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Research paper

Antimicrobial PLGA ultrafine fibers: Interaction with wound bacteria

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ABSTRACT

The structure and functions of polymer nanofibers as wound dressing materials have been well investigated over the last few years. However, during the healing process, nanofibrous mats are inevitably involved in dynamic interactions with the wound environment, an aspect not explored yet. Potential active participation of ultrafine fibers as wound dressing material in a dynamic interaction with wound bacteria has been examined using three wound bacterial strains and antimicrobial fusidic acid (FA)-loaded electrospun PLGA ultrafine fibers (UFs). These were developed and characterized for morphology and in-use pharmaceutical attributes. In vitro microbiological studies showed fast bacterial colonization of UFs and formation of a dense biofilm. Interestingly, bacterial stacks on UFs resulted in a remarkable enhancement of drug release, which was associated with detrimental changes in morphology of UFs in addition to a decrease in pH of their aqueous incubation medium. In turn, UFs by allowing progressively faster release of bioactive FA eradicated planktonic bacteria and considerably suppressed biofilm. Findings point out the risk of wound reinfection and microbial resistance upon using non-medicated or inadequately medicated bioresorbable fibrous wound dressings. Equally important, data strongly draw attention to the importance of characterizing drug delivery systems and establishing material–function relationships for biomedical applications under biorelevant conditions.

1. Introduction

Cutaneous wound healing is a dynamic process involving overlapping phases of hemostasis, inflammation, tissue regeneration, and remodeling with scar formation [1,2]. The healing process can be delayed by diverse factors, mainly bacterial colonization and infection of the wound [3]. Healing acceleration with functional and esthetic results remains the main goal of efficient wound care. Wound dressings play a key role in this respect by providing a mechanical protective effect, an optimum microenvironment for tissue regeneration and by controlling bacterial infection [4]. A wide range of passive, interactive, and bioactive wound dressing materials with different clinical merits have been developed [4,5]. Since the turn of the millennium, ultrafine fibers and nanofibers with a diameter ranging from several micrometers down to tens of nanometers have evolved as a soft porous scaffold for tissue regeneration and wound healing applications [5,6]. Their unique structural and functional properties have demonstrated the potential to revolutionize wound management. Nanofibers are fabricated by different methods, most commonly electrospinning using well-documented processing and characterization methodologies [7,8]. A wide variety of natural and synthetic polymers, polymer blends, copolymers as well as hybrid and composite polymer systems are used for their production [5,7].

As wound dressing biomaterials, nanofibrous mats perform two important functions, temporary substitute for the native ECM and potential carrier system for the controlled delivery of antibacterial agents and other wound healing enhancers. Because of their resemblance to the fibrillar highly porous structure and size scale of the native ECM, plain nanofibers inherently promote the hemostasis phase of wound healing and initiate tissue repair by facilitating cell attachment and proliferation [5,9]. They reduce wound scarring by giving cells a better roadmap for self-repair [5]. Moreover, nanofibrous mats promote wound cleanliness by restricting bacterial invasion via the sieve effect. The role of nanofibrous wound dressings can be further enhanced by functionalization with antimicrobial drugs and other wound healing promoters such as silver nanoparticles [10] and bioactive agents [11]. The large surface area of the nanofibers results in efficient drug release by mass transfer [7], a process that can be modulated by controlling characteristics of the nanofibrous membrane [12,13] and by functionalization with drug-loaded nanoparticles incorporated or adsorbed on nanofibers [14,15] as well as surface graft polymerization [16].

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Studies dealing with polymer nanofibrous wound dressing materials usually focused on the implication of their structure and antimicrobial function in effective wound healing [17,18]. However, eventual infection control and tissue repair involve an inevitable dynamic interaction of the fibrous mat with the wound environment including bacteria. Irreversible attachment of bacterial cells to a substratum or interface resulting in colonization and biofilm formation has been recently reviewed [19]. Colonization of nanofibers with wound bacteria might considerably affect their characteristics as wound dressing materials. Surprisingly, there have been virtually no literature reports documenting the interaction of nanofibers with wound bacteria, particularly the potential effect of wound bacteria on the structural integrity and functions of nanofibrous mats.

The objective of the study was to gain more insight into potential nanofibers-wound bacteria interactions. To this end, antimicrobial biodegradable electrospun fusidic acid (FA)-loaded PLGA ultrafine fibers as wound dressing material were developed and characterized. PLGA has been selected as an FDA-approved biodegradable and biocompatible copolymer. PLGA with different glycolic acid to lactic acid ratio produces fibers with suitable mechanical properties and a wide range of diameters and degradation rates [5]. Because of PLGA hydrophobicity, it is electrospun from organic solvents which allows faster electrospinning at a lower voltage compared to electrospinning settings required for water soluble polymers such as PVA [5,7]. The developed electrospun fibrous mats were used to examine in vitro interaction with three wound bacterial strains: *Pseudomonas aeruginosa*, *Staphylococcus aureus* standard strain, and methicillin-resistant ($MRSA_1$) clinical isolate. The study addressed the effect of wound bacteria on the structural integrity and function of the ultrafine fibrous mat, mainly in terms of matrix degradation and drug release properties and the effect of the FA-loaded mat characteristics on the in vitro antimicrobial activity.

2. Materials and methods

2.1. Materials

The following materials were used: Fusidic acid (gift of Pharaonia Pharm. Co., Alexandria, Egypt), Medisorb® Poly (lactide-co-glycolide) (PLGA) 50:50 DL 3A, MW 50 kD and inherent viscosity 0.36 dl g^{-1} (Alkermes, Inc., Cincinnati, Ohio, USA), dichloromethane (DCM), Biotech. grade 99.9% (Sigma. Aldrich, USA), absolute ethanol, potassium dihydrogen orthophosphate, sodium hydrogen phosphate dibasic, sodium chloride, and sodium hydroxide, analytical grade (Adwic, El-Nasr Pharmaceutical Co., Egypt), and nutrient agar (Oxoid Ltd., Basingostok, Hampshire, England). Bacterial strains used were standard *S. aureus* ATCC 6538P (Sa_{st}) and *Ps. aeruginosa* ATCC 9027 (Ps_{st}) strains and a methicillin-resistant *S. aureus* clinical isolate ($MRSA_1$) isolated 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.

