Full Length Article

Star poly(ε-caprolactone)-based electrospun fibers as biocompatible scaffold for doxorubicin with prolonged drug release activity

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ABSTRACT

In this work, a novel drug delivery system consisting of poly(ε-caprolactone) (PCL) electrospun fibers containing an ad-hoc-synthesized star polymer made up of a poly(amido-amine) (PAMAM) core and PCL branches (PAMAM-PCL) was developed. The latter system which was synthesized via the ring opening polymerization of ε-caprolactone, starting from a hydroxyl-terminated PAMAM dendrimer and characterized by means of ^{1}H NMR, IR and DSC, was found to be compatible with both the polymer matrix and a hydrophilic chemotherapeutic drug, doxorubicin (DOXO), the model drug used in this work. The preparation of the dendritic PCL star product with an average arm length of 2000 g/mol was characterized using IR and ^{1}H NMR measurements. The prepared star polymer possessed a higher crystallinity and a lower melting temperature than that of the used linear PCL. Electrospun fibers were prepared starting from solutions containing the neat PCL as well as the PCL/PAMAM-PCL mixture. Electrospinning conditions were optimized in order to obtain defect free fibers, which was proven by the structural FE-SEM study.

PAMAM moieties enhanced the hydrophilicity of the fibers, as proved by comparing the water absorption for the PCL/PAMAM-PCL fibers to that neat PCL fibers. The drug-loaded system PCL/PAMAM-PCL was prepared by directly introducing DOXO into the electrospinning solutions. The DOXO-loaded PCL/PAMAM-PCL showed a prolonged release of the drug with respect to the DOXO-loaded PCL fibers and elicited effective controlled toxicity over A431 epidermoid carcinoma, HeLa cervical cancer cells and drug resistant MCF-7 breast cancer cells. On the contrary, the drug-free PCL/PAMAM-PCL scaffold demonstrated no toxic effects on human dermal fibroblasts, suggesting the biocompatibility of the proposed system which can be used in cellular scaffold applications.

1. Introduction

Among the methods used to limit the risk of tumor recurrence, the controlled/localized delivery of anticancer drugs has been proposed as a promising and alternative approach to classic applied procedures, such as radiation and chemotherapy. In general, local drug delivery can be performed by applying various biodegradable polymer systems, including drug-eluting films [1], hydrogels [2,3], wafers [4], nanoparticles [5,6] and nanofibrous scaffolds [7–9]. In the latter case, the peculiar architecture of the support, which is characterized by a highest surface/contact area, that favors a robust drug loading capability together with a prolonged drug release

that consequent kills of the tumor cells [10–12]. Moreover, the delivery of the drugs can be controlled by tuning the fiber morphology, porosity and composition. Electrospun polymer fibers have been widely applied as delivery devices of several anticancer drugs such as carmustine [13], paclitaxel [7], cisplatin [8,9], polyphenol [14], camptothecin [15] and doxorubicin (DOXO) [16–20]. Despite the potentialities of these systems, the simple electrospinning of the drug and the polymer mixture leads to an undesirable initial burst release of the drug, which is often inevitable [21]. In order to overcome this limitation and attain a slow, sustainable release, several inorganic nanocarriers were inserted into the electrospun nanofibers. In particular, composite nanofibers were developed by combining the polymer with silica based nanoparticles [17,18,22–24], and hydroxyapatite [25], as well as a combination of silica and hydroxyapatite [26]. Even though such nanofillers can be considered ideal drug carriers, mainly due to their high surface area and good biocompatibility, their application in
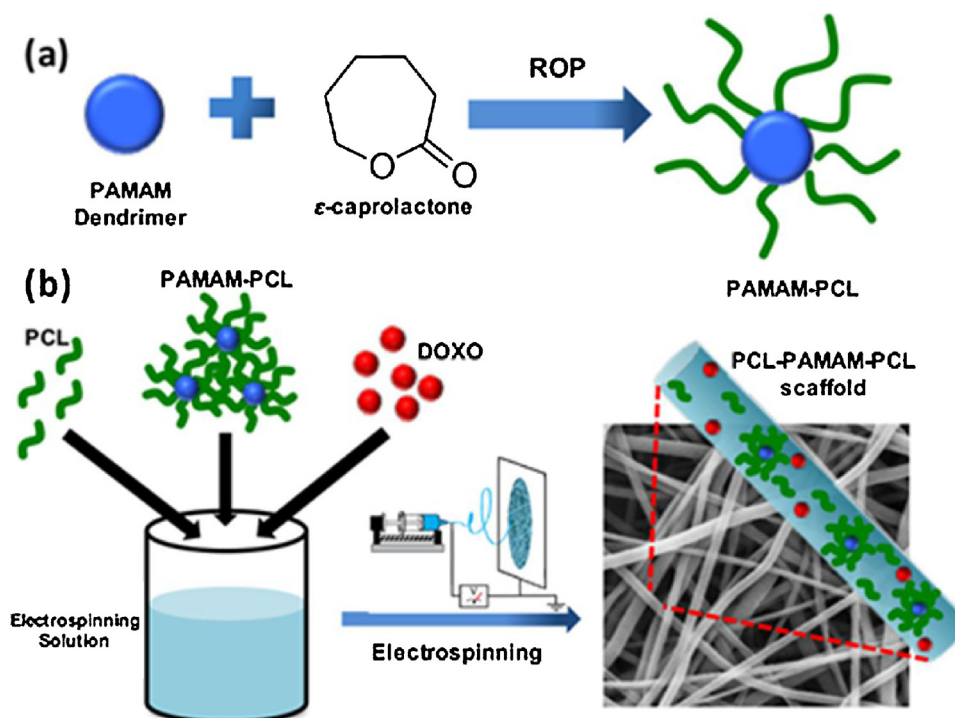
Scheme 1. Schematic representation of the preparation of PCL–PAMAM moiety (a) and DOXO-loaded PCL/PAMAM-PCL fibers prepared by electrospinning PCL, PAMAM-PCL and chemotherapeutic drug DOXO (b). PCL: Poly (ε-caprolactone), PAMAM: poly (amido amine); DOXO: doxorubicin; PAMAM-PCL: the star polymer synthesized containing PCL and PAMAM; ROP: ring opening polymerization.

