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Fibrous wound dressings encapsulating essential oils as natural antimicrobial agents

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I. Liakos,[*a] L. Rizzello,[b,c] H. Hajiali,[a,d] V. Brunetti,[b] R. Carzino,[a] P. P. Pompa,[b] A. Athanassiou[*a] and E. Mele[*a]

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Preventing infections is one of the main focuses of wound care. The colonisation of wounds by microorganisms can in fact have negative consequences on the healing process, delaying it. Here, we propose the use of essential oils as natural antimicrobial agents for cellulose-based fibrous dressings. We demonstrate the production of composite electrospun fibres that effectively encapsulate three different types of essential oils (cinnamon, lemongrass and peppermint). The fibrous scaffolds are able to inhibit the growth of *Escherichia coli*, even when small amounts of essential oils were used. At the same time, they are not cytotoxic, as proved by biocompatibility assays on skin cell models. The created dressings are promising as advanced biomedical devices for topical treatments.

Introduction

Physical or chemical traumas are often responsible for injuries of the human skin, with the consequent disruption of its normal physiology and the initiation of specific healing processes to restore the tissue functions.[1] Acute (e.g. incisions, lacerations, burns or irritations) or chronic wounds (e.g. ulcers caused by tumours, pressure, venous diseases or diabetes) affect millions of people annually. For instance, over 6 million patients endure severe burns each year,[2] and currently over 250 million adults have diabetes.[3] The incidence of chronic wounds is expected to increase in the next years mainly due to the ageing of global population, and changes in lifestyle. Therefore, advances in medical devices for wound care are required.

One strategy for the development of efficient medical devices deals with the realization of nanofibrous scaffolds or mats by hydrodynamic methods, including centrifugal spinning,[4] pressurised gyration,[5] and electrospinning (ES).[6-7] In particular, in wound care the ultrafine size of the electrospun fibres guarantees mechanical flexibility and consequently excellent conformability of the non-woven mat to the wound site. In this way, the nanofibrous bandage provides complete coverage of the injured tissue, protection against infections and dehydration, as well as thermal insulation. On the other hand, the high porosity of the electrospun mesh facilitates the permeation of gases, the transport of nutrients, the retention of moisture, and the absorption of exudates. Furthermore, the high surface-to-volume ratio of the nanofibres favours the adhesion, proliferation and differentiation of cells during tissue regeneration.[8-9] Nanofibres can also work as vehicles for delivering therapeutic substances (e.g. antioxidant, anti-inflammatory, and antimicrobial agents) to the wound.[2, 10]

Since infection is the most common complication that can occur during wound healing, dressing systems encapsulating antiseptic agents are fundamental for delaying or blocking the growth of microorganisms.[11] Many studies have demonstrated that essential oils (EOs), usually extracted from various aromatic plants, have intrinsic antibacterial, antifungal and insecticidal properties.[12-15] EOs present advantages over other synthetic antimicrobial agents, because they are widely available natural compounds with low degree of toxicity. Moreover, they can be efficiently combined with polymeric matrices to create composite materials with exceptional antimicrobial properties.[16]

So far, only few groups have reported on the incorporation of natural antimicrobial compounds into electrospun nanofibres. For instance, poly(ε-caprolactone) (PCL) and poly(lactic acid) (PLA) nanofibres incorporating thymol, the most important component of thyme oil, have been proposed for their antimicrobial activity against *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*).[17] In another study, herbal extracts from *Tecomella undulate*, a plant from Thar Desert, were added in PCL and polyvinyl pyrrolidone (PVP) electrospun fibres. The induced antimicrobial properties were evaluated against three model organisms, i.e. *Pseudomonas aeruginosa* (*P.aeruginosa*), *S. aureus* and *E. coli*.[18]

Here, we report on the production of electrospun cellulose-based nanofibres encapsulating essential oils (cinnamon, lemongrass and peppermint), as natural antimicrobial agents. We analysed two different concentrations of EOs, and we

demonstrated that the created composite scaffolds effectively inhibited the growth of *E. coli*. Moreover, the biocompatibility of the fibrous constructs was investigated for skin cell models (fibroblasts and keratinocytes), showing that the fibres were biocompatible. The combination of antibacterial effect and biocompatibility makes these fibrous scaffolds attractive as biomedical devices for topical wound care.