2.2. Development and characterization of plain and FA-loaded PLGA ultrafine fibers (UFs)

PLGA ultrafine fibers (UFs) were prepared using the electrospinning technique [17,18] in an air-conditioned laboratory at an ambient temperature of ≈25 °C and relative humidity of <65%. The electrospinning apparatus was equipped with 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). The distance between the spinneret and the fiber collector was kept constant at 10 cm. PLGA solution (5 ml) in DCM was gravity-fed to the spinneret. A copper collector covered with aluminum foil and a nonwoven synthetic porous mat for the ease of peeling of the electrospun mat was used. For the preparation of FA-loaded UFs, the drug was dissolved in the DCM polymer solution. Preliminary trials were made to adjust the processing parameters. The effect of two formulations variables, polymer content and initial drug loading, was examined.

2.3. Characterization of PLGA ultrafine fibers (UFs)

2.3.1. Scanning Electron Microscopy (SEM)

Samples of UFs were mounted on metal stubs using double-sided adhesive tape onto which the UF fibrous meshes were fixed. The samples were then coated with gold using an ion sputtering coater (JFC-1100E, JEOL, Japan) and the gold coated samples scanned using SEM (JEOL, model JFC-1100E, Japan). The mean fiber diameter was determined using image analysis software and at least 10 randomly selected fiber segments.

2.3.2. Determination of drug content and % entrapment efficiency

FA was extracted from UFs by shaking an accurately weighed amount (20 mg) of drug-loaded UFs in 10 ml absolute ethanol in well-closed screw-capped 20 ml vials at ambient temperature (≈25 °C). Vials were shaken intermittently for 24 h, time proven sufficient for complete drug extraction. A 1-ml sample was diluted threefold with absolute ethanol, and FA concentration was determined spectrophotometrically in absolute ethanol at λmax, 220 nm using UV–Visible spectrophotometer (Thermospectronic, Helios alpha, NC 9423 UV A 1002E, England). The % entrapment efficiency was calculated using the following equation:

$$\text{Entrapment efficiency } \% = \frac{\text{Weight of drug in UFs}}{\text{Theoretical drug loading}} \times 100$$

Results are the average of three determinations.

2.3.3. Differential scanning calorimetry (DSC)

The thermal behavior of the electrospun fibers was investigated by DSC (DSC-6, CT, Perkin Elmer instruments, USA) under a nitrogen atmosphere. DCS traces were recorded between 25 and 400 °C at a constant rate of 10 °C/min. Indium standard was used to calibrate the DSC temperature and enthalpy scale. An empty pan was used as reference.

2.3.4. Degradation of PLGA UFs

Degradation of plain and FA-loaded PLGA UFs was assessed at 37° in PBS pH 7.4 as degradation medium by monitoring the change in pH [20] using a digital pH-meter (Schott Geräte CG-820, Germany). Samples of PLGA UFs mats were immersed in 10 ml of the degradation medium in 20-ml screw-capped glass vials for 60 days without agitation. Prior to each measurement set, the pH-meter was calibrated and data reproducibility checked by replicate pH measurement of selected samples. Results are the average of two measurements.

2.3.5. In vitro drug release

Samples of FA-loaded UFs mats, 2×2 cm^2, were immersed in PBS pH 7.4/1% ethanol as release medium in capped Erlenmeyer flasks containing 30 ml of the medium. The medium was selected based on a preliminary solubility study. 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 the time specified. At scheduled time intervals, 3 ml of the release medium was withdrawn, filtered using 0.45-μm Millipore filter, and replaced with the same volume of fresh medium adjusted to 37 °C. The concentration of released FA was measured spectrophotometrically at λmax 207 nm [21]. UV spectra were recorded to check for reproducibility and lack of interference. All release experiments were run in triplicate. The effect of medium compensation on release data was corrected for. Kinetics of FA release from PLGA UFs were investigated.

The release procedure was used to study the effect of polymer content and initial drug loading on FA release from test UFs. It was also used for the modulation of release characteristics by surface deposition of free FA on UFs and UV exposure as well as for the release stability of UFs.

2.3.5.1. Effect of surface deposition of free FA on preformed UFs. A $4 \times 4\ cm^2$ electrospun 10% FA-loaded 25% PLGA ultrafine fibrous mat was electrosprayed with a FA solution, containing 20% of the amount of drug-loaded in the test mat in the least volume of absolute ethanol using the electrospinning apparatus. A 0.7-mm-diameter spinneret set at an angle of 45° was used. The test UF mat was fixed to the collector at a 10-cm distance from the spinneret.

2.3.5.2. Effect of UV exposure. Electrospun 10% FA-loaded 25% PLGA UFs mats were irradiated using a UV lamp (λ = 254 nm) at a distance of about 1.25 m for 1, 2, and 3 h prior to the release study.

2.3.5.3. Release stability of FA-loaded UFs. Five percent of FA-loaded 25% PLGA UFs mat samples were stored in well-sealed containers in the refrigerator (2–8 °C), protected from light and humidity. Drug release was assessed at zero time, 6 months and 12 months. The difference in release data obtained at 6 and 12 months was tested for statistical significance using Kruskal–Wallis (nonparametric ANOVA). This was followed by Dunn's multiple test to compare each release profile with that obtained at zero time using GraphPad InStat Software.

2.4. Interaction of PLGA UFs with wound bacteria

2.4.1. Effect of wound bacteria on PLGA UFs

Three bacterial strains likely to infect wounds were used in the study. These include *S. aureus* ATCC 6538P (Sa_{st}), *Ps. aeruginosa* ATCC 9027 (Ps_{st}), and a methicillin-resistant *S. aureus* clinical isolate ($MRSA_1$) from an infected wound.

2.4.1.1. Bacterial colonization of UFs and biofilm formation. This was investigated by exposing plain UFs samples to overnight cultures of $MRSA_1$ wound isolate and determining the viable count of adhering bacteria. Samples of PLGA UFs ($2 \times 2\ cm^2$) were left in contact with 3 ml nutrient broth inoculated with $MRSA_1$ ($\sim 10^8$ CFU/ml) for 24 h at 37 °C. At the end of the incubation period, UFs samples were removed aseptically, washed twice with sterile saline to remove planktonic bacteria attached to the fibers' surface, and sonicated for 10 min in 5 ml sterile saline. The dispersions obtained were serially diluted tenfold with sterile saline for viable count determination. The results were presented as % bacterial viability calculated from the maximal viability counts. UFs were also examined morphologically using SEM.

