivo wound healing in animal models. However, both types of assessment do not provide direct evidence on the inevitable dynamic interaction of nanofibrous membranes, notably of the biodegradable type, with the biochemical wound environment. The effect of such an interaction on the structure and drug release characteristics under biorelevant conditions presents a knowledge gap that is worth investigating.

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In an earlier study [18], we have shown that incubation of antimicrobial fusidic acid-laden PLGA ultrafine fibrous mats with overnight cultures of wound bacteria (10^7 CFU/ml) resulted in massive bacterial colonization and the formation of a dense biofilm throughout the mat. This was associated with a marked enhancement of initial drug release. In turn, the progressively faster release of bioactive FA allowed eradication of planktonic bacteria and considerable suppression of biofilm. Such a dressing material-wound milieu interaction may have important clinical implications as pristine fibrous mats, by sequestering bacteria, could prevent infection dissemination, though reinoculation of the wound becomes highly probable. On the other hand, enzymes and toxins produced by colonizing bacteria may affect the structural integrity and drug release properties of biodegradable drug-loaded wound biomaterials.

A better understanding of the interaction of the wound biomaterials with the wound environment would allow optimization of wound dressings to conform to in-use structural and biochemical requirements. It would also allow the utilization of internal microbial stimuli generated in the wound microenvironment to elicit structurally-based functional responses [19,20]. A "response" in this context may be described as a combination or sequence of events involving reception of a microbial signal followed by chemical reaction of the matrix material or changes of the material's properties and transduction of the changes into macro/microscopically significant events including release of a drug cargo [21].

The objective of the present study was to extend earlier findings [18] in order to gain deeper mechanistic insights into the interaction of antimicrobial PLGA ultrafine fibers with cultures of wound bacteria. The impact of such an interaction on the structural integrity, drug release characteristics, and wound healing properties of an antimicrobial ultrafine fibrous mat in a rat model was investigated. Another objective was to assess the potential polymer-based bioburden-responsiveness of the ultrafine fibers under study.

2. Materials and methods

2.1. Materials

PLGA (Medisorb® PLGA 50:50 DL 3A, MW 50 kDa, inherent viscosity 0.36 dL g^{-1}, Alkermes Inc., Cincinnati, OH, USA), dichloromethane (DCM) (Biotech. grade 99.9%, Sigma–Aldrich, St. Louis, MO, USA). Fusidic acid (Courtesy of Pharaonia Pharm. Co., Alexandria, Egypt), nutrient broth (Oxoid Ltd.; Basingostok; Hampshire, England), Tween 80 (Guangdong Guanghua Chemical Factory Co., Ltd., Shanghai, China), and Tris–HCl buffer (Sigma–Aldrich, St. Louis, MO, USA) were used.

2.2. Preparation and characterization of plain and fusidic acid-loaded PLGA ultrafine fibers (UFs)

Ultrafine fibers (UFs) prepared using a 25% PLGA solution in DCM were used throughout the study. Fibers were fabricated as reported earlier [18]. Briefly, the polymer solution was electrospun in an air-conditioned laboratory at an ambient temperature of ~25 °C and relative humidity of <65%. A high voltage DC power supply (ALE 402, TDK-Lambda Americas, Inc., USA) set to 25 kV and a syringe with a blunt-tip stainless steel spinneret (0.9 mm diameter) kept at 10 cm from the fiber collector (copper plate covered with aluminum foil and a nonwoven synthetic porous mat) were used. For the preparation of fusidic acid (FA)-loaded UFs, FA was dissolved in the DCM polymer solution.

For the determination of drug content and % entrapment efficiency (% E.E.), FA was completely extracted from UFs with absolute ethanol and assayed spectrophotometrically in absolute ethanol at λ_{max}, 220 nm using UV–Visible spectrophotometer (Thermospectronic, Helios alpha, NC 9423 UV A 1002E, England).

2.3. Microbiological study

Three bacterial strains were used: two standard strains: *Staphylococcus aureus* ATCC 6538P (Sa_{st}) and *Pseudomonas aeruginosa* ATCC 9027 (Ps_{st}), in addition to a methicillin-resistant *Staphylococcus aureus* clinical isolate ($MRSA_1$) obtained from an infected wound (Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt). Bacteria were maintained at 4 °C as slant cultures of sterile nutrient agar for a maximum of 1 month. Long-term preservation was performed by freezing in 15% glycerol broth. The effect of pre-exposure to overnight cultures (24 h) of different inoculum size of the three bacterial strains on the characteristics of FA-PLGA UFs was investigated.

2.3.1. Effect of in vitro pre-exposure to wound bacteria on drug release

Samples (2×2 cm^2) of FA-loaded PLGA UF mats of known drug content, determined based on % E.E and UFs sample weight were disinfected with 70% ethanol and pre-exposed to bacteria in vitro by incubation with overnight broth cultures of the test strains, adjusted to three inoculum sizes: 10^3, 10^5, and 10^7 CFU/ml, for 24 h at 37 °C. Adequate controls were included in which the UFs were incubated in sterile nutrient broth diluted with sterile saline. Test and control UFs were removed and washed with sterile saline. Drug release was assessed by immersing the sample mats in 50 ml capped Erlenmeyer flasks containing 30 ml of the release medium (PBS, pH 7.4, containing 1% ethanol), a medium selected to increase FA solubility [18]. The flasks were shaken at 50 rpm in a thermostatically controlled shaking water bath (GFL, type 1083, Germany) at 37 °C for 2 h daily for 14 days. At pre-determined time intervals, 3 ml of the release medium was withdrawn, filtered using 0.45 μ Millipore filter, and replaced with the same volume of fresh medium adjusted to 37 °C. The concentration of eluted FA was measured spectrophotometrically (UV–Visible spectrophotometer, Thermospectronic, Helios alpha, NC 9423 UV A 1002E, England) at λ_{max} 207 nm. UV spectra were recorded to check for reproducibility and lack of interference. Release experiments were run in triplicate, and mean values of % drug released were calculated. The effect of medium compensation on release data was corrected for.