Experimental

Chemicals and materials

Essential oils (EOs) of cinnamon, lemongrass and peppermint (100% pure) were purchased from Maitreya-Natura (Italy). Cellulose Acetate (CA) (acetyl content of 39.8%; Mw = 30 kDa), acetone, Dulbecco's Modified Eagle's Medium (DMEM), Bovine Calf Serum (BCS), Fetal Bovine Serum (FBS), Trypsin- ethylenediaminetetraacetic acid (EDTA) solution, Penicillin-Streptomycin, Phosphate Buffered Saline (PBS), Fluoroshield with DAPI, and Thiazolyl Blue Tetrazolium Bromide (MTT) were obtained from Sigma Aldrich. Alexa Fluor® 647 Phalloidin was purchased from Life Technology. All compounds were used as received. *Escherichia coli* (*E. coli*) and *Candida albicans* (*C. albicans*) were purchased from ATCC (US). *E. coli* were grown onto Luria–Bertani (LB) medium, composed of: 1% tryptone (Sigma Aldrich), 0.5% yeast extract (Sigma Aldrich), and 1% NaCl (Sigma Aldrich). For the inhibition assays, *E. coli* cells were inoculated onto solid LB media, namely, LB+1.5% bacteriological agar (Sigma Aldrich), pre-loaded in 92×16 mm^2 (diameter×height) Petri dishes (Sarstedt). *C. albicans* were grown onto Sabouraud (dextrose) broth, composed of 1% bacteriological peptone (Sigma Aldrich), and 4% dextrose (Sigma Aldrich), and then spread onto 92×16 mm^2 (diameter×height) Petri dish (Sarstedt) with solid Sabouraud, namely Sabouraud + 1.5% bacteriological agar (Sigma Aldrich). NIH3T3 fibroblast cells from murine embryo were purchased from ATCC, and normal human keratinocyte cell line (HaCaT) from CLS Cell Lines Service GmbH (Deutsches Krebsforschungszentrum, Stiftung des öffentlichen Rechts "DKFZ", Heidelberg, Germany).

Fabrication of the composite CA/EOs fibrous mats

Solutions of CA with cinnamon or lemongrass or peppermint oil were prepared by firstly dissolving 15% w/v CA in acetone and then adding 1 or 5% v/v of the selected EO. Conductivity measurements were acquired using a Mettler Toledo FiveEasy^{TM} conductivity FE30 meter by immersing the probe into the CA/EO solutions at room temperature. The conductivity values for three prepared solutions containing 5% v/v EO are reported in the Supporting Information (Table S1). Plastic syringes with stainless steel 23-gauge needles were filled with the CA/EO solutions and connected to a syringe pump (NE-1000, New Era Pump Systems Inc.) that was working at flow rates of 3 or 5 ml h^{-1}. The solutions were electrospun by using a high voltage power supply (EH40R2.5, Glassman High Voltage Inc.). The produced fibres were collected on an aluminium plate, placed at a distance of 15 cm from the needle. The following parameters of flow rate and voltage were used for the ES process, ensuring the best conditions to provide fibres free of defects and inhomogeneities: 5 ml h^{-1} and 15 kV for pristine CA solutions, 3 ml h^{-1} and 25 kV for CA solutions containing cinnamon EO, 5 ml h^{-1} and 20 kV for CA solutions containing lemongrass or peppermint EO. The resulting electrospun mats had a thickness of about 0.2 mm.

Morphological and chemical characterisation of the fibres

The morphology and the size of the electrospun fibres were analysed by Scanning electron microscopy (SEM). A JEOL JSM-6490LA microscope working in high vacuum mode, with an acceleration voltage of 15 kV, was used. A coating of 10 nm Au/Pd was required to prevent charging effects.
The chemical analysis of the CA and CA/EOs fibres was carried out by Raman spectroscopy, using a Horiba Jobin-Yvon μRaman operating with a He-Ne laser source. The wavelength of the laser radiation was 632 nm and the objective used was a 50× with a slit aperture about 200 μm.