2.4.1.2. Effect of in vitro pre-exposure to wound bacteria on drug release and UFs degradation. The effect of pre-exposure of 5% FA-loaded 25% PLGA UFs to the three bacterial strains under study on the release of FA was assessed using sample mats, $2 \times 2\ cm^2$, with known drug content, determined based on % EE and UFs sample weight. The mats were pre-exposed to wound bacteria in vitro for 24 h at 37 °C by incubation with overnight broth cultures of the test strains properly diluted with sterile saline to obtain inocula containing about 10^7 CFU/ml. Adequate controls were included in which the UFs were incubated in sterile broth diluted with sterile saline. Test and control UFs were removed and washed with sterile saline. Drug release from UFs pre-exposed to wound bacteria was assessed as described under release studies. To test the effect of in vitro pre-exposure to wound bacteria on UFs degradation, plain and 5% FA-loaded 25% PLGA UFs samples weighing 50 mg were pre-exposed to the three test bacterial strains under similar conditions. The UFs degradation procedure was completed as described under degradation study.

2.4.2. Effect of FA-loaded UFs on wound bacteria

Preliminary investigations were carried out to assess the sensitivity of the 3 test organisms to FA by determining MIC using the broth macrodilution method. Further, the bioactivity of FA released from PLGA UFs was tested by comparing the antibacterial effect of a solution of FA released from 10% FA-loaded 15% PLGA UFs after determining its concentration with that of a solution of the free drug of equal concentration using the agar diffusion method. Further, the effect of FA loading (5%, 10%, and 15%) of 25% PLGA UFs on their antibacterial activity was determined against the three test bacterial strains using the agar diffusion technique. Weights of ~10 mg of FA-loaded and plain UFs were disinfected by brief immersion (~20 s) in 70% ethanol and drying in closed sterile Petri dishes. The UFs mats were applied to the surface of Mueller–Hinton agar plates. Release of FA from the UFs was allowed for 24 h at 37 °C. The plates were then inoculated and incubated at 37 °C for 24 h. The diameters of inhibition zones were measured in millimeters. Results are the average of two experiments.

2.4.2.1. Effect of FA-loaded UFs on planktonic bacteria. The antibacterial effect of 10% FA-15% PLGA UFs on planktonic bacteria of the test strains was investigated using the dynamic viable count method. Test UFs samples weighing ~20 mg were disinfected with 70% ethanol and immersed in 13.5 ml sterile nutrient broth in sterile flasks. Pre-incubation release of FA from the UFs was allowed for 24 h in nutrient broth at 37 °C. Each flask was then inoculated with 1.5 ml of diluted inoculum ($\approx 10^7$ CFU/ml) of the test strains at the end of the pre-incubation release period. The systems were mixed well and incubated at 37 °C for 24 h. Samples, 0.5 ml each, were aseptically withdrawn from each flask and serially diluted tenfold with sterile saline for viable count determination using over-dried nutrient agar plates. The plates were incubated at 37 °C for 24 h. The % bacterial killing was calculated from the maximal viability counts determined from control bacterial cultures not exposed to UFs. The procedure was used to assess the sustainability of antibacterial activity over a 48-h incubation period.

2.4.2.2. Effect of FA-loaded UFs on bacterial colonization and biofilm. This was investigated by exposing $2 \times 2\ cm^2$ UFs samples to $MRSA_1$ wound isolate and estimating the count of adhering bacteria using the viable count technique as described above. Four types of UF samples were used: PLGA UFs as control, 10% FA-loaded UFs, plain UFs impregnated with sodium fusidate solution (2 mg/ml) for 2 h at 37 °C, and 10% FA-loaded UFs similarly impregnated with the sodium fusidate solution. Results were presented as % bacterial viability calculated from the maximal viability counts. UFs were also examined morphologically using SEM.

3. Results and discussion

3.1. Development and characterization of plain and FA-loaded PLGA ultrafine fibers (UFs)

3.1.1. Morphology by SEM

Plain electrospun fibers obtained had a smooth surface and showed a relatively broad diameter distribution. Their mean diameters ranged from 200 nm to 2 μm. Fibers in both the micron and sub-micron ranges can be referred to collectively as ultrafine fibers, UFs [22]. Fig. 1 shows the effect of PLGA content (15%, 20%, and 25%) on the morphology of plain PLGA UFs. As demonstrated previously [23], increasing PLGA content resulted in progressively reduced beading, increased fiber diameter, and reduced diameter distribution.

Increasing initial drug loading of 25% PLGA UFs led to in a slight increase in fiber diameter (Fig. 2). Entrapment of unionized drugs generally results in increased nanofiber diameter [24]. However, change in fiber diameter of electrospun membranes was shown to have a little effect on in vivo wound healing efficiency [25]. SE micrographs showed no FA crystals on the surface or within the UFs mesh. This precludes phase separation upon rapid solvent evaporation during electrospinning as the drug tends to remain inside the fibers where enough solvent is left [26].

3.1.2. Entrapment efficiency percent (% EE)

The % EE values were relatively high. Data for 10% FA-loaded UFs with 15%, 20%, and 25% polymer content ranged from 92.4% to 86.6%. On the other hand, increasing drug loading at a fixed 25% PLGA content resulted in lower % EE (89.7%, 86.6%, and 72.0% corresponding to 5%, 10%, and 15% initial drug loading, respectively) because of the lower FA-saturated solubility in the electrospinning polymer solution. An inverse relationship between initial loading of lipophilic drugs and % EE has been observed by Zamani et al. [27].

3.1.3. Differential scanning calorimetry (DSC)

Fig. 3 shows DSC thermograms for FA, PLGA, FA-PLGA physical mixture (1:9), and 10% FA-25% PLGA UFs. Thermogram for FA showed a sharp endothermic peak at 190.97 °C (melting) while that of PLGA showed an endothermic inclination at 47.34 °C (glass transition temperature, Tg) [28] and a peak at 336.28 °C corresponding to thermal decomposition. The thermal curve for the physical mixture showed peaks for individual components. However, thermogram for FA-loaded UFs revealed disappearance of FA endothermic peak, suggesting molecular dispersion of FA in the fiber matrix and lack of phase separation.

3.1.4. Degradation of PLGA UFs

The effect of polymer content and initial drug loading on the hydrolytic degradation of plain PLGA UFs in PBS pH 7.4 at 37 °C was assessed by monitoring pH lowering for 60 days and by SEM imaging. Hydrolytic degradation of 15% PLGA UFs with the release of acid products resulted in a progressive decrease in pH from 7.4 to 5.4 in 60 days (Fig. 4a), a behavior consistent with the chemical stability of ester polymers [29,30]. Increasing polymer content slightly increased the fiber resistance to hydrolysis. A slightly more rapid decline in pH was noted upon incorporation of FA (Fig. 4b). Different types of drugs were observed to affect the degradation of PLGA matrices to various extents [31,32]. Matrix degradation data are crucial, particularly when bioresorbable wound dressing materials are considered.