2.3.2. Effect of in vitro pre-exposure to wound bacteria on degradation of PLGA UFs

UFs samples similar to those used for the in vitro release study were exposed to test bacterial cultures under the same conditions. UF samples were removed from the cultures, washed with sterile saline, and immersed in 10 ml PBS, pH 7.4, in 20 ml screw-capped glass vials kept at 37 °C without agitation. A pH value of 7.4 has been reported for artificial wound fluid [22]. The degradation of PLGA UFs in PBS was assessed by monitoring the change in the medium pH, a simple surrogate method indicating release of PLGA acid degradation products. The pH was measured at pre-determined time intervals over a period of 56 days. Experiments were run in duplicate, and the mean pH values were calculated.

2.3.3. Effect of incubation with bacterial cultures on the morphology of UFs (SEM)

This was investigated by immersing samples 2×2 cm^2 of FA-loaded PLGA UF mats (45 μg FA/mg fibers) in equal volumes of the supernatant obtained by centrifugation (13,148*g* for 10 min) of overnight bacterial cultures of $MRSA_1$ wound isolate adjusted to contain ~10^7, 5×10^7, and 10^8 CFU/ml. Control tubes containing sterile saline were used. UFs samples were removed aseptically

after 7 days, washed twice with sterile saline, and prepared for scanning electron microscopy.

2.3.4. Relationship between inoculum size and esterase activity of the test bacterial strains

Quantitative estimation of the esterase activity produced in bacterial cultures was determined using Tween 80 as a water soluble hydrolysable substrate [23]. The method is based on the determination of calcium oleate formed by reaction of oleic acid, the Tween 80 hydrolysis product produced by bacterial enzymatic hydrolysis, with $CaCl_2$ added to the medium. Tween 80 solution 2% w/v in 20 mM Tris–HCl buffer containing 80 mM $CaCl_2$ was distributed into Widal tubes. The solution was thoroughly mixed with an equal volume of the supernatants obtained by centrifugation at 13,148*g* for 10 min (High speed centrifuge EBA 12, Hettich, Germany), of overnight bacterial cultures adjusted to contain from $\sim10^3$ to $\sim10^8$ CFU/ml. Control tubes containing sterile saline were included. The reaction mixtures were incubated at 37 °C for 1, 3, and 7 days. Calcium oleate as an indirect measure of esterase activity was determined spectrophotometrically at 450 nm, and correction was made for absorbance of the control tubes. Data are the average of three determinations.

2.4. Wound healing in a rat model

The effect of plain and FA-loaded PLGA UFs (15 mm × 5 mm mats weighing ~5 mg with a drug content of 45 μg FA/mg UFs) on the healing of both initially clean wounds (lightly contaminated with autogenous skin microorganisms) and heavily infected full thickness wounds (inoculated with 50 μl of *Sa*$_{st}$ culture, 10^6 CFU/ml), was assessed in a rat model for 14 days. Wound healing was evaluated microbiologically, morphologically, and histologically.

2.4.1. Experimental animals

Young female albino rats 2 month-age weighing 150 ± 20 g were used. All experiments were performed in strict accordance with institutional guidelines on ethical conduct in the care and use of research animals. The rats were housed individually in cages with mesh galvanized wire and were acclimatized for 1 week prior to the study.

2.4.2. Study protocol

The effect of plain and FA-loaded UFs on the healing of lightly contaminated incisions (Experiment 1) and *Sa*$_{st}$-infected incisions (Experiment 2) was investigated. For each experiment, rats were divided into three test groups, six rats each. Group 1 (control group): four untreated incisions/rat; Group 2 (Plain UFs group): two incisions were treated with plain UFs while the other two were left untreated (internal control). Group 3 (FA-loaded UFs group): two incisions were treated with FA-loaded UFs while the other two were left untreated.

Rats were weighed and anesthetized with thiopentone sodium (50 mg/kg body weight) intraperitoneally. Four standardized full skin thickness linear incision wounds of initial length 20 mm were generated on the shaved back of each rat parallel to the vertebral column. Two incisions were made directly beneath the scapulae, and the other two just above the hip joints. Incisions were washed immediately with sterile normal saline and tapped dry with disposable sterile cotton pads. For induction of infection, 50 μl of *Sa*$_{st}$ (10^6 CFU/ml) was inoculated into each incision using a calibrated micropipette. An ultrafine fibrous mat was applied to each incision, which was then sutured with 4-0 waxed silk to prevent removal by the rat.

Wound healing was assessed by three methods: (a) Microbiologically: the outer side of the wound was swabbed on days 2, 7, and 14 and the swabs cultured on nutrient agar plates to detect residual infection. The number of infected wounds per group of rats was determined, and the percentage fatalities at day 2 in all groups were calculated using Eq. (1):

$$\%\text{Fatality} = \frac{\text{Total No. of rats} - \text{No. of survivors}}{\text{Total No. of rats}} \times 100 \quad (1)$$

(b) Morphologically: incisions were photographed on days 2, 7, and 14; and (c) Histologically: incisions were examined on day 14 after sacrificing the rats. Skin tissue biopsies were fixed in formol saline, processed, and embedded in paraffin. Sections were stained using the routine procedures hematoxylin and eosin (H and E) for general analysis and Masson's trichrome for assessment of collagen formation, a parameter indicative of wound healing.

2.5. Statistical analysis

Statistical analysis of data for microbiological assessment of wound healing was performed using Student's *t*-test and GraphPad