designing composite nanofibers for drug delivery has some drawbacks. Indeed, there are important issues to be taken into account: the interactions between the drug molecules and the inorganic nanoparticles are weak [16] the dispersibility of the nanofillers into the polymer matrix is not always easy [27] and their bio-absorption capacity is slow. Clearly, the development of a nanofibrous scaffold that contains more specific drug carriers, as an alternative to classic inorganic nanofillers, that is characterized by a good affinity with both the drug molecules and the polymer matrix, represents an ideal target in the development of materials to be applied as a local drug delivery system. With this in mind, we have developed a novel drug delivery system, consisting of fibers, which is based on a biocompatible and re-absorbable polymer, containing an *ad-hoc*-synthesized star polymer as drug carrier, and was designed having taken into account the specific characteristics of the polymer matrix, and the chosen drug that is DOXO. Star polymers are a class of regular branched polymers in which the branches radiate from a central core, having physio-chemical properties completely different from linear analogues [28]. Thanks to their peculiar structure, which has relatively short chains but also a high mass, a more compact shape and an increased concentration of functional end-groups with respect to linear polymer, star polymers are characterized by enhanced solubility, low viscosity, low crystallizability and thermal/hydrolytic stability [28]. Star structures made up of a dendritic-type core and branches of biocompatible/bio-absorbable polymers, such as poly(lactide) [29,30] and poly(ε-caprolactone) (PCL) [30–33], have been addressed as potential systems for the encapsulation/controlled release of drug molecules, because of their amphiphilic core-shell structure. In particular, poly(amido-amine) (PAMAM) dendrimers, a family of dendrimers, built up of polyamide branches with tertiary amines as focal point, have been used as multifunctional macro-initiators in the ring-opening polymerization of lactide [30] and ε-caprolactone [30,31–33], with the arm length being tuned through the monomer-to-initiator-molar ratio. As for the PCL/PAMAM system, which is the subject of the

present work, the dendrimer was mainly used to build up star block copolymers, whose arms consist of an inner block of PCL and an outer block of an hydrophilic polymer, such as poly(ethylene glycol) [30–32]. The above systems, used in micelle form, were proven to solubilize hydrophobic molecules, such as pyrene, in an aqueous solution [30]. Besides PEG, poly(D-gluconamidoethyl methacrylate) (PGAMA) was also used to develop PAMAM/PCL/PGAMA star block copolymers [34]. It is worth underling that PCL-based star polymers were used as micelles or particles in all the above mentioned works, as in their neat form they are totally unsuitable for exploitation as structural materials. Clearly, their formulation as self-standing materials, for example their insertion into an easily processable, biocompatible and bio-absorbable polymer support, represents an important advantage regarding their applicability. In this work, the novel drug delivery system was developed by first synthesizing a star-like polymer, made up of a PAMAM core and PCL arms (PAMAM-PCL), via ROP of ε-caprolactone starting from a hydroxyl-terminated PAMAM dendrimer (Scheme 1). Then, electrospun fibers were prepared by mixing in solution the commercially available high molecular mass PCL with the here-synthesized star polymer. The chemical features and the peculiar architecture of the PAMAM-based macromolecule being characterized by arms whose chemical nature is identical to that of the polymer matrix may favor its dispersion. On the other hand, the hydrophilic PAMAM core may promote specific interactions with DOXO, which is a clinically used hydrophilic anticancer drug. Therefore, the insertion of the above molecule into PCL should allow us to combine the features of the star molecule with those of the processable polymer support. As shown in Scheme 1, DOXO was directly introduced in the electrospinning solution and the obtained fibers were characterized in terms of morphology, thermal properties and water absorption. PCL alone and in combination with other well know polymers such as PLA [34], chitosan [35,36], gelatin [37–40], etc. have been successfully used for the past few decades as three dimensional matrices for tissue engineering, especially in

bone [34,36] and nerve [35,38], regeneration and also in wound healing [39,40] applications, used bared or loaded with stimulating drugs and growth factors. Only a few examples are available on their use as drug delivery agents in cancer therapeutics when made of complex responsive systems [20,41,42]. In this study, the biocompatibility of the here synthesized drug/free PCL/PAMAM-PCL scaffold was tested on adult human dermal fibroblasts (HDFa). On the DOXO-loaded scaffolds, cytotoxicity assays were conducted on three different human cancer cell lines and the PCL/PAMAM-PCL were found to exhibit a slower release profile than standard PCL fibers only. The biocompatibility of the material and the drug release profile are features that are both interesting for the application of such drug deliver scaffolds in cancer therapy.

2. Experimental

2.1. Materials

For the preparation of PAMAM-PCL: ε-caprolactone (97%), which was purchased from Sigma-Aldrich and distilled under reduced pressure; second generation PAMAM dendrimer containing 16 amidoethanol surface groups (structure reported in Fig. 1S), commercialized from Sigma-Aldrich as a 20 wt.% solution in methanol, was evaporated under vacuum at 50 °C for 24 h prior to use, in order to completely remove the solvent. Tin(II) 2-ethylhexanoate (Sn(Oct)$_2$) (95%; from Sigma-Aldrich) was used without further treatments. Anhydrous toluene ($\geq$99.7%), chloroform and methanol were purchased from Sigma-Aldrich and used as received.

For the preparation of the PCL (DOXO) and PCL/PAMAM-PCL (DOXO) nanofibers: PCL with an average molecular weight of 80.000, N,N-dimethylformamide (DMF), dichloromethane (DCM) and doxorubicin hydrochloride (DOXO) were purchased from Sigma-Aldrich.