The change in morphology of FA-loaded UFs upon exposure to the degradation medium for 30 days is shown in Fig. 5. A marked increase in fiber diameter with fiber fusion at some crossing points was obvious, an observation reported recently by Puppi et al. [30] for retinoic acid-loaded electrospun fibrous meshes. Further, loss of structural integrity was associated with drug crystals expulsed to the fibers' surface.

3.1.5. Drug release studies

The effect of polymer content and initial drug loading on the release of FA at 37 °C in PBS pH 7.4 containing 1% ethanol is shown in Fig. 6a and b, respectively. FA release was sustained for at least 9 days with a limited burst effect and complete FA liberation. Absence of extensive burst release pointed out drug compatibility with the polymer/solvent electrospinning solution while the sustained release pattern confirmed adequate UFs structural integrity throughout the study.

Interestingly, increasing PLGA content of UFs resulted in a change in release pattern (Fig. 6a). Biphasic release profiles were obtained for UFs with 15% and 20% PLGA with complete drug release at 9 and 11 days, respectively. The initial faster release phase relates to diffusion of drug molecules lodged near the fiber surface, facilitated by the large surface area of UFs and the presence of ethanol in the medium. The second slower release phase represents Fickian drug diffusion through the polymer matrix in addition to diffusion through water-filled pores generated progressively by drug release [24]. Appearance of a third phase in the release profile of UFs with the highest PLGA content (25%) was noteworthy (fig. 6a). This possibly reflected further formation of water-filled channels upon diffusion of nearly 60% of FA by the end of the second phase (≈9 days) in addition to progressive matrix erosion as revealed by degradation data. Release data were generally in accordance with the mechanisms of drug release from biodegradable cylindrical matrices [27,33].

Release profiles for 25% PLGA UFs with increasing drug loading were expectedly triphasic as implied by the polymer content (Fig. 6b). A greater % of entrapped drug led to faster initial drug release and earlier complete drug liberation. Incorporation of larger amount of drug into nanofibers impelled drug molecules to migrate toward the surface during electrospinning as reported by

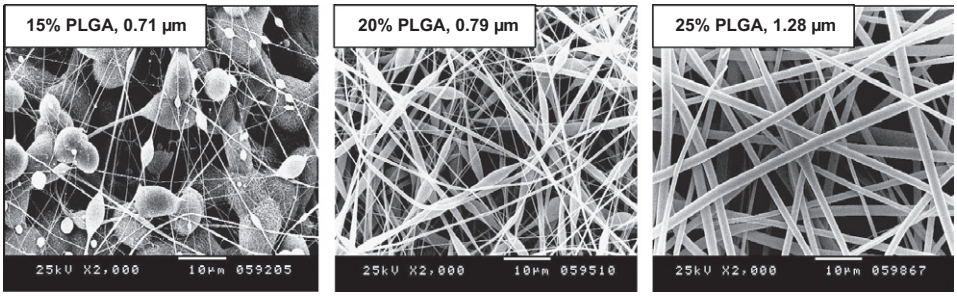

Fig. 1. SE micrographs showing the effect of PLGA content on fiber morphology and average diameter.

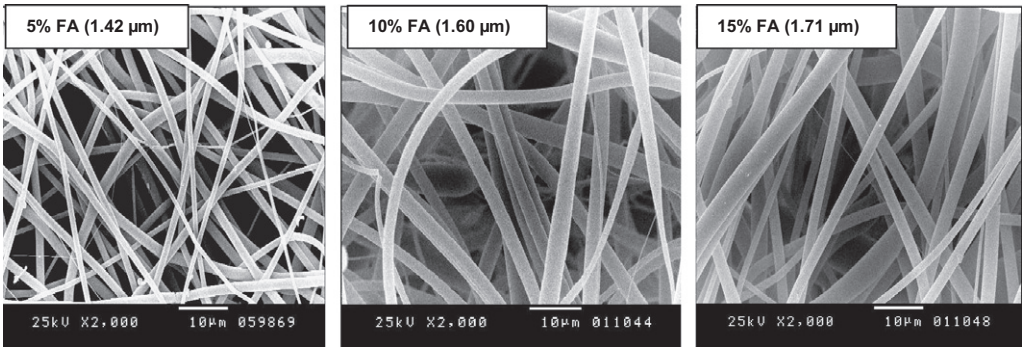

Fig. 2. SE micrographs showing the effect of initial fusidic acid (FA) loading on fiber morphology and average diameter.

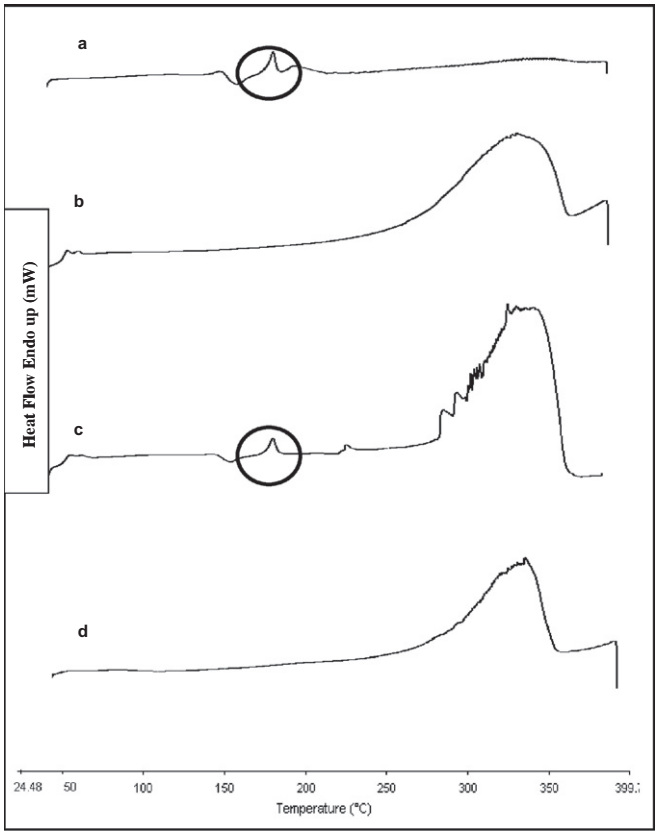

Fig. 3. DSC thermograms of (a) FA, (b) PLGA, (c) 10% FA-PLGA physical mixture, and (d) 10% FA-25% PLGA UFs.

Zamani et al. [27]. This effect combined with enhanced matrix porosity induced by drug depletion explained the data obtained.

FA release kinetics were examined by subjecting release data to zero-order, first-order and Higuchi's square root of time mathematical models. The largest r^2 values were obtained for the Higuchi model (Table 1) indicating a diffusion-controlled release mechanism. The relatively smaller r^2 value for 25% PLGA UFs can be accounted for by faster drug release during the third release phase.