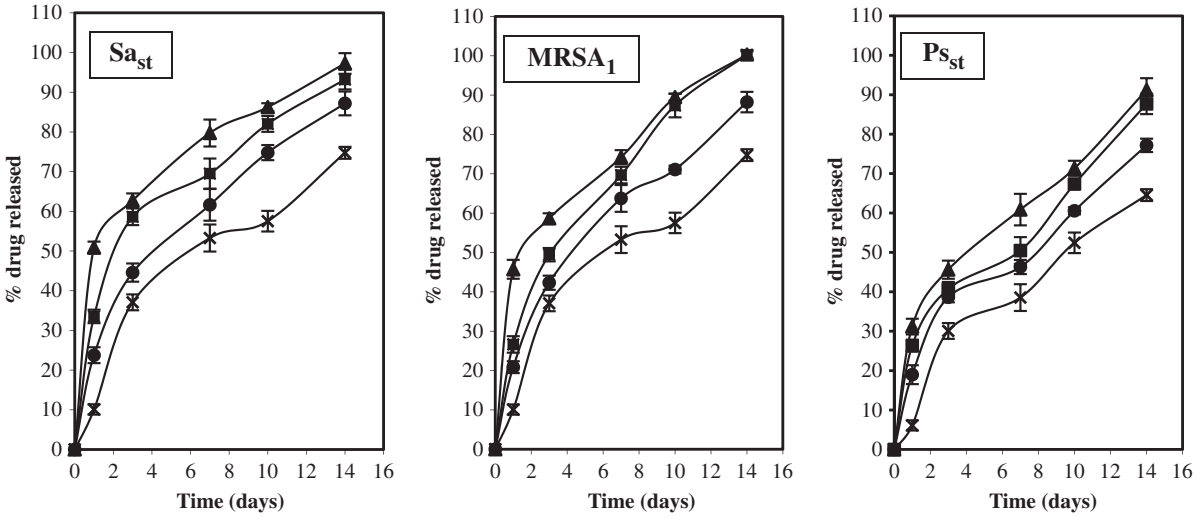

Fig. 1. Release profiles of FA-loaded PLGA UFs pre-exposed to overnight bacterial cultures of three wound bacteria at three inoculum sizes for 24 h, in PBS, pH 7.4/1% ethanol at 37 °C. × Control, ● ≈10^3, ■ ≈10^5, and ▲ ≈10^7 CFU/ml. Error bars represent mean ± SEM (n = 3).

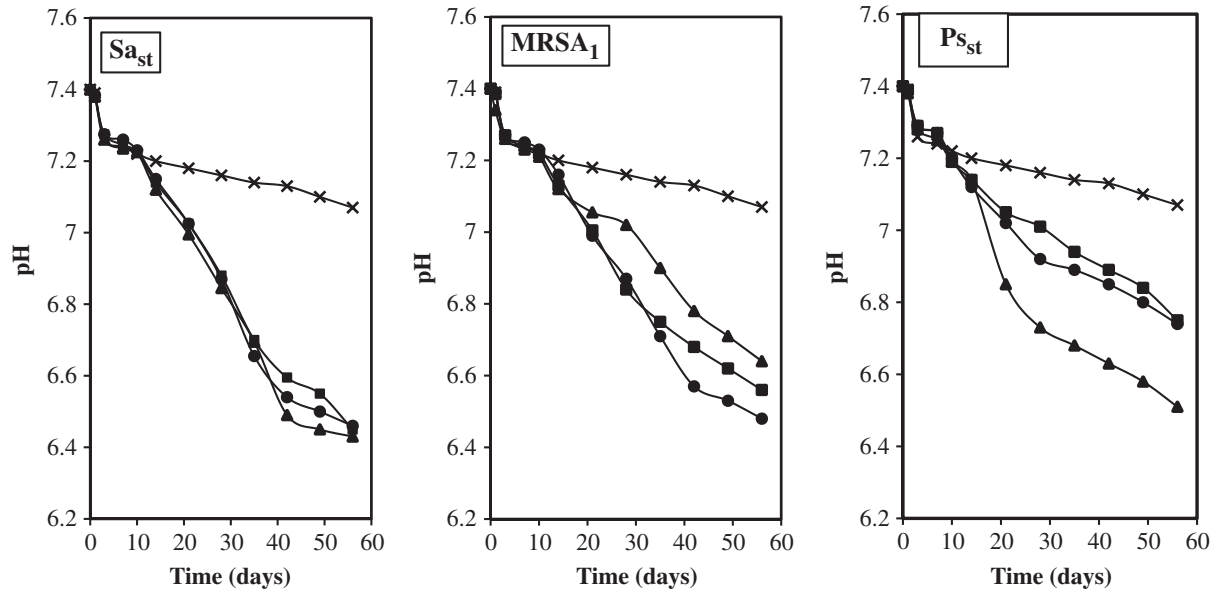

Fig. 2. pH lowering of phosphate buffered saline, pH 7.4, upon incubation of FA-loaded PLGA UFs pre-exposed to wound bacterial cultures at three inoculum sizes. × Control, ● ≈10^3, ■ ≈10^5, and ▲ ≈10^7 CFU/ml (n = 2).

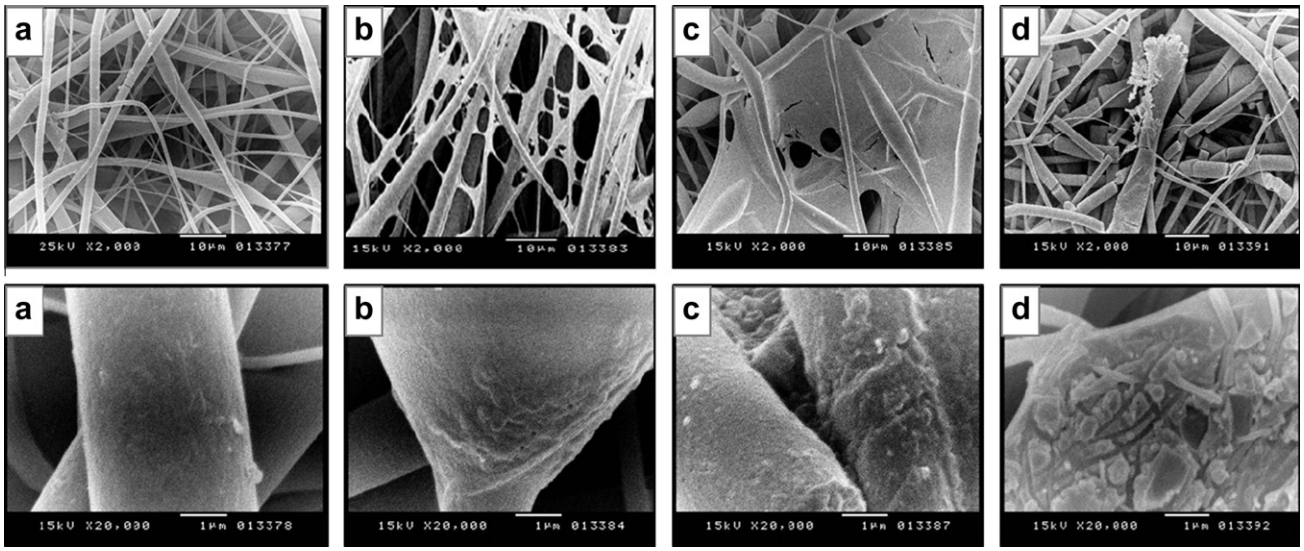

Fig. 3. The effect of incubation with overnight MRSA$_1$ cultures of different inoculum size for 7 days at 37 °C on the morphology of PLGA UFs. (a) Control, (b) 1 × 10^7, (c) 5 × 10^7, and (d) 1 × 10^8 CFU/ml. Magnification power: Upper 1 × 2000 and lower 1 × 20,000.