Antibacterial activity of the CA/EO fibrous dressings

The antiseptic properties of the produced CA/EO fibres were tested against two model microorganisms: the bacterium *E. coli* (Gram-negative), and the fungus *C. albicans*. Before use, fibrous mats, having a weight of 15 mg, were sterilized by ultraviolet irradiation overnight inside a biohazard hood. We estimated that the concentration of EO (in dry basis) contained inside the analysed samples was of 6.2 and 25.0 w/w% for fibres obtained from CA solutions with 1 or 5 v/v% of EO, respectively. For the antibacterial assays, a culture of *E. coli* was grown overnight in a 37 °C incubator, then 200 μL of a 10^6 CFUs/ml inoculum was spread onto LB medium agar plates. The plates were placed in the incubator at 37 °C for 2 hours to allow a proper evaporation of the residual liquid medium. Then, the electrospun mats were sterilized overnight under ultraviolet (UV) irradiation, and positioned on the top of the solidified medium. To investigate the antifungal activity of the fibres, the initial 10^8 CFUs/ml inoculum of *C. albicans* was diluted to 10^5 cells/ml, and 200 μL of this solution were spread onto freshly prepared Sabouraud dextrose agar (SDA). The plates were then incubated at 37 °C for 2 hours, as previously described, followed by the positioning of the fibrous substrates. After 24 h of incubation time, the plates were removed from the incubator, and the inhibition effect was analyzed.

Biocompatibility assays

The proliferation of fibroblast cells (NIH3T3-cells) and HaCaT cells was analysed on the produced nanofibrous constructs.
NIH3T3 cells were cultured in DMEM supplemented with 10% of BCS and 1% of antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin), in incubator at 37 °C with 5% CO_2. The culture

medium was replaced every 3 days. The electrospun samples were cut into 14 mm diameter circles, and sterilized under UV light for 30 minutes; then they were washed using a PBS solution. Fibroblast cells were detached from the culture flask using trypsin-EDTA solution, when 80% of confluence was reached, and seeded onto the electrospun mats. Adhered and proliferated cells, cultured for 24 hours, were analysed by a colorimetric MTT assay. In order to observe the morphology of cells, the samples were fixed in 3.7% formaldehyde for 15 min before rinsing them with PBS for 3 times. Samples were then permeabilised with 1% Triton X-100, and stained with alexa-fluor 647 phalloidin and DAPI for actin cytoskeleton and nucleus, respectively. The confocal microscope Nikon A1 was utilized to visualize the morphology of fluorescent-stained 3T3 fibroblasts on the electrospun fibres.

HaCaT cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. After reaching 90% confluency, the cells were detached by trypsin–EDTA, and viable cells were counted using a hemocytometer. Cells were further seeded onto the UV sterilized fibrous scaffolds, placed in a 96-well plate at a seeding density of 10^4 cells/well. The cell–scaffold constructs were then cultured for 24h in 200 µL of DMEM complete, and then processed for cell proliferation assay. To study the cell proliferation on different substrates, viable cell counts were determined by WST-8 cell counting assay kit (Sigma Aldrich) following the manufacturer's instruction. In brief, at predetermined time points (24, 48, 72, and 96 hours), the cell-seeded scaffolds were incubated in WST-8 solution (1:10 v/v in cell culture medium) in an incubator at 37 °C and 5% CO_2 for 2h. The intense yellow-colored formazan dye generated by the activities of dehydrogenases in cells is soluble in the tissue culture media, and the amount of the formazan dye is directly proportional to the number of living cells. The absorbance was then measured at 450 nm by using Fluo Star Optima. Data were collected by Control Software and elaborated with MARS Data Analysis Software (BMG LABTECH). Data presented are expressed as mean±standard deviation.