2.2. Synthesis of PAMAM-PCL

Star-like PCL was synthesized by the ring-opening polymerization of ε-caprolactone, at 120 °C, using the hydroxyl-terminated PAMAM dendrimer as macroinitiator and Sn(Oct)$_2$ as a catalyst. In detail, 1.14 ml of PAMAM solution (corresponding to 0.2 g of PAMAM) was introduced into a 50 ml two-neck round-bottomed flask equipped with a magnetic stirrer and in-situ dried; then, 3 g of ε-caprolactone (which was previously distilled) was charged into the flask under argon flow. The reaction vessel was immersed into a thermostatically controlled oil bath (initially set at 140 °C, in order to favor the solubilisation of the dendrimer in the monomer), under vigorous stirring. As soon as the mixture was completely homogenized, about 0.3 ml of a freshly prepared solution of Sn(Oct)$_2$ in toluene ([ε-caprolactone]/[Sn(Oct)$_2$] = 5·10^3) was added through a micropipette. The reaction was then allowed to proceed for 24 h at 120 °C, always in an inert atmosphere. After this, the polymerization was stopped by cooling down the reaction mixture, which was subsequently dissolved in chloroform and poured into an excess of cold methanol. The as-purified polymer (PAMAM-PCL) was recovered as a fine white powder by filtration and dried in vacuum at 40 °C until the powder reached constant weight.

2.3. Fiber preparation

Electrospinning solutions were prepared by dissolving 15 wt.-% of PCL or PCL containing 10 wt.-% of PAMAM-PCL (PCL/PAMAM-PCL fibers) in the DMF and DCM solvent mixture with a 30:70 v/v ratio. Regarding the preparation of fibers containing the drug, the concentration of DOXO in the polymer solution was 0.015 mg/ml in

order to have a final total amount of 0.5 µg in the electrospun mats, which were used for each of the wells in the cytotoxicity testsg.

The fibers were electrospun using a conventional electrospinning system [43]. The solutions were loaded in a syringe (model Z314544, diameter d = 11.6 mm, Aldrich Fortuna Optima) and were placed in a horizontal direction. A Gamma high-voltage research power supply (Model ES30P-5W) was used to charge the solution in the syringe with a positive DC voltage. The positive electrode was connected to the needle (diameter d = 0.45 mm) of the syringe and the negative electrode was attached to the grounded collector, an aluminium sheet wrapped on a glass cylinder (height 4 cm, diameter 14.5 cm). The distance between the tip and the collector was 20 cm. A syringe pump (Harvard Apparatus Model 44 Programmable Syringe Pump) was used to feed the needle. The needle tip and the ground electrode were contained in a hollow plastic cylinder (height 30.5 cm, inner diameter 24 cm, and thickness 3.5 mm), internally coated with a polytetrafluoroethylene sheet (thickness 1 mm). A glass Brooks rotameter was used to keep the air flow constant in the enclosed electrospinning space. The air flow was fed in the chamber at atmospheric pressure from an inlet placed behind the collector.

The established conditions were: voltage tension = 15 kV, tip-collector distance = 20 cm, air flow = 2 l/min and temperature = 21 °C.

2.4. Measurements

FTIR spectra were recorded on a Bruker IFS66 spectrometer in the spectral range 400–4000 cm^{-1}. ^{1}H NMR spectra were collected with a Varian NMR Mercury Plus instrument, at a frequency of 300 MHz, in CDCl$_3$ solutions containing tetramethylsilane as internal standard.

Differential scanning calorimetry (DSC) measurements were performed with a Mettler-Toledo TC10A calorimeter calibrated with high purity indium and operating under a flow of nitrogen. The sample weight was about 5 mg and a scanning rate of 10 °C/min was employed in all the runs. The samples were heated from 25 °C to 100 °C, then the melt was allowed to relax for 1 min at this temperature before being cooled down to −100 °C, and finally heated up again to 100 °C (second heating scan). The degree of crystallinity (Xc) was calculated by using an enthalpy of fusion for a 100% crystalline PCL of 136 J/g [44].

The water absorption was measured by immersing electrospun mat specimens having area of 1.5×1.5 cm^2 in distilled water at room temperature for 48 h, and expressed as increase in weight percent according to the formula [45]:

$$\text{wateradsorption}(\%)[(W_{wet} - W_{dry0})/W_{dry0}] \times 100 \qquad (1)$$

in which W_{wet} is the wet weight measured (immediately – to avoid evaporative losses) after withdrawing the films from water and gently wiping off the surface water with a tissue, and W_{dry0} is the initial weight of the specimens, measured after vacuum-drying the films for 24 h at 40 °C.

With regards to the sample surface morphology, a Zeiss Supra 40 VP field emission scanning electron microscope (FE-SEM) was used to examine the fiber morphologies. All samples were thinly sputter-coated with carbon using a Polaron E5100 sputter coater. The fiber diameters and their distribution were measured using an image analyzer, with ImageJ 1.41 software.

Confocal analysis was performed to check the presence of DOXO in the PCL and PCL/PAMAM-PCL mats. The DOXO-loaded-PCL and DOXO-loaded-PCL/PAMAM-PCL mats were cut into 1×1 cm^2 pieces. Each piece was then mounted on a flat sample holder and viewed under a confocal Nikon Eclipse Ti microscope equipped with an argon laser source with an excitation and emission wave-

length of 488 and 530 nm, respectively, to view the doxorubicin loading in the electrospun mats.

2.5. In-vitro viability assay for testing of toxicity caused by pristine and DOXO-loaded PCL or PCL/PCL-PAMAM electrospun mats

Three different cancer cell lines were used: - A431 epidermoid carcinoma cells (ATCC® CRL-1555™), HeLa-WT cervical cancer cells (ATCC® CCL-2™) and MCF-7 breast cancer cells (ATCC® HTB-22™). All these cell lines were cultured using DMEM media supplemented with 10% Fetal Bovine Serum, 2% Penicillin-streptavidin and 1% L-Glutamine (Gibco™) under 37 °C, 5% CO₂ and 95% relative humidity. These cells were then harvested and plated in 24 multi-well-plate at a cell number of 100,000 cells in 1 ml cell media per well and allowed to adhere overnight. Given the interference of phenol-red in the optical read out of the cytotoxicity assay prior to performing the viability assays applied in this work, the media in the well plates was replaced with 1 ml of freshly prepared Phenol-Red free DMEM media supplemented with 10% Fetal Bovine Serum, 2% Penicillin-streptavidin and 1% L-Glutamine. Meanwhile, the electrospun mats, pristine PCL, pristine PCL/PAMAM-PCL, DOXO-loaded-PCL and DOXO-loaded-PCL/PAMAM-PCL were cut into 1×1 cm² pieces (each single piece of scaffold was manipulated to contain 0.5 µg DOXO during the electrospinning process). These 1×1 cm² scaffold pieces were peeled off from their aluminium substrate and dropped on top of the media in the 24 multi-well-plate that contained the cells and incubated for 1, 3, 5, 7 and 9 days. After the respective incubation periods, PrestoBlue® and AlamarBlue® Cell Viability Reagents (Molecular Probes™) were used to access the viability of the cancer cells. For each condition, the assays were performed three times.