Instat 3.10. Probability values less than 0.05 were considered indicative of statistical significance.

3. Results and discussion

Antimicrobial fusidic acid-laden electrospun PLGA ultrafibrous mats prepared using different polymer solution concentration and drug loading have been developed earlier [18]. They were characterized for pharmaceutical attributes including size, entrapment efficiency, drug release, and storage stability. As the diameter of the developed fiber formulations was generally in the submicron to micron range (710 nm to 1700 nm), the term ultrafine fibers was used [24]. Microbiological assessment indicated full bioactivity upon FA entrapment and release from PLGA UFs and antimicrobial activity against three bacterial strains. Data also indicated interaction of these UF fibers with wound bacteria resulting in rapid colonization of the fibrous mats and biofilm formation that was associated with a marked increase in initial FA release.

3.1. PLGA ultrafine fibers (UFs) under study

Electrospun UFs prepared using 25% PLGA solution have been selected for the present study based on morphological and pharmaceutical properties reported earlier [18]. These fibers were used to investigate the effect of bacterial burden on the physicochemical characteristics of the ultrafibrous mats and their wound healing properties.

3.2. Effect of pre-exposure to bacterial cultures on antimicrobial release characteristics and in vitro degradation of FA-loaded UFs

The release of FA from control UFs (45 µg FA/mg UFs) not pre-exposed to bacterial cultures was generally triphasic (Fig. 1),

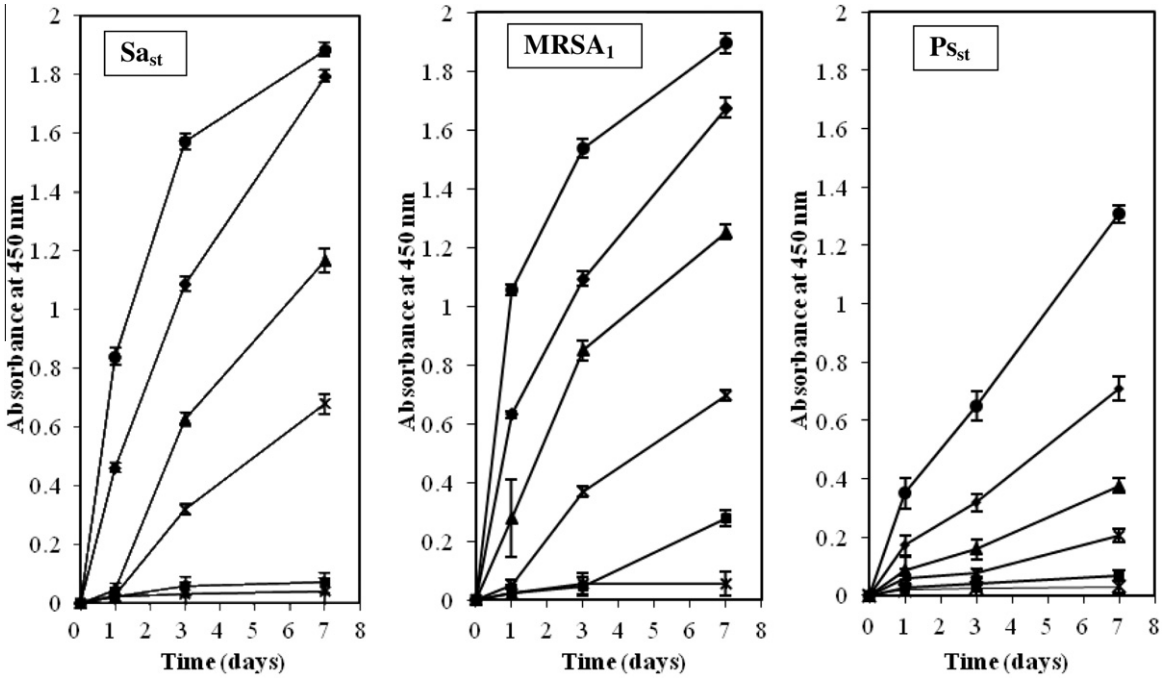

Fig. 4a. Effect of initial inoculum size of test bacteria on the enzymatic hydrolysis of Tween 80 upon incubation with bacterial culture supernatants at different inoculum size for up to 7 days at 37 °C. ● ≈10^8, ◆ ≈5×10^7, ▲ ≈2×10^7, × ≈10^7, ■ ≈10^5, and * ≈10^3 CFU/ml. Error bars represent mean ± SEM (n = 3).

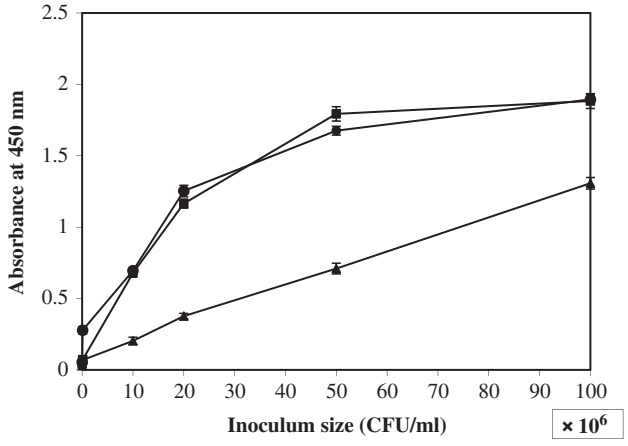

Fig. 4b. Relationship between inoculum size of test bacteria and the hydrolysis of Tween 80 (expressed as absorbance of calcium oleate at 450 nm) upon incubation with supernatants of bacterial cultures at different inoculum size for 7 days at 37 °C. ■ Sa_{st}, ● MRSA$_1$ wound isolate and ▲ Ps_{st}. Error bars represent mean ± SEM (n = 3).