Two attempts have been made to modulate FA release from PLGA UFs aiming at enhancing initial drug release to provide an early antimicrobial effect crucially required during wound healing to eradicate intruding bacteria before they proliferate. In the first attempt, FA was electrosprayed on preformed UFs. This resulted in drug deposition on UFs surface with a ≈50% increase in initial release on day one (Fig. 7a). Inclusion of a free drug fraction proved to be a useful approach to modulate drug release from various drug delivery systems [34,35]. The second approach was based on exposure of FA-loaded UFs to UV radiation at 254 nm for 1, 2, and 3 h. Enhanced drug release was noted (Fig. 7b), the effect being maximal following 2-h UV exposure. SEM showed marked changes in fiber morphology (Fig. 7b inset) including surface coarsening, micropore formation, cracking, and appearance of drug crystals on the fibers' surface. Yixiang et al. [36] reported a 46% reduction in the average molecular weight of PLGA and a 26% reduction in the tensile strength of nanofibers upon UV irradiation for 1 h. These data, combined with our release data, point to the influence of exposure of polymer nanofibers to biophysical agents on their structural and functional properties in different applications.

Release stability of UFs was examined as information on structural integrity of fibrous structures upon storage is fundamental for various applications. The effect of aging on the release stability of electrospun fibers has not been documented. Storage of FA-loaded PLGA UFs in well-sealed containers in the refrigerator at (2–8 °C) protected from light and humidity for 6 and 12 months resulted in a moderate increase in drug release (Fig. 8) probably caused by aging-induced cracking and fragmentation although the fibrous structure was maintained (Fig. 8 insets). Statistical analysis of release stability data was performed using Kruskal–Wallis (nonparametric ANOVA) test and Dunn's multiple test [37] to assess the significance of differences between release data obtained at 6 and 12 months and storage release data with their corresponding values at zero time, respectively. Differences proved to be insignificant, which was evident from the overlapping standard deviation of data points (Fig. 8). Despite statistical insignificance, aging-induced structural changes may affect the function of PLGA UFs. Storage of PLGA nanofibers at refrigerator temperature protected from humidity is recommended. Moreover, external conditions should be well controlled during electrospinning to improve nanofibers consistency as demonstrated recently by Hardick et al. [38].

3.2. Interaction of FA-loaded PLGA UFs with wound bacteria

FA-loaded PLGA UFs developed as wound dressing material with good pharmaceutical attributes were used to investigate potential interactions with wound bacteria.

3.2.1. Effect of wound bacteria on PLGA UFs

Incubation of PLGA UFs samples in a broth culture of the wound isolate $MRSA_1$ ($\approx 10^8$ CFU/ml) at 37 °C for 24 h resulted in the retention of a significant number of bacterial cells on the fibers' surface and inside the mesh pores forming a dense biofilm (Fig. 9). Bacterial population of the biofilm reached $\sim 4.375 \times 10^7$ CFU/cm^2, which corresponded to 100% viable count. Data obtained indicated that pristine ultrafine fibers are good templates for the rapid proliferation of bacteria and biofilm formation.

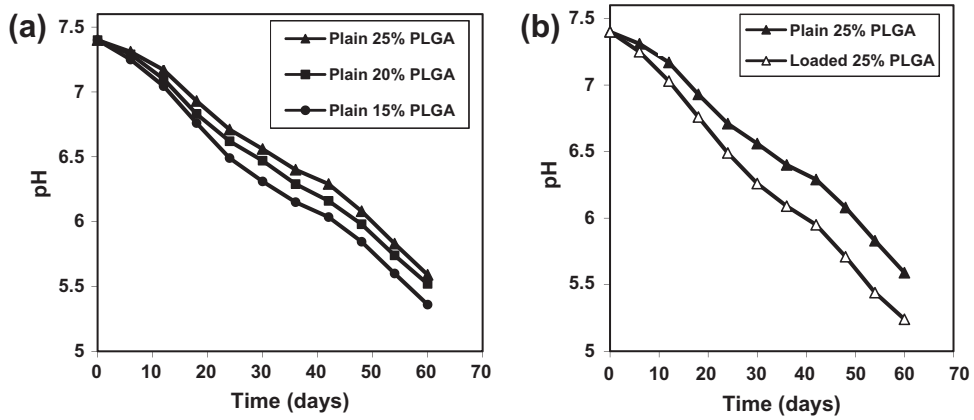

Fig. 4. pH lowering of PBS saline pH 7.4 at 37 °C upon incubation of PLGA electrospun UFs: (a) Plain UFs with different polymer content, (b) 10% FA-25% PLGA UFs.

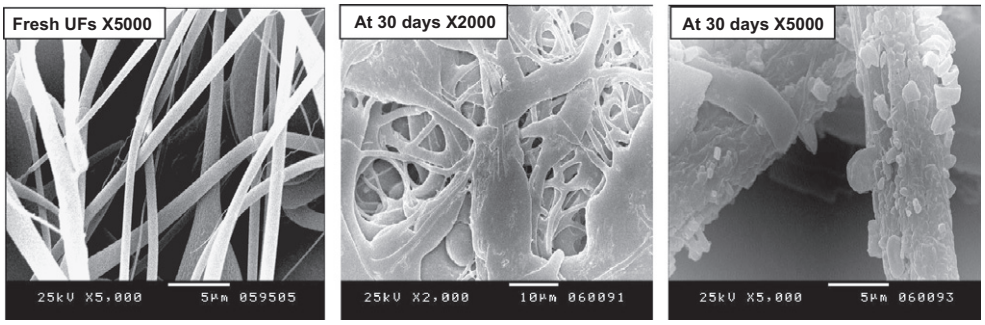

Fig. 5. SE micrographs of samples of FA-loaded PLGA UFs before and after degradation for 30 days in PBS pH 7.4 at 37 °C.