2.6. PrestoBlue® viability assay

The 24 multi-well-plates containing the cells incubated with the scaffolds floating in their media, were washed after their incubation period with Phosphate Buffer Saline (PBS) and incubated with fresh Phenol-Red free media containing 10% PrestoBlue® reagent for 30–40 min at 37 °C. Then 100 µl of supernatants from each well were transferred in a new 96 multi-well-plate and absorbance was read using a Multiskan™ GO Microplate Spectrophotometer (ThermoFisher Scientific™) at 570 and 600 nm. The viability of each experimental condition was normalized to the viability of the cells cultured under the same conditions and never exposed to the materials which were considered to be 100% viable. The experiment was done four times and the mean value with its deviation (SD) was plotted using GraphPad.

2.7. AlamarBlue® viability assay

After the respective incubation periods previously mentioned, 100 µl of AlamarBlue reagent (10% AlamarBlue reagent in media) was added to each of the 24 multi well-plate containing the cells incubated with the floating scaffolds. The well plates were then incubated at 37 °C for 4 h. Next, 100 µl of supernatants from each well were collected in a new 96 multi well-plate and absorbance was read using a Multiskan™ GO Microplate Spectrophotometer (ThermoFisher Scientific™) at 570 and 600 nm (Ab_{570}-Ab_{600}). The viability of each experimental condition was normalized to the viability of the control cells which were considered to be 100% viable. The experiment was done four times and the mean value with its deviation (SD) was plotted using GraphPad.

2.8. Confocal microscopy characterization to study the attachment and proliferation of adult human dermal fibroblasts (HDFa) on PCL/PAMAM-PCL electrospun scaffolds

The HDFa Adult human dermal fibroblasts (ThermoFisher Scientific™ C0135C) were grown in special fibroblast media, Medium 106 (M106500) supplemented with 10 ml low serum growth supplement (LSGS_S00310). The cells were maintained in culture at 37 °C in a sterile incubator with 5% CO₂ and 95% relative humidity. These cells were then harvested using 0.1% trypsin-EDTA, counted and plated in the 24 MW-plate containing the PCL/PAMAM-PCL scaffolds at density of 10,000 cells/well in 1 ml complete media to check for the ability of this normal and healthy cell line to attach and proliferate on the drug free-PCL/PAMAM-PCL scaffolds. In order for the scaffolds to remain at the bottom of the well, a soft hand-made PDMS ring was placed over the scaffold. The scaffolds used were neither physically (heat, plasma treatment, etc.) nor chemically (using proteins or adhesion factors) pre-treated.

These cells, were washed carefully with PBS after 24 h and fixed using 4% sterile formaldehyde (Sigma) for 10 min, then washed thoroughly and permeated with 0.1% Triton X-100 for 2–3 min. Following fixation and permeation, the scaffolds containing the cells were washed 3 times with PBS. They were the incubated with 1% bovine serum albumin (BSA) for 30–40 min, which provides a blocking step for the upcoming F-actin staining. Next, they were stained using Alexa Fluor® 488 phalloidin (Invitrogen™ A12379) for 30 min, washed with PBS 3 times and stained using NucBlu® Fixed Cell ReadyProbes® Reagent (Invitrogen™ R37606) for 15 min. Then the scaffolds containing the cells were washed and carefully inverted in the same 24 MW-plate and filled with 1 ml fresh PBS for the purpose of imaging using a Confocal Microscope (Nikon) at an excitation and emission wavelength of 495/518 nm for F-actin phalloidin and 360/460 nm for nuclei DAPI. The same was repeated after 48 and 72 h. The analysis was done three times.

3. Results and discussion

3.1. Synthesis and characterization of dendritic star-like PCL (PAMAM-PCL)

In this work, at first, the star-like polymer, made up of a PAMAM core and PCL arms, was synthesized via ROP of ε-caprolactone using the generation 2 PAMAM (fitted with 16 hydroxyl end groups) as macro-initiator (Fig. 1S).

The dendrimer-graft architecture of the obtained product was established through spectroscopic measurements. Fig. 1a shows the FTIR spectrum of PAMAM-PCL: together with the intense absorption band for the stretching of the ester carbonyl at 1720 cm⁻¹, which is characteristic of PCL, two new peaks with a lower intensity can be observed at 1650 and 1545 cm⁻¹ (corresponding to amide carbonyl stretching and amide N—H bending, respectively) [46], which is related to the poly-(amidoamine) structure of the PAMAM molecule. Since the sample was purified by precipitation in methanol, which is a good solvent for PAMAM, the presence of these peaks is indicative of the successful incorporation of PAMAM as the core onto which the PCL arms were grown.