Results and discussion

Either single solvent (acetone, chloroform, dimethylformamide and dichloromethane) or mixtures of them have been used in the ES process of CA.[19] In this work, we selected acetone as single solvent, because both CA and EOs are highly soluble in it. In fact, the prepared solutions were completely homogeneous, without phase separation or presence of solute precipitates. This positively affected the morphology of the produced electrospun composite fibres. As shown in Figure 1a, large area mats were produced. They are easy to handle, and they are characterised by mechanical flexibility. SEM images of fibrous mats of bare CA (Fig. 1b), or obtained from a CA solution with 5% v/v of cinnamon (CA/5-CN, Fig. 1c), lemongrass (CA/5-LG, Fig. 1d) or peppermint (CA/5-PM, Fig. 1e) oil are shown as representative cases. The SEM analyses clearly revealed that all the fibres were free from defects or beaded structures, and characterised by a ribbon-like shape. Fibres of pure CA exhibited a diameter of (4.2±2.1) µm, whereas fibres of CA/5-CN, CA/5-LG, and CA/5-PM had a size of (0.9±0.3) µm, (2.8±1.1) µm and (2.3±0.8) µm, respectively. This can be principally attributed to the different experimental parameters used in the ES process (flow rate and voltage). The length of the fibres is in the range of 6-8 cm.

We analysed the effective encapsulation of the EOs, and most importantly of their active principles, inside the fibres by Raman spectroscopy. In Figure 2a, the comparison between the Raman spectra of CA fibres (continuous black line), bare cinnamon oil (dotted purple line), and CA/5-CN fibres

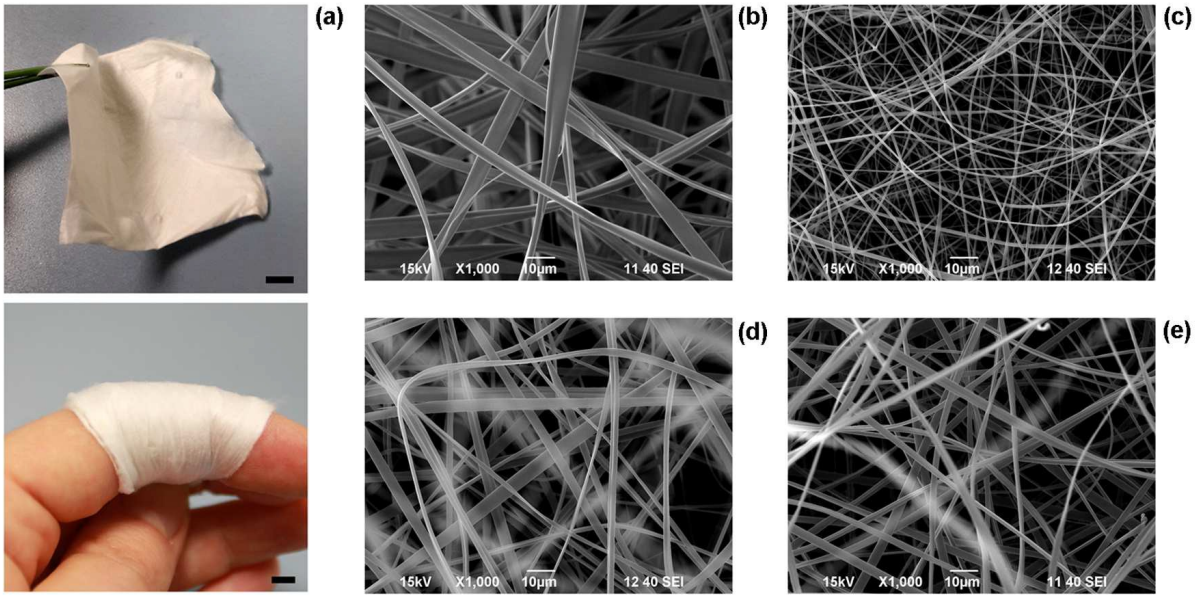

Fig. 1 (a) Photographs of the CA/cinnamon mat: free standing (top picture, scale bar=1cm) and used as a bandage (down picture, scale bar=0.5 cm). SEM images of fibrous electrospun substrates of (b) CA, (c) CA/5-CN, (d) CA/5-LG, and (e) CA/5-PM.