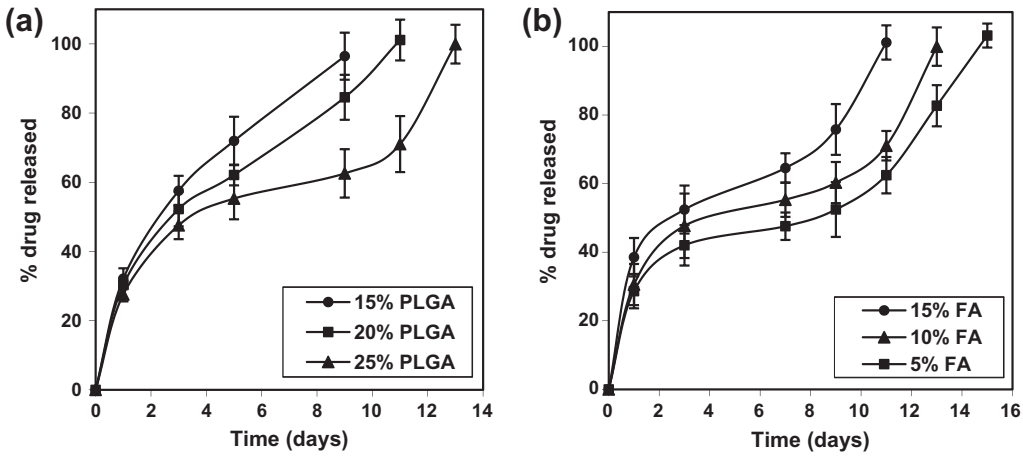

Fig. 6. Release profiles of FA from PLGA UFs with: (a) Different polymer content and (b) Different initial drug loading. Error bars indicate mean±SD.

Table 1
Release kinetics of 10% FA-loaded PLGA UFs with different PLGA content in PBS pH 7.4 containing 1% ethanol at 37 °C.

Model	Zero order		First order		Higuchi	
Equation	$\% Qt = kt$		$\log(100 - \% Qt) = kt$		$\% Qt = kt$	
% PLGA content	r^2	K	r^2	k	r^2	k
15	0.895	8.925	0.968	0.176	0.996	30.990
20	0.922	8.500	0.726	0.150	0.996	28.936
25	0.874	5.886	0.524	0.156	0.935	23.533

Bacterial immobilization by removable non-medicated hydrogel dressings for the management of wounds that are in bacterial balance is considered advantageous based on the minimization of bacterial dispersion upon removal of the dressing [39,40]. How-

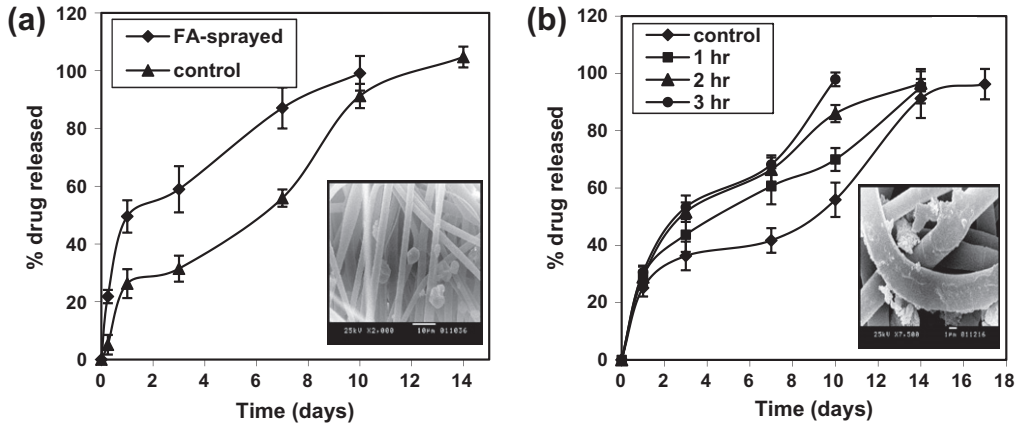

Fig. 7. Modulation of FA release from UFs in PBS pH 7.4 containing 1% ethanol at 37 °C by: (a) electrospraying of 10% FA-25% PLGA UFs with free FA and (b) UV irradiation for 2 h. Insets: SE micrographs of treated UFs. Error bars indicate mean±SD.

ever, for wounds that are infected or at risk of infection and for bioresorbable wound dressings intended to be left in the wound, bacterial sequestration may promote infection and bacterial resistance with delayed wound healing. This must be taken into account when considering non-medicated nanofibers as wound dressing mats.

The effect of in vitro pre-exposure to wound bacteria on the structure and function of FA-PLGA UFs was investigated using drug release and PLGA degradation data. Release profiles for 5% FA-loaded 25% PLGA UFs samples pre-exposed to cultures of the three test bacterial strains at $\sim 10^7$ CFU/ml inoculum size for 24 h at 37 °C showed a marked enhancement of drug release rate, despite the presence of a considerable barrier of bacterial stacks (Fig. 9). Enhanced release rate was notable at earlier time points (Fig. 10a). It could be observed that FA release at day 1 increased from 7.8% (control UFs pre-exposed to sterile broth diluted with sterile saline) to 31.5%, 44.6%, and 51.8% upon incubation with $MRSA_1$, Ps_{st}, and Sa_{st}, respectively. The time for 50% FA release was 6.5, 4.5, 2, and 1 day for control UFs and UFs colonized with $MRSA_1$, Ps_{st}, and Sa_{st} strains, respectively. Almost complete FA release was achieved at day 14, especially for Ps_{st} and Sa_{st} containing systems.

The unpredictably large increase in initial FA release led to the hypothesis that bacterial colonization and/or the associated biochemical changes taking place in the bacterial culture during pre-exposure exerted a considerable effect on surface characteristics or structural integrity of the UFs. Indeed, both bacterial species under study (*S. aureus and Ps. aeruginosa*) were reported to secrete lipolytic esterases [41,42] which may initiate the biocatalytic hydrolysis of PLGA. This was assessed by SEM and pH measurements (Fig. 10b). SEM revealed obvious detrimental changes in UFs morphology. However, reduction in the pH of degradation