This was further confirmed by means of ¹H NMR spectroscopy (Fig. 1b), in which the characteristic peaks of PCL (at δ 1.40, 1.65, 2.31, 3.64 and 4.06 ppm – assigned to CH₂—CH₂—CH₂—CH₂—CH₂, CH₂—CH₂—CH₂—CH₂—CH₂, CO—CH₂, terminal CH₂—OH, and CH₂—O—CO protons, respectively) are accompanied by several signals in the region of δ 2.60-3.60 ppm, which is ascribable to the presence of the PAMAM units [46]. Furthermore, the triplet at δ 3.60 ppm for the terminal methylene
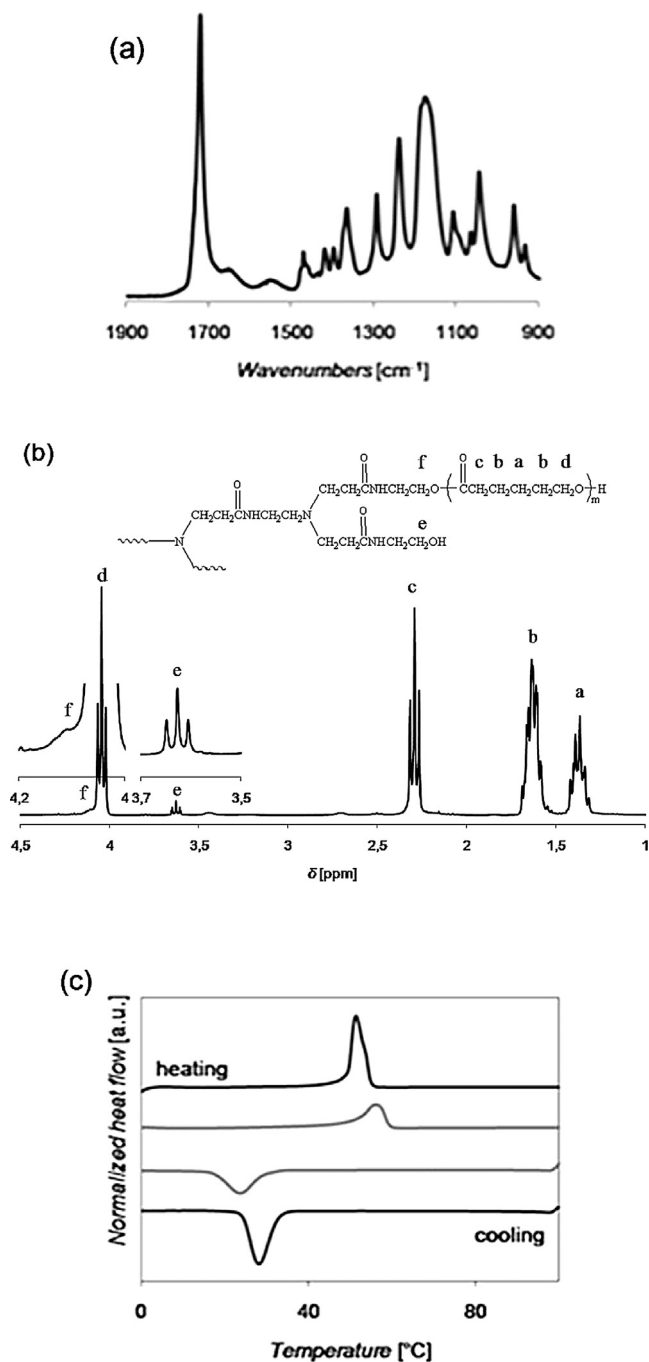
Fig. 1. (a) FTIR spectra of the sample PAMAM-PCL in the spectral region 1900–900 cm^{-1}, (b) ^{1}H NMR spectrum of PAMAM-PCL (region at δ 4.5-1.0 ppm) with enlargement insets (at δ 3.7-3.5 and 4.2-4.0 ppm) and (c) DSC thermograms of PAMAM-PCL (black lines) and PCL (grey lines) on cooling and subsequent heating at 10 °C/min after one minute relaxation in the melt at 100 °C (the heat flow is normalized to the PCL content).

PCL chain with those at the chain end (at δ 2.31 and δ 3.64 ppm, respectively), ascertaining it is about 18. Thus, the spectroscopic measurements enabled us to assess the successful preparation of a dendritic PCL star product, with 16 arms, each of which has an average length of about 2000 g/mol, and thence an overall number average molecular weight of about 35000 g/mol − corresponding to a PAMAM content of about 9 wt.%.

Fig. 1c presents the DSC cooling and (second) heating curves of PAMAM-PCL, together with those of the linear high-molecular-weight PCL employed for electrospinning. In spite of the branched nature of PAMAM-PCL, which would be expected to disturb ordering, its crystallizability is enhanced with respect to PCL: the melt-crystallization is anticipated, as well as the final crystallinity is found higher (Xc ∼ 55% against ∼37%) than that of the linear polymer. As reported in the literature [47], this suggests that the improved chain mobility is promoted in the short-armed star system, compared to high-molecular-weight PCL and prevails over the hindering effect caused by branching. Contrariwise, the temperature of melting is lower for the PAMAM-PCL than for the linear PCL (Tm ∼51 °C against ∼56 °C), which is a general observation for star polymers, and it would be imputed to the short arm length and branched architecture of PAMAM-PCL, both of which are factors that negatively affect crystal thickness and perfection.
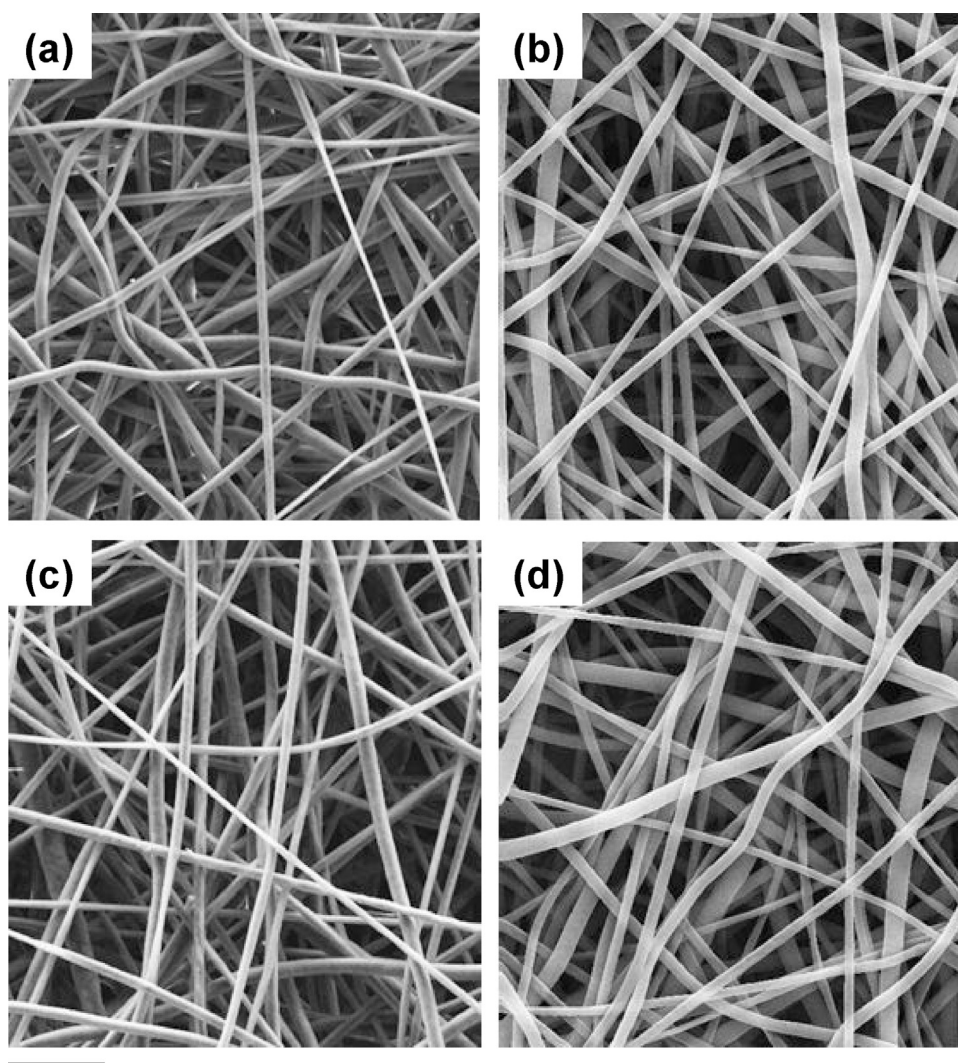
3.2. Preparation and characterization of PCL, PCL/PAMAM-PCL, PCL/PAMAM-PCL/DOXO and PCL/PAMAM-PCL/DOXO fibers