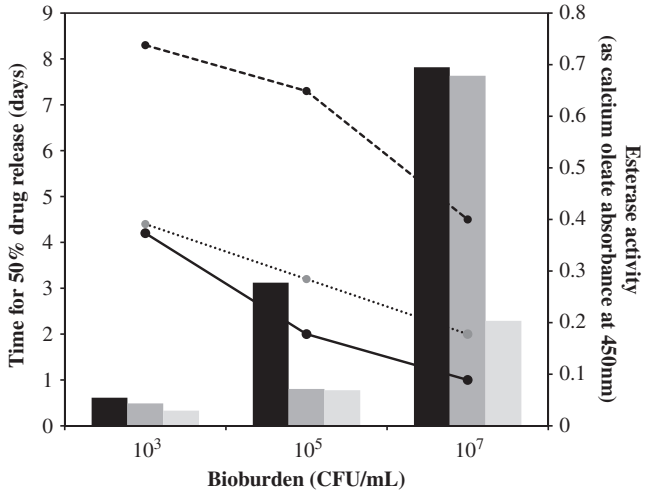

Fig. 5. Correlation between initial bioburden of the three test bacterial strains, esterase activity at day 7 and time for 50% FA release. Esterase activity of ■ Sa_{st}, ▩ MRSA and ▩ Ps_{st}. Time for 50% drug release for (---) Ps_{st}, (. . .) MRSA and (–) Sa_{st}.

suggesting a diffusion/bulk erosion mechanism typical of biodegradable polymer matrices [25]. Drug release was sustained for more than 14 days. Release profiles were characterized by a relatively limited burst effect (~10% at day 1), an intermediate slower release phase representing mainly drug diffusion through the polymer matrix and water-filled channels formed by progressive drug elution and a late relatively fast release phase. This was probably associated with more formation of water-filled channels and bulk matrix erosion. Pre-exposure of FA-PLGA UFs to overnight cultures of Sa_{st}, MRSA$_1$ wound isolate and Ps_{st} at three initial inoculum size (10^3, 10^5, and 10^7 CFU/ml) produced a marked enhancement in initial FA release (up to sixfold at day 1) and faster achievement of complete drug elution with no major changes in the overall release pattern. It was worth noting that release enhancement was in proportion to inoculum size and was influenced by the bacterial strain. The least effect was produced by Ps_{st}.

The notable increase in initial release rate even at the lowest bioburden level (10^3 CFU/ml) in all cases combined with the bioburden-triggered initial drug release enhancement strengthened the postulation that these effects were mediated by surface degradation and erosion of PLGA UFs. This could be attributed to catalysis by bacterial enzymes acting at the solid–liquid interface in addition to autocatalysis by the acid degradation products [26]. Bacteria are known to secrete hydrolytic enzymes [27,28], and the susceptibility of PLGA as aliphatic polyester to enzymatic degradation is well documented [29–31]. Polymer-based bioburden-triggered drug release enhancement data implied a perceivable response of PLGA UFs to internal microbial stimuli generated in the culture media. This finding is of interest in biomedical applications.

Table 1
The percentages of infected wounds and fatalities during the wound healing study in rats using plain and FA-loaded PLGA UFs.

Type of UFs (number of incisions)	Percentage of incisions showing bacterial burden at study days:			Percentage fatalities at day 2
	Day 2	Day 7	Day 14	
Experiment 1: Lightly contaminated wounds				
Control (24)	83.3	83.3	66.7	0
Plain UFs (12)*	50	16.7	0	0
Plain UFs internal control**	83.3	16.7	16.7	0
FA-loaded UFs (12)*	0	0	0	0
FA-loaded internal control*	0	0	0	0
Experiment 2: Heavily Sa_{st} infected wounds				
Control (8)	100	100	50	67.0
Plain UFs (8)***	50	0	0	33.0
Plain UFs internal control***	50	50	25	0
FA-loaded UFs (12)***	0	0	0	0
FA-loaded UFs internal control***	0	0	0	0

* Significant difference from the control group in Experiment 1 at $p < 0.05$ of the two-tailed paired *t*-test.
** Non-significant difference from the control group in Experiment 1 at $p < 0.05$.
*** Significant difference from the control group in Experiment 2 at $p < 0.05$ of the two-tailed paired *t*-test.

For a better understanding of the mechanism of bioburden-triggered drug release, the effect of pre-exposure of FA-loaded UFs to bacterial cultures under conditions similar to those of the release study was investigated using pH measurements. Lowering of pH, indicative of PLGA degradation and release of acid products, was observed for test relative to control UFs, notably after ~14 days (Fig. 2), though pH change was not generally proportionate to inoculum size. Enzymatic hydrolysis of polymeric biomaterials is a complex process involving diffusion of the enzyme to the solid–liquid interface where a substrate-enzyme complex is formed [26]. Hydrolysis is catalyzed at the surface because of the large size of enzyme molecules, a step followed by diffusion of degradation products into the medium [32]. The exact mechanism is not yet fully elucidated, and the role of enzymes in polymer degradation has been considered either primary by directly acting on the polymer backbone or secondary, by accelerating chemical hydrolysis [31,33]. The second mechanism based on assimilation of the released oligomers by microorganisms as a carbon-source has been more advocated. This was reported to result in loss of weight of the polymer device, enhanced porosity, and dispersion of polymer degradation products into the medium [34]. Such events may explain the pH change data obtained (Fig. 2) as assimilation of PLGA acid degradation products by bacteria might have obscured proportional pH lowering. The large increase in initial drug release rate, however, can be attributed to the large surface area of UFs subject to enzymatic hydrolysis.