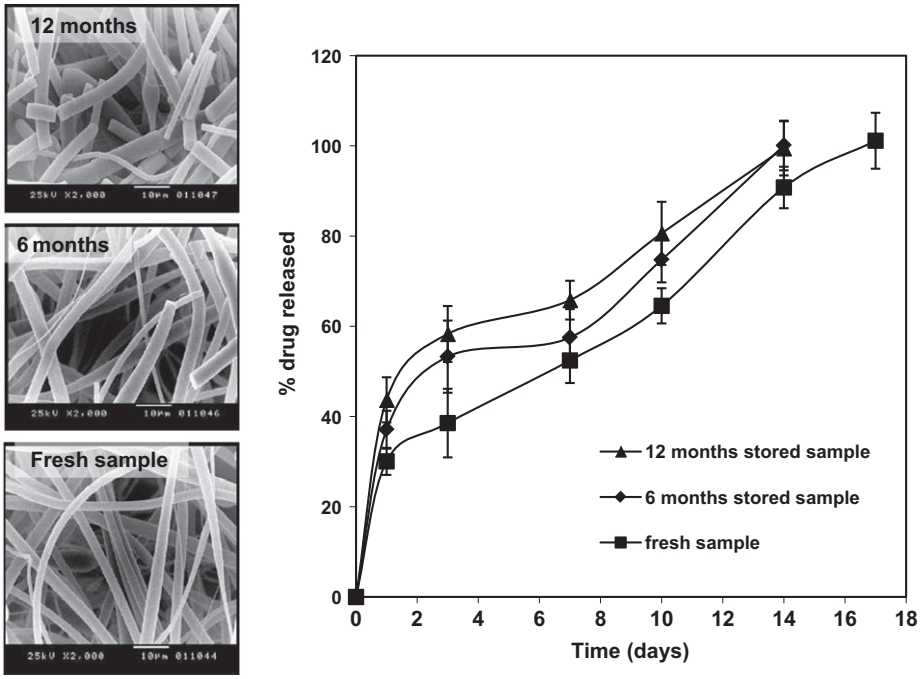

Fig. 8. Effect of aging of 5% FA-25% PLGA UFs at 4 °C on fiber morphology and drug release. Error bars indicate mean±SD.

medium over a 30-day study period (Fig. 10b) did not match the marked enhancement in initial drug release and deterioration of the fibers' structural integrity. An explanation may be the restriction of enzymatic polymer degradation to the UFs surface as the relatively large size of enzymes molecules precludes their penetration into the polymer matrix [43]. Moreover, possible consumption of the acid degradation products by bacteria [44] may obscure the effect of polymer degradation on the pH of degradation medium. Incubation of PLGA nanofibers with porcine smooth muscle cells culture for up to 3 months was reported by Dong et al. [45,46] to accelerate surface erosion of the nanofibers without affecting their mechanical strength.

3.2.2. Effect of FA-loaded PLGA UFs on wound bacteria

The MIC of FA against the $MRSA_1$ isolate, Sa_{st}, and Ps_{st} was 7.8 μg/ml, 3.9 μg/ml and 7.8 μg/ml, respectively. MIC values for the Staph strains were consistent with reported data [47]. The antibacterial effect of a solution of FA released from 10% FA-loaded 15% PLGA UFs against Sa_{st} and Ps_{st} was similar to that of a solution of the free drug of equal concentration (diameter of inhibition zones was 45.3 mm and 33.5 mm against Sa_{st} and Ps_{st}, respectively). Moreover, UV spectra of pure FA solution and FA released from UFs were similar (data not shown). This confirmed that the electrospinning process and entrapment of FA in PLGA UFs did not affect its bioactivity and structural integrity, an essential requirement for antimicrobial delivery systems. The effect of the fibers on antimicrobial activity is shown in fig. 11a and b. Increasing FA loading enhanced the antimicrobial activity of the fibrous mats against the three test organisms.

The effect of FA-loaded UFs on planktonic bacteria was assessed by incubating 10% FA-15% PLGA UFs with bacterial cultures (≈10^7 CFU/ml) of the three test bacterial strains for 8 h following a 24-h pre-exposure drug release period. This resulted in a generally significant bactericidal effect. The % killing ranged from 97.8 to 99.6, Ps_{st} being relatively the least susceptible. A further progressive increase in % killing (>99.9%) was observed by increasing the incubation time to 24 and 48 h. Data indicated that the concentration of FA released during the 24-h preincubation period was bactericidal and that the killing effect was sustained during the 48-h study. This has important practical consequences as antimicrobial wound dressings should retain their bioactivity for in-use relevant periods upon exposure to proliferating bacteria and the associated changes in the wound environment.

The effect of UFs on bacterial colonization and biofilm formation is shown in Fig. 12. While exposure of plain 15% PLGA UFs to an overnight broth culture of $MRSA_1$ for 24 h resulted in massive bacterial colonization and biofilm formation, loading of UFs with 10% FA markedly suppressed the biofilm. Bacterial population of the biofilm was lowered to ∼10% of the maximal biofilm count.

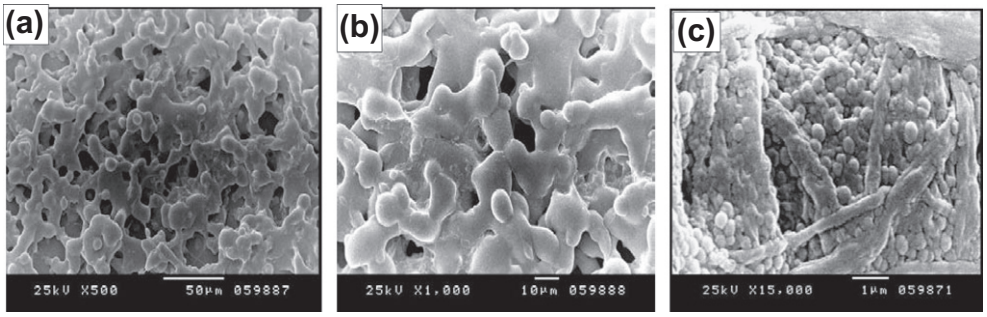

Fig. 9. SE micrographs of plain PLGA UFs samples showing bacterial colonization upon incubation with an overnight $MRSA_1$ broth culture for 24 h at 37 °C. Magnification power: (a) ×500, (b) ×1000, and (c) ×15,000.

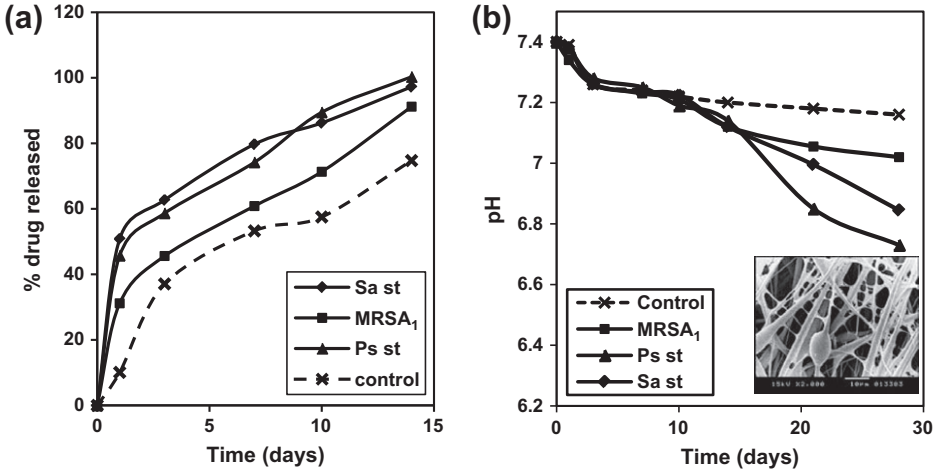

Fig. 10. Effect of pre-exposure of FA-PLGA UFs to an overnight culture of wound bacteria (10^7 CFU/ml) for 24 h at 37 °C on (a) drug release profiles and (b) degradation profiles. Inset: SE micrograph of UFs exposed to an overnight $MRSA_1$ culture (10^7 CFU/ml) for 7 days.