As shown in Scheme 1, DOXO was directly introduced to the electrospinning solution and the obtained fibers were characterized in terms of morphology using SEM imaging technique and confocal microscopy.

The electrospinning conditions were tailored in order to obtain defect-free and homogeneous fibers. Indeed, in Fig. 2a , a typical SEM micrograph of a PCL mat with an average diameter of 1.5 μm, prepared by applied the optimized electrospinning conditions, is shown. Also, the addition of PAMAM-PCL (10 wt.-%) turned out not to modify the fibers morphology, as by applying the same conditions used in the preparation of the neat PCL fibers with an average diameter similar to that of the PCL fibers were obtained (Fig. 2b). The addition of DOXO within both the PCL and PCL/PAMAM-PCL mats did not change the fiber morphology, as shown in Figs. 2c and 2d. The incorporation of the hydrophilic PAMAM moieties into the hydrophobic PCL fibers is expected to enhance the hydrophilicity of the resulting materials, which should be reflected in their capability to absorb water and, at the same time, interact with hydrophilic drug molecules like DOXO. The percentage of water absorbed by the mats containing PAMAM-PCL resulted in being 30%, while the neat PCL fibers do not show any tendency to uptake water. This finding demonstrates the capacity of the star PAMAM-PCL polymer to modify the performances of the fibers, increasing their affinity for hydrophylic molecules, which is a feature that happens be relevant for the formulation of the novel drug delivery system based on the hydrophylic DOXO.

The DOXO loading in the PCL/PAMAM-PCL (Fig. 3) and PCL (Fig. 2S a and c) fibers was analyzed using confocal microscopy at an emission wavelength of 620 nm. Considering the confocal microscopy images, the DOXO-loaded fibers result in being characterized by a uniform red fluorescence, thus indicating a fine dispersion of the drug into the mats.

To test the drug release from the various mats, an indirect cell viability study was performed on different cancer cell lines (A431 adenocarcinoma, HeLa-WT cervical cancer and MCF-7 breast cancer cells). The cytotoxicity effects of the DOXO were tested by using two sensitive resazurin based-viability assay kits (PrestoBlue® and AlamarBlue®), that assess the mitochondrial activity of cells exposed to the scaffolds at different time points post-incubation

protons of unbound PAMAM becomes hardly discernible, while a signal appears at δ ∼4.12 ppm (see the insets of Fig. 1b), which is consistent with the resonance shift upon the formation of ester linkages. This confirms that the formation of the desired star-like PCL with the ROP started from the hydroxyl end groups of PAMAM, and also indicates that all of these groups were active initiators, thus giving rise to a dendritic star-like PCL having on average 16 polyester arms. Finally, ^{1}H NMR spectroscopy was exploited to calculate the mean degree of polymerization of the PCL arms by comparing the peak integral of the methylene protons in the

Fig. 2. FE-SEM micrographs of: (a) PCL, (b) PCL/PAMAM-PCL, (c) PCL/DOXO and (d) PCL/PAMAM-PCL/DOXO fibers. Scale bar represents 5 μm.

(1, 3, 5, 7 and 9 days). To demonstrate that the toxicity was caused by the release of DOXO and not by the materials constituting the electrospun mat itself, the pristine PCL and PCL/PAMAM-PCL fibers were tested in parallel with the corresponding DOXO-loaded fibers (PCL/DOXO and PCL/PCL-PAMAM/DOXO). As can be seen in Fig. 3, PCL and PCL/PAMAM-PCL showed good biocompatibility with all the cancer cells lines studied, for up to 9 days of incubation. The HeLa-WT cells showed an abrupt reduction in viability from day 1–9 when treated with PCL/DOXO fibers whereas the same cell-line treated with PCL/PAMAM-PCL/DOXO showed a subtler profile in the reduction of viability over a few days (Fig. 3b and c). Controls were untreated cells grown in cell media and kept under the same culture conditions. In case of A431 cancer cells, also a similar trend was also observed (Fig. 3S). The prolonged and slow release of the new fibers could be due to the fact that the amphiphilic nature of the PAMAM-PCL contained in the mats increases the interactions and good mixing of the hydrophilic drug that in turn, was released in a more controlled manner over a few days, with respect to the most hydrophobic PCL mats. Indeed, the latter released DOXO at a much higher rate causing such sudden decrease in viability. We also tested the scaffolds on a DOXO resistant MCF-7 cell line, which showed much more resistance to toxicity caused by the release of DOXO in comparison to the other two cell lines studied, but a gradual reduction in viability was also observed which followed the

same comparative trends as for the other two cell lines (Fig. 3d and e). Overall the cytotoxicity study suggests that the PCL/PCL-PAMAM scaffolds enable a slower release of the drug with respect to the PCL mats thus allowing a prolonged drug treatment. When considering the drug release profile with other hydrid-based fibers prepared by electro-spun techniques, we could assess that in our study we looked at longer periods of drug release (up 9 days) than studies on polyethylene oxide/chitosan/graphene oxide fibers (up to 48 h) [41] and to the polymeric-based fibers (72 h) [42]. The drug release profile of our hybrid system was closely in line with that of multi-wall carbon nanotubes/poly(lactic-co-glycolic acid) polymer fibers in which release over several days has been also achieved (up to 42 days) [48] This prolonged release feature makes the nano-based systems appealing as an implantable post-surgical drug delivery scaffold.