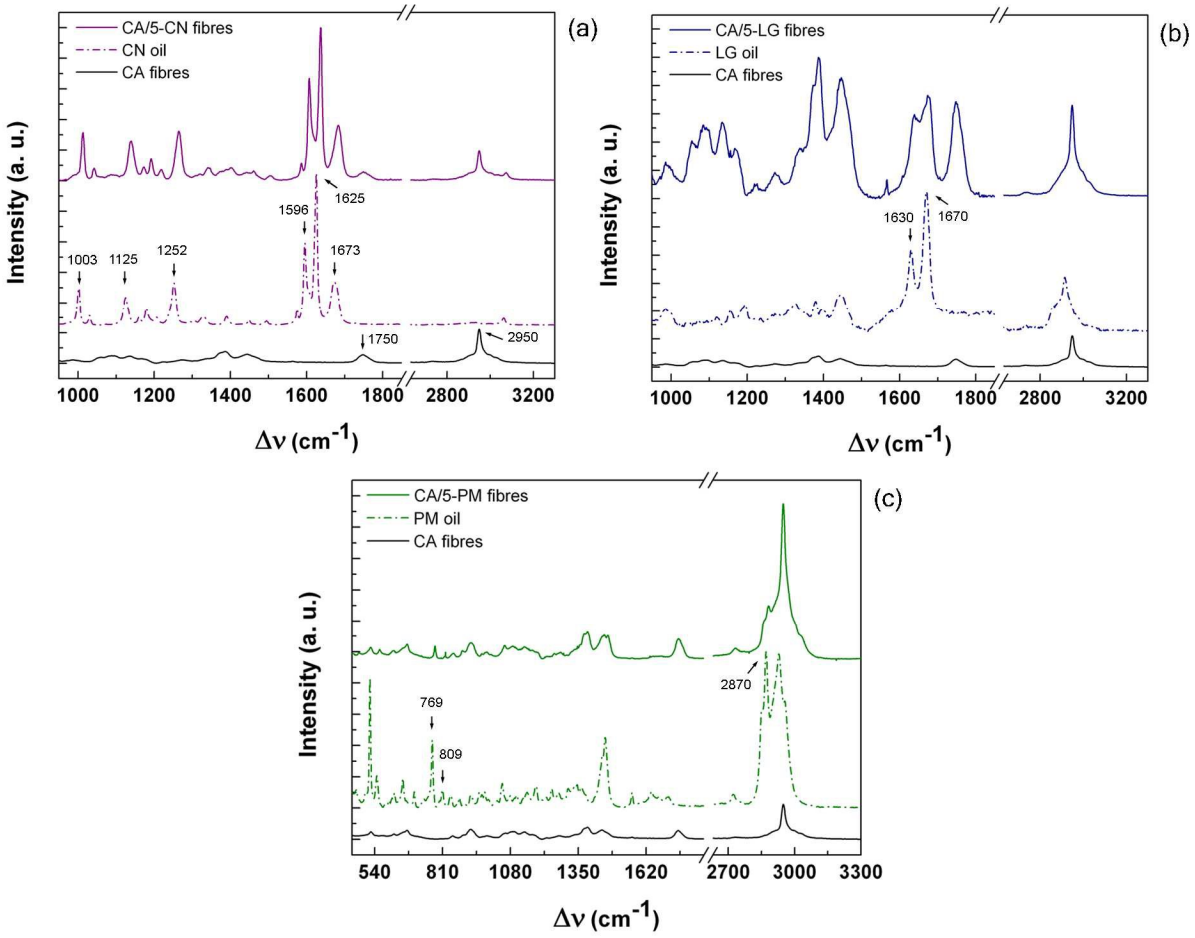

Fig. 2 Comparison between the Raman spectra of the CA electrospun fibres (continuous black line), essential oils and composite CA/EO fibres: (a) pristine cinnamon oil (dotted purple line) and CA/5-CN fibres (continuous purple line); (b) pristine lemongrass (dotted blue line) and CA/5-LG fibres (continuous blue line); (c) pristine peppermint oil (dotted green line) and CA/5-PM fibres (continuous green line).