This denoted adequately fast release of bioactive FA in inhibitory concentrations. Surface antimicrobial deposition by impregnation of plain UFs with sodium fusidate solution 2 mg/ml for 2 h resulted in a more or less similar effect (Fig. 12). The bacterial suppression by surface bioactivity observed in the present study was in agreement with the prevention of bacterial colonization of polymer catheters by impregnation with fusidic acid [48]. Further, a dual approach involving 10% FA loading combined with impregnation of UFs with sodium fusidate resulted in further lowering of biofilm survivor count to less than 2% (Fig. 12). These data were supported by SE microscopical investigation of the four test samples for surface adhering bacteria.

Data obtained indicate the ability of FA-loaded PLGA UFs to sequester and inhibit the proliferation of potentially pathogenic wound micro-organisms. Functionalization of wound dressings with antimicrobial agents could prevent turning the positive feature of bacterial sequestration into a microbial resistance and reinfection hazard that may lead to wound healing retardation.

4. Conclusions

This study has shown for the first time that biodegradable polymer ultrafine fibrous wound dressing materials, apart from providing structural support for wound repair and an antimicrobial

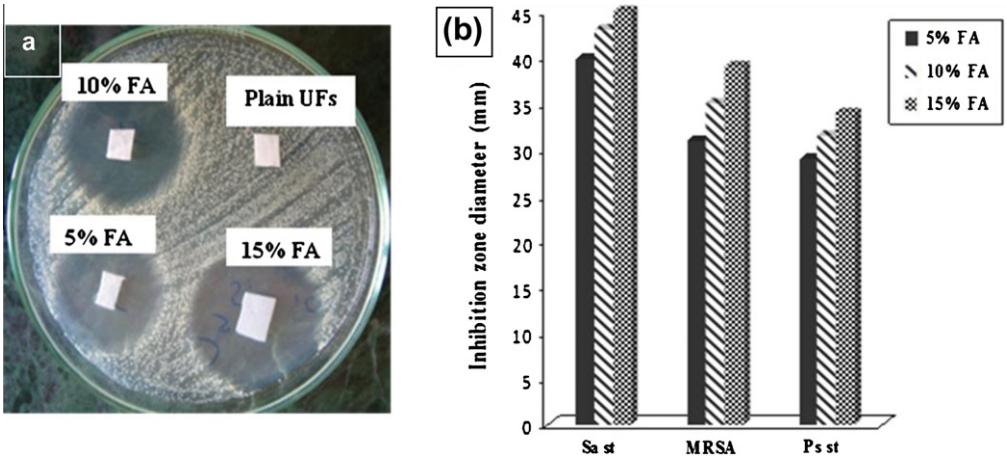

Fig. 11. Effect of FA loading (5%, 10%, and 15%) on the antibacterial activity of 25% PLGA UFs using the agar diffusion method. (a) A sample agar plate showing antibacterial activity against Ps_{st}; (b) Antibacterial activity against the three test bacterial strains.

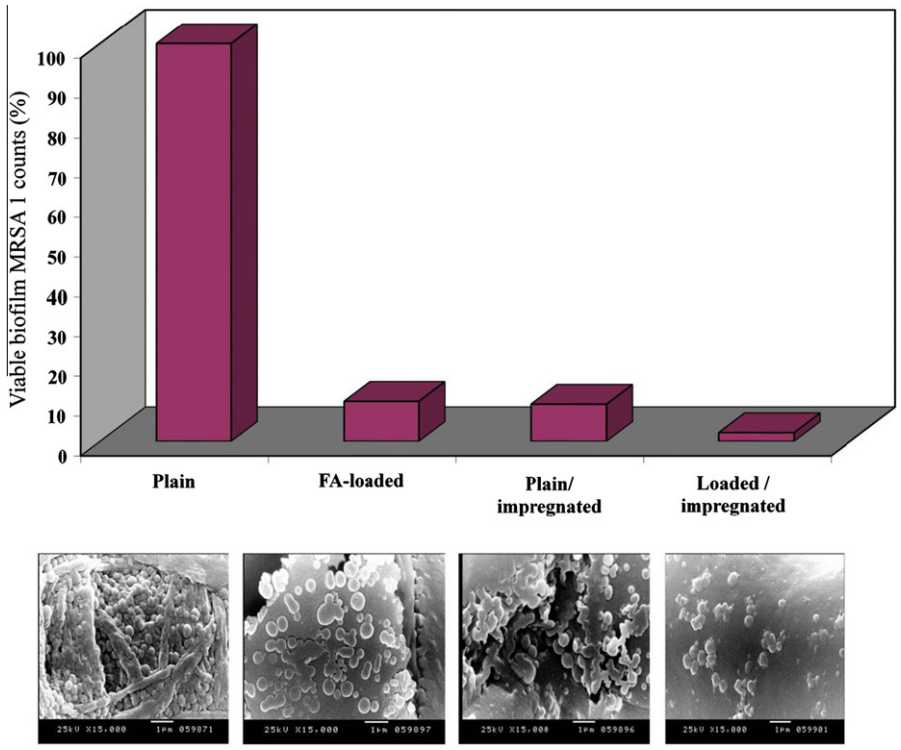

Fig. 12. Effect of 10% FA loading and/or impregnation of PLGA UFs with 0.2% sodium fusidate solution on $MRSA_1$ biofilm density (SEM) and biofilm viable counts %. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reservoir function for infection control of the wound, are actively participating in a dynamic interaction with the wound milieu. Changes observed in mat characteristics would intuitively have important implications in the development and clinical use of nanofibrous wound dressings based on biodegradable controlled release systems. Main concerns in this respect include bacterial colonization and acquisition of resistant surface stacks of bacteria in case of non-medicated mats or antimicrobial release in subinhibitory concentrations; faster degradation of the polymer matrix in addition to faster antimicrobial release. Findings strongly draw attention to the importance of establishing biomaterial characteristics–function relationships under biorelevant conditions. Further work is carried out to gain more insight into the mechanism of interaction of antimicrobial biodegradable PLGA ultrafine fibers with the biochemical wound environment. From a practical standpoint, fusidic acid-loaded PLGA UFs developed in the study offer great pharmaceutical potentials as bioresorbable antimicrobial material for easier wound care.

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