The drug-free PCL/PCL-PAMAM scaffolds did not show any toxicity towards any of the tumor cell lines. This prompted us to investigate the biocompatibility of the mats with healthy cells with the idea of applying such mats as tissue scaffolds. To this aim adult human dermal fibroblasts (HDFa) were grown directly on PCL/PAMAM-PCL scaffolds. Confocal imaging characterization was done to study the attachment and growth of these cells (Figure 4 and Fig. 4S). It was evident that HDFa cells attached firmly already after day 1 of incubation, which were characterized by their multi-
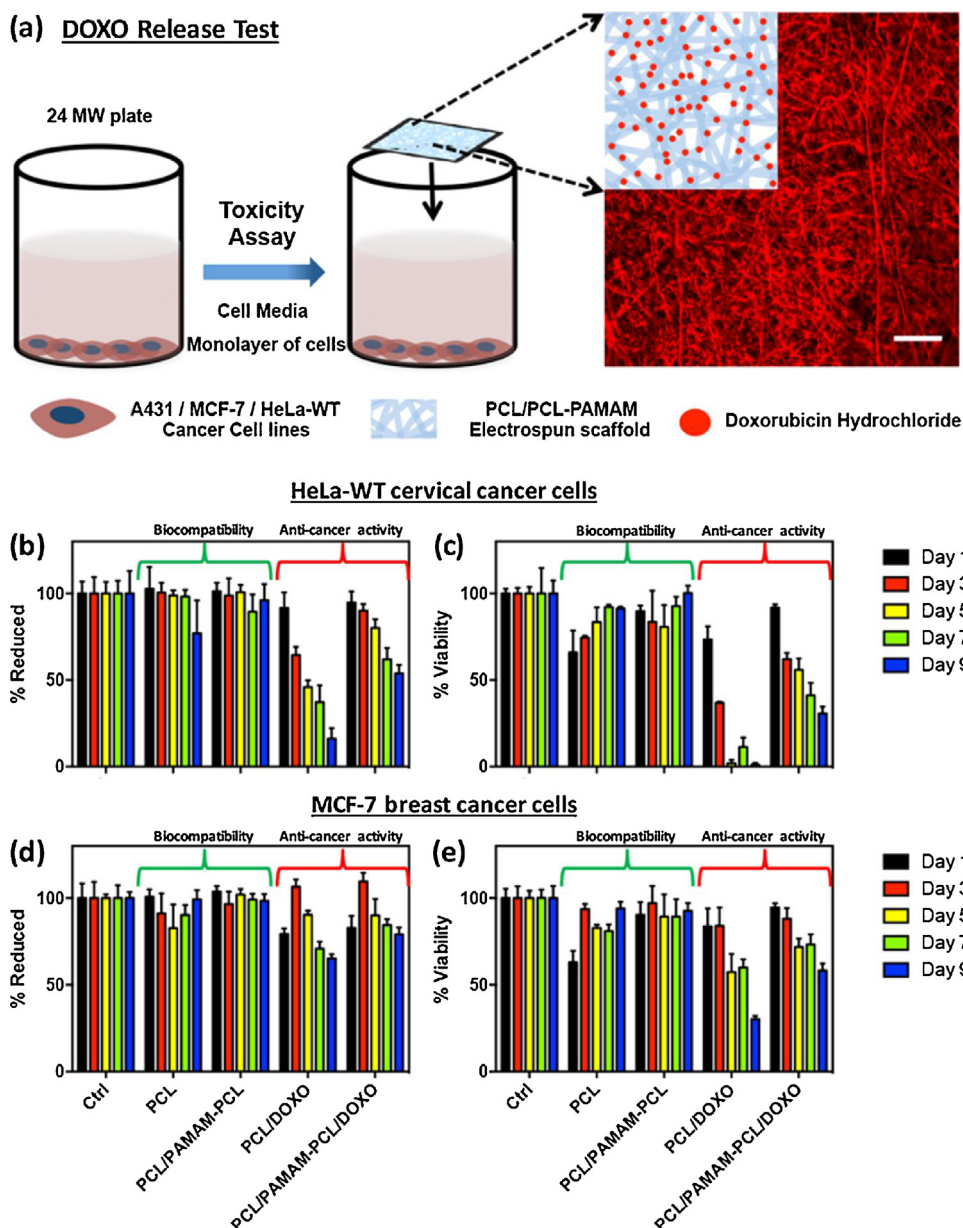
Fig. 3. Scheme representing the drug release assay when using the mats as drug scaffolds and typical confocal imaging of a red fluorescent DOXO loaded PCL/PAMAM-PCL fibers ((a) – scale bar corresponds to 50 μm). AlamarBlue® Viability assay on HeLa-WT (b) and MCF-7 (d) tumor cells treated with pristine and DOXO-loaded PCL or PCL/PCL-PAMAM mats. PrestoBlue® Viability assay on HeLa-WT (c) and MCF-7 (e) tumor cells treated with pristine and DOXO- loaded PCL or PCL/PCL-PAMAM mats. (Error bar represents Sd and n = 4 for all groups studied). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ple finger-like projection (F-actin stained in green by Alexa Fluor® 488 phalloidin, Fig. 4b). By day 3 these cells were proliferating rapidly and by day 5, they spread well and formed their typical bipolar shape. An interesting note is that these cells were able to penetrate deep into the PCL/PAMAM/PCL scaffold, as shown in the zeta-stack analysis of the fibers (Fig. 5S), even without any physical or chemical pre-treatment of the scaffolds, which might be contributed to the hydrophilic PAMAM moiety in the scaffold.