3.3. Morphological study (SEM)

The effect of incubation with bacterial cultures of $MRSA_1$ on the morphology of UFs was visualized by SEM following exposure to bacterial cultures at different inoculum size for 7 days. Compared to control UFs, detrimental morphological and structural effects

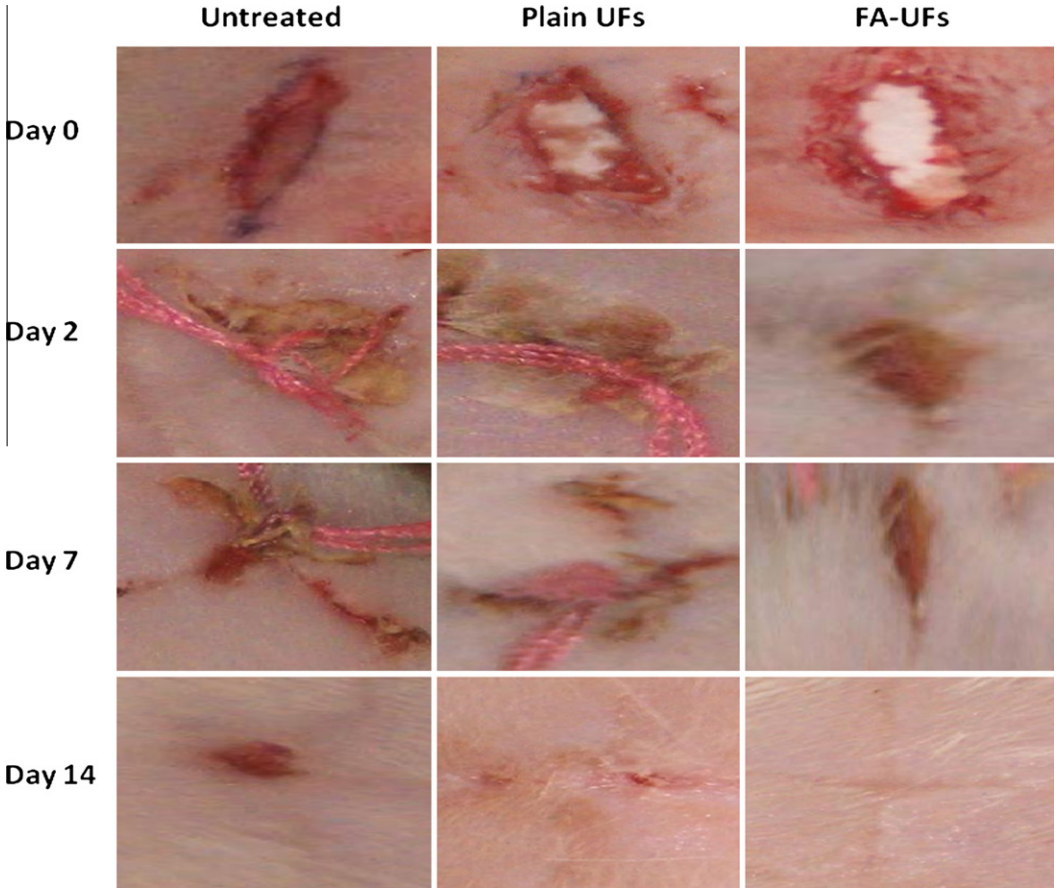

Fig. 6. Effect of plain and FA-loaded PLGA UFs on the healing of lightly contaminated wounds in rats.

in test samples were obvious particularly at larger inoculum size (Fig. 3). Coarsening and fissuring of the UFs surface confirmed initiation of UFs enzymatic degradation at the surface [26,35]. Surface erosion was further enhanced by more roughening and cracking of the eroding surface because of increased surface area [26].

3.4. Relationship between bioburden and esterase activity of the 3 bacterial strains under study

The overall esterase activity in cultures of the three bacteria was assessed quantitatively using Tween 80 as a water soluble esterase/lipase substrate [23] and calcium oleate concentration as indirect measure of Tween 80 hydrolysis. Data obtained (Fig. 4a) confirmed secretion of a considerable amount of microbial hydrolytic enzymes during the 24 h-incubation period notably by Sa_{st} and $MRSA_1$ isolate. Hydrolysis of Tween 80 increased considerably by increasing exposure time and bacterial bioburden. Different *Pseudomonas* and *staphylococcus* species and even strains of the same species were reported to produce several types of esterases and lipases with variable hydrolytic activity toward ester substrates [27,28,36]. Inoculum size-esterase activity relationships for the three test bacteria at 7 days are shown in Fig. 4b. Profiles for both *staphylococci* were almost similar and characterized by non-linearity with tapering off at larger inoculum size.

Data obtained so far are strongly supportive of an interaction between PLGA UFs and bacterial cultures initiated by adherence of bacteria to the ultrafibrous mat. Enzymatic surface degradation and erosion resulted in partial loss of structural integrity and enhanced drug release. This was evident from the relationship between bacterial bioburden, esterase activity, and rate of drug release from UFs (Fig. 5). The time for 50% drug release was reduced from 7 days (control) to 4.6, 2.3, and 1.2 days upon exposure to 1×10^7 CFU/ml bacterial cultures of Ps_{st}, $MRSA_1$, and Sa_{st}, respectively. Positive correlation suggested active involvement of bacterial enzymes in bioburden-triggered drug release enhancement based on a change in material properties. Such an enzyme-responsive mechanism may be of value as far as the use of PLGA and other aliphatic polyesters in drug delivery matrices for wound healing or other biomedical applications is concerned.

In wound healing, an infection responsive approach has been used to develop antimicrobial delivery systems responsive to microbial enzymes as internal stimuli inherent to infected wounds. For instance, an insoluble polymer–drug conjugate in which gentamicin was bound to poly(vinyl alcohol) hydrogel through a proteinase-sensitive or a thrombin-sensitive peptide linker [19,37] released gentamicin upon incubation with *Staphylococcus aureus*-infected wound fluid or exposure to two simultaneous enzyme activities. Ciprofloxacin covalently linked to polyurethane was released upon polymer degradation by esterases generated by macrophages and neutrophils present during the inflammatory response of wound healing [38]. Another approach based on the lysis of antimicrobial-loaded vesicles in response to toxins of pathogenic bacteria has been reported recently [20]. However, these systems release the antimicrobial agent after bioburden increases, which may allow early intruding bacteria to proliferate, or in response to wound inflammation rather than infection.