(continuous purple line) is reported. The spectrum of the CA fibres presents a characteristic peak around 1750 cm^{-1} that arises mainly from the ester C=O of the polymer chains, whereas the bands at 2950 and 2980 cm^{-1} are assigned to the symmetric and asymmetric CH_2 vibrations.[13] The analysed cinnamon oil exhibits characteristic lines at 1003, 1125, 1252, 1596, 1625 and 1673 cm^{-1}, in accordance with the results reported in literature for cinnamon bark and cinnamyl aldehyde.[20] In particular, the peaks at 1003 and 1596 cm^{-1} originate from the mono-substituted benzene rings, as well as the band at approximately 3070 cm^{-1}. The peaks at 1625 and 1673 cm^{-1} are instead ascribed to C=C and C=O bonds, mainly contained in cinnamaldehyde and eugenol that are the main components of cinnamon oil [13] and responsible for its antimicrobial activity.[14] The characteristic bands of CA and cinnamon oil have been found also in the Raman spectrum of CA/5-CN fibres, indicating the presence of the oil inside them. In Figure 2b, the Raman spectrum of lemongrass EO (dotted blue line) and of the realised electrospun composite fibres (continuous blue line) were compared. The peaks at 1630 and 1670 cm^{-1} observed in the spectrum of the bare LG oil are associated to C=C and C=O bonds, respectively. In particular, they are connected to the existence of two terpenes:[20] neral and geranial that are the principal components of LG oil, and confer it the well-known antiseptic properties.[13] Finally, peppermint oil (dotted green line in Fig. 2c) exhibits Raman lines at 548, 769, 809 and 2870 cm^{-1}. The peaks at 769 and 2870 cm^{-1} were due to δ_{C-H} and to the hydrocarbon vibrations, respectively.[21] In this case, the terpenes of interest for the antimicrobial effect are menthone and menthol.[13, 22]. These bands are evident also in the spectrum of CA fibrous mats encapsulating PM oil (solid green curve in Fig. 2c). Therefore, we can state that the EOs and their corresponding antibacterial substances are well encapsulated within the composite electrospun webs.

In order to demonstrate the efficiency of the produced composite fibres against pathogens, we carried out several antimicrobial assays using *E. coli* and *C. albicans* as model microorganisms. We first analysed the bare CA electrospun fibres as reference sample, and we noted that they were not capable of blocking the growth neither of *E. coli* nor of *C. albicans*. In particular, as shown in Figure 3a, the CA fibrous mats were easily colonised by *E. coli*, and the presence of the