4. Conclusions

In this work, a composite polymer material designed to be applied as a localized/sustainable delivery system of anticancer drugs was developed. The system consists of poly(ε-caprolactone) (PCL) electrospun fibers, a material which combines the suitable features of the polymer for its implantation in the body, such as

the bioabsorbibility and biocompatibility, with those of the fibrous architecture, for example the high surface area and the easy application at the targeted area. In order to enhance the affinity of the fibers for the drug, doxorubicin, and tune its release, an *ad-hoc*-synthesized star polymer, made up of a poly(amido-amine) (PAMAM) core and PCL branches (PAMAM-PCL), was added into the electrospun fibers. In contrast to the inorganic nanofillers, which are commonly applied as drug carriers in the polymer fibers, our star system has a good affinity with PCL, since the PAMAM-branched PCL has arms whose chemical nature is identical to that of the polymer matrix. Moreover, the PAMAM core of the star polymer, which renders the fibers more hydrophilic, by interacting with the chosen hydrophilic drug (DOXO), improves its dispersion in the fibers and allow its prolonged and controlled release (we have here extended the study to 9 days). These fibers can be used for delivery systems as implantable patches with a prolonged

(a) PCL/PAMAM-PCL Scaffolds- Biocompatibility Study
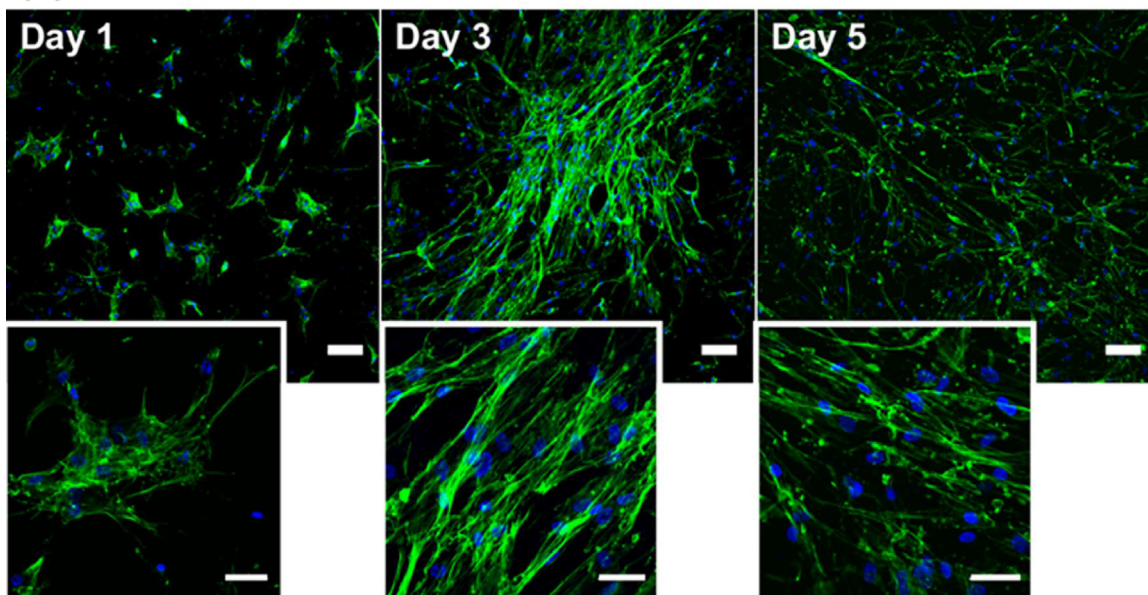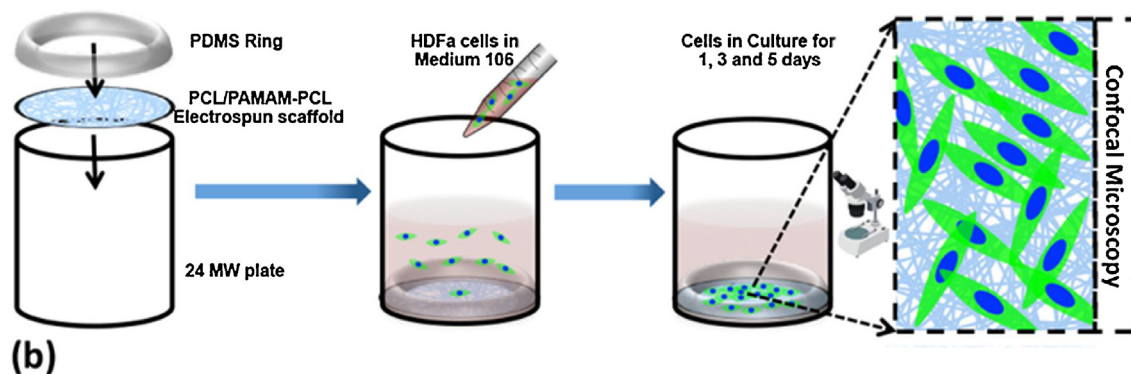
Fig. 4. Scheme representing the culturing of healthy dermal fibroblast (HDFa) cells on PCL/PAMAM-PCL scaffold (a). Confocal images showing the attachment and growth of HDFa cells on a PCL/PAMAM-PCL scaffold between 1 and 5 days (b). Scale bar represents 100 μm (50 μm in the inserts). F-Actin stained using Alexa Fluor® 488 Phalloidin (green) and nuclei stained with DAPI (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

release period, to avoid the development of drug resistance in cancer therapy due to an unnecessary burst and uncontrolled release of the anti-cancer drug used in conventional chemotherapies. The described PCL/PAMAM-PCL system could be used in various applications just by replacing the therapeutic agents incorporated within the fibers. Both hydrophilic as well as hydrophobic drugs can be incorporated into this system due to their amphiphilic nature, which makes them more versatile than most available systems. Remarkably, the biocompatibility study showing the attachment and growth of adult Human Dermal Fibroblast (HDFa) studied on PCL/PMAM-PCL scaffolds suggests their use as transdermal drug delivery patches for wound healing and faster regeneration of the damaged tissue (stem cell delivery with appropriate signaling molecules) [49–51], as biodegradable patches to be used post-surgery to avoid infections when loaded with antibiotics (Biteral®, gentamicin sulphate, etc.) [52,53] or eventually as bandages when loaded with immuno-suppressants to prevent organ rejection in post organ transplantation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.colsurfb.2017.11.014.

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