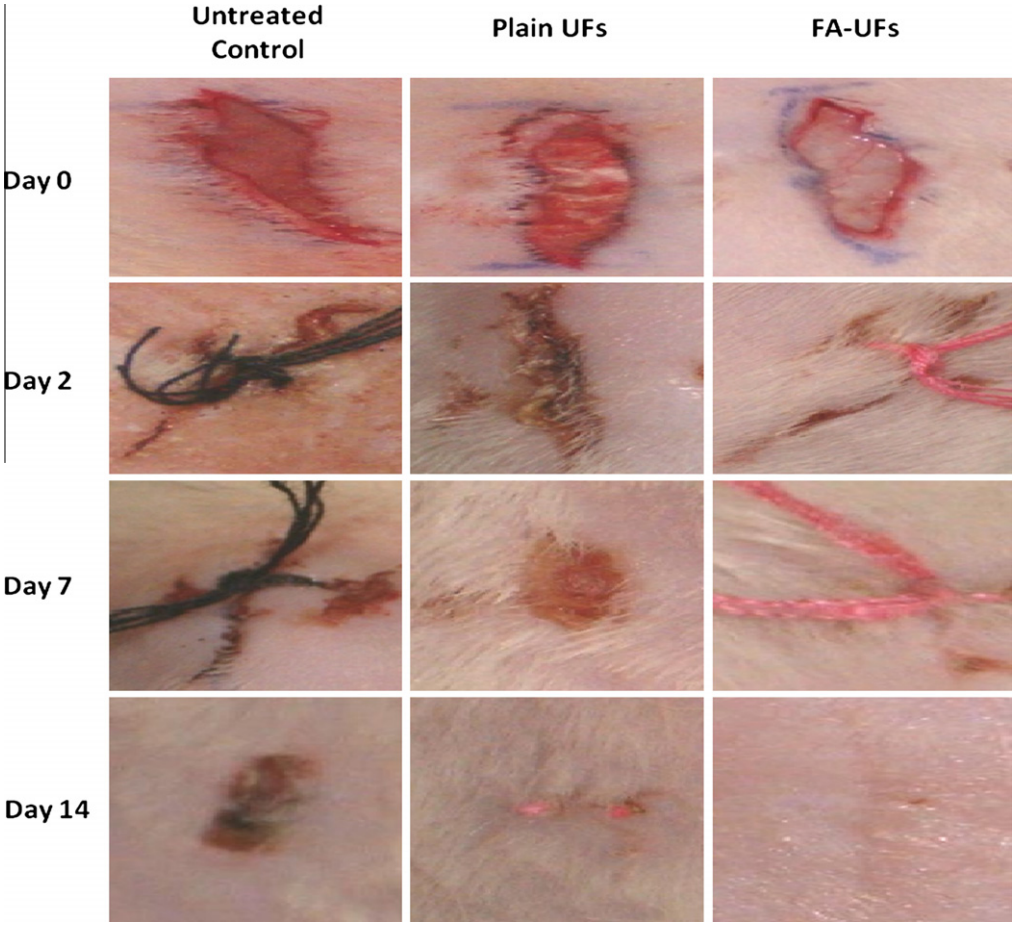

Fig. 7. Effect of plain and FA-loaded PLGA UFs on the healing of heavily Sa_{st} infected wounds in rats.

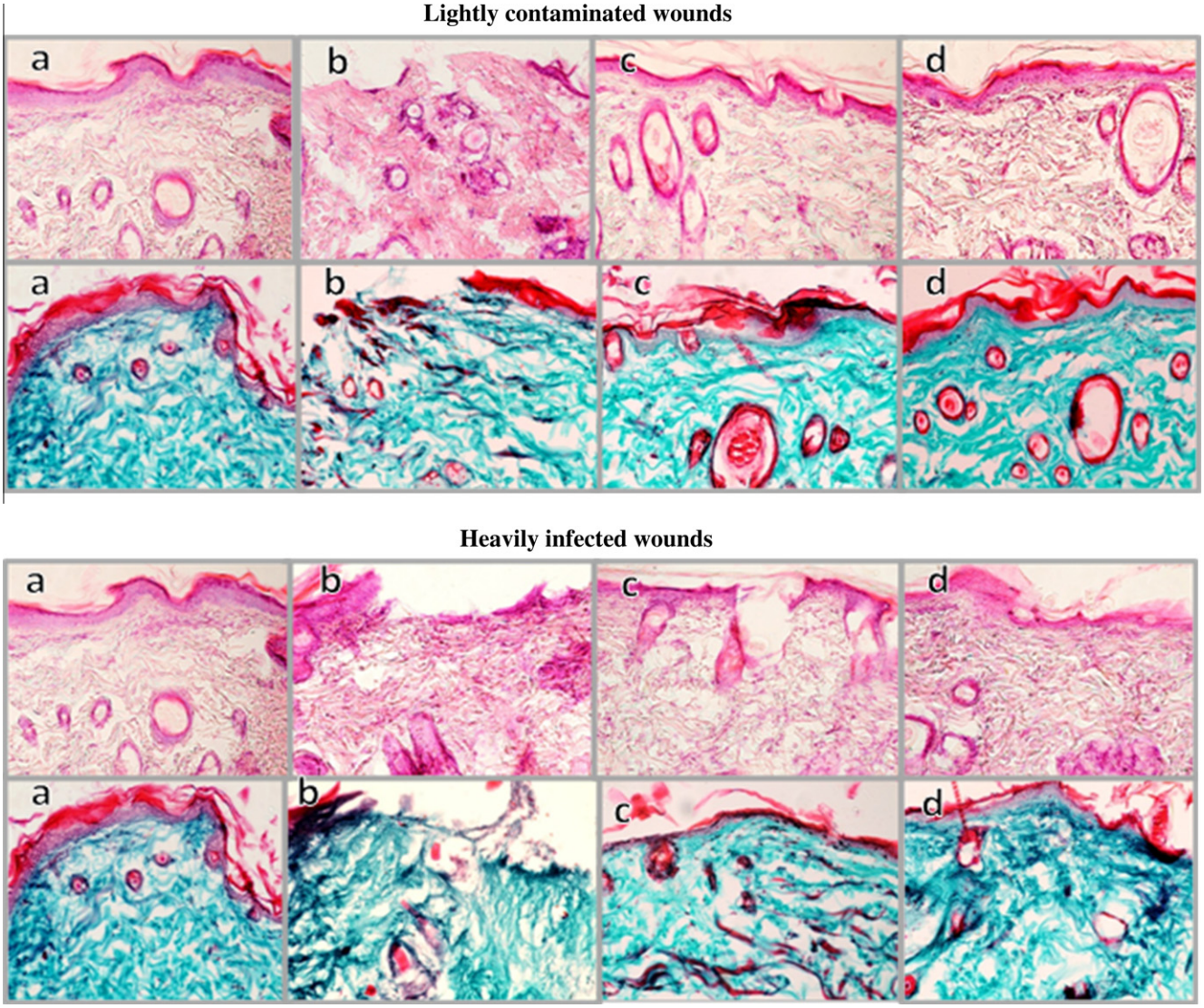

Fig. 8. Histological examination of lightly contaminated and heavily Sa_{st} infected wounds in rats at day 14. (a) Normal skin, (b) control wound, (c) plain UFs and (d) FA-loaded UFs. Upper: H and E stain ×200; lower: Masson's trichrome stain ×200.

According to the general mechanism of responsive behavior of polymer devices [21,39], bioburden responsiveness of FA-loaded PLGA UFs in the present study can be described as a polymer-based triggered drug release mechanism in response to microbial stimuli inherently present in wounds. The mechanism depends on two requirements, enzyme sensitive biodegradability of the polymer substrate and microbial enzymes capable of directing the interactions that lead to macroscopic polymer transitions [35].

3.5. In vivo wound healing properties of FA-loaded PLGA UFs in rats

The effect of the UFs under study on the healing of lightly contaminated and heavily Sa_{st}-infected wounds in female albino rats was assessed by microbiological, morphological, and histological examination of the wounds following a single application of plain or FA-loaded 5 mg-samples of UFs (45 μg FA/mg UFs).

Results of microbial examination are shown in Table 1. In general, swabs of initially clean wounds (Experiment 1) showed light contamination with autogenous normal skin bacteria that persisted throughout the study. These were suppressed upon treatment with plain UFs pointing to effective sequestering of bacteria and prevention of bacterial dissemination. A mild inflammatory response known for PLGA [14] appeared as slight swelling. This faded out by time and did not retard wound healing. Incisions treated with FA-loaded UFs and their internal controls remained clean throughout the study (Table 1). This was indicative of early and sustained eradication of bacteria that reflected in-wound relevant antimicrobial release characteristics. Internal control incisions interestingly remained microbially clean. No fatalities were recorded in the lightly contaminated wound experiment. Statistical analysis of data of all groups compared to the control group (No UFs) was carried out using two-tailed paired *t*-test and GraphPad Instat 3.10. In all cases, differences were statistically significant at $p < 0.05$ except for the mean of the differences between the control group and the internal control group of plain UFs.

A generally similar pattern was observed for Sa_{st}-infected incisions (Experiment 2, Table 1) though infection was expectedly much heavier. *Staphylococcus aureus* was selected being the most common pathogen isolated from wounds [40]. Untreated control wounds showed widespread infection dissemination and 67% fatalities during the first 2 days. However, infection subsided toward the end of the study due to the natural antimicrobial defense

mechanisms of the rats [41]. A marked containment of infection was noted upon application of plain UFs, most probably on account of bacterial sequestration and control of infection dissemination. Crucially, FA-loaded UFs totally prevented infection during the early stage of wound healing and throughout the process providing 100% protection of the rats. This implied initial and sustained FA release in concentrations sufficient to respond to the increasing demand of the wound, protecting new tissues from reinfection. Differences between means of the control group and all test groups were statistically significant at $p < 0.05$. Immobilization of potentially pathogenic bacteria by antimicrobial wound dressings of the electrospun polymer ultrafine fibrous [18] or hydrofiber type [42] was shown to reduce wound bioburden, which contributes to an environment supportive to wound healing.

Morphological examination of lightly contaminated and heavily infected wounds by digital photography generated the photographs' arrangements in Figs. 6 and 7, respectively. Untreated incisions did not close in all rats during the study period. Wound healing was enhanced by plain UFs and much more enhanced by FA-loaded UFs in terms of speed, quality (minimal scarring), reduced infection dissemination, and fatalities (Table 1). Such a superior performance can be attributed to the multifunctionality of antimicrobial UFs mats as cell regeneration guiding structure and a responsive antimicrobial controlled delivery system up-regulated by bioburden-triggered drug release. In all cases, UFs were integrated within the wound during healing.

Results of histological examination using H and E and Masson's trichrome staining are shown in Fig. 8 for lightly contaminated and heavily infected wounds. Control lightly contaminated wounds showed discontinuous epidermis associated with dermal infiltration of a large number of leukocytes. Application of plain UFs led to relative restoration of epidermal and dermal tissue with a mild increase in collagen content. Application of FA-loaded UFs resulted in remodeling of collagen fibers toward normal architecture, an effect usually visualized by Masson's trichrome staining [43]. For heavily infected wounds, control wounds showed interrupted epidermis with cellular infiltration and early scar development. Treatment with plain PLGA UFs restored normal epidermis though inflammatory cellular infiltration was visible and healing was incomplete. Importantly, FA-loaded UFs enhanced skin epithelialization with full development of the epidermis, appearance of multiple hair follicles and remodeling of collagen fibers. This implied lack of cytotoxicity, functional activity of fibroblasts, and keratinocytes in addition to effective utilization of various cells and growth factors in the wound milieu in accelerating the healing process [44].

Microbiological, morphological, and histological in vivo wound healing results following a single application of FA-UFs showed successful prevention of wound infection that killed 67% of control rats and maintenance of microbiological cleanliness of both lightly contaminated and heavily infected wounds throughout the healing period. In addition, the speed and quality of healing were enhanced, and UFs were completely bioresorbed. In vivo data coupled with bioburden-triggered drug release data (Fig. 1) provide strong evidence of the infection responsive antimicrobial activity of the FA UFs under study.

4. Conclusions

Findings obtained contribute to a better understanding of the material properties-function relationship of PLGA UFs as wound healing material under biorelevant conditions. The polymer-based bioburden-triggered drug release enhancement effect observed might be useful in the development of antimicrobial wound dressing materials capable of up-regulating their function in response to the severity of wound infection. With further advancement in polymer engineering, it may be possible to synthesize biomaterials that incorporate enzyme-sensitive linkages to mediate inherent dynamic processes in diverse biomedical applications. From a practical standpoint, antimicrobial PLGA UFs in this study offer promise as wound dressing with polymer-based bioburden responsive properties.

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