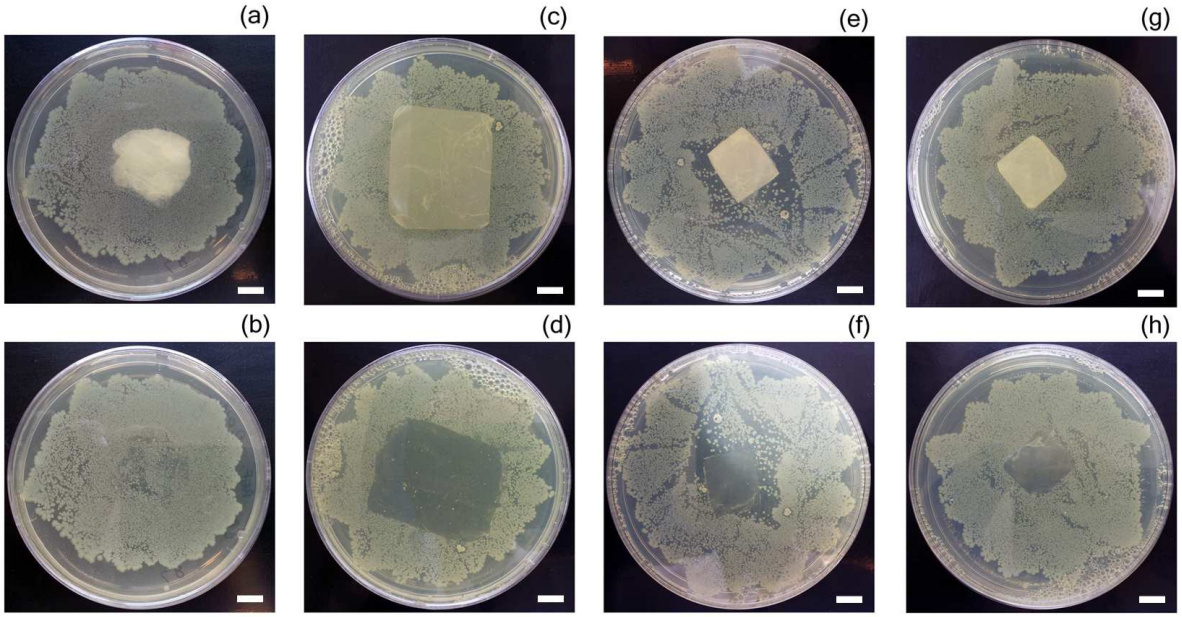

Fig. 3 Photographs of the electrospun mats during the *in-vitro* assays for proving their activity against *E. Coli*: fibres of (a, b) CA without EOs, (c, d) CA/5-CN, (e, f) CA/5-LG, (g, h) CA/5-PM. Biofilm formation (a, c, e, g) before and (b, d, f, h) after the removal of the fibrous scaffolds. Scale bar = 0.5 cm.

biofilm is clearly visible in the agar medium after the removal of the electrospun nonwoven fabric (Fig. 3b). The same behaviour was also observed for *C. albicans* (Fig. S1). On the contrary, fibres encapsulating 6.2 and 25.0 w/w % of EOs were able to effectively stop the proliferation of *E. coli*. In Fig. S2 and in Fig. 3 photographs of the agar plates before and after the removal of the fibrous mats with EOs are shown. Since the CA matrix swells just slightly inside the agar medium, the encapsulated EOs are not expected to be released intensely in the aqueous-based environment. Therefore, a clear inhibition zone around the samples was hardly detected (Figs. 3c, 3e, and 3g). On the other hand, when the CA/EOs fibrous samples were removed from the bacteria culture, the area previously occupied by them is totally free from *E. coil* (Figs. 3d, 3f, and 3h), indicating that the EOs exerted their antibacterial activity. Therefore, the produced fibrous networks were antimicrobial mainly upon direct contact with the microorganisms.

The use of fibrous networks, instead of planar films, strongly enhances the antibacterial effect of the investigated composite CA/EOs materials. This is mainly due to the high exposed surface area of the fibres compared to uniform films, and also to the possibility for the microorganisms to penetrate inside the fibrous network, perceiving better the presence of EO active principles. If we consider *E. coli* cells, their size is of approximately of 1.5 μm,[23-24] and every 20 minutes they start a division cycle. Therefore, at any time, inside the agar medium *E. coli* cells have a size smaller than the gaps between the neighbouring fibres. From the SEM pictures in Fig. 1, it is possible to estimate that the empty spaces in the fibrous network have a dimension of approximately 2-10 μm. Therefore, *E. coli* cells were able to penetrate inside the matrix of the fibres (Fig. S3a), and the direct contact with the surrounding antibacterial material inhibited their growth and proliferation. For this reason, we achieved antimicrobial activity even encapsulating small amounts of EOs inside the fibres, whereas in another previous study from our group the same concentration of EOs within compact alginate films could not inhibit the *E. coli* growth.[16]

In order to strengthen this hypothesis, we tested the electrospun mats with microorganisms having dimensions bigger that *E. coli*. To this aim, we selected *C. albicans* cells that have diameters of around 4 μm,[25] hence more than four times larger than *E. coli.* Even if in a previous study we have demonstrated that the EO of cinnamon, lemongrass and peppermint strongly reduce the proliferation of this fungus,[16] here we observed that CA fibres containing both 6.2 and 25 w/w % of EOs were not effective. We observed that even 40 w/w% of EOs in the CA solution was not capable to successfully block the growth of *C. albicans* (Fig. S4). We believe that one possible explanation for this different behaviour is related to the dimensions of *C. albicans* compared with the morphology of the electrospun mats. Due to their size, *C. albicans* cells difficultly penetrate inside the meshes of the web, and therefore they can only sense the EOs loaded in the outermost layers of the fibrous network (Fig. S3b). The small percentage of active agents contained in the surface of the fibres was not enough to stop their growth, resulting in the observed high resistance of *C. albicans* with respect to *E. coli*. Antifungal activity was observed for EOs concentrations higher than 40 w/w%, but in this case the quality of the electrospun fibres was negatively affected.

Since for real applications it is important to investigate over time the stability and durability of the antimicrobial effect of the produced composite fibres, the CA/EO fibres were also stored for two months under ambient conditions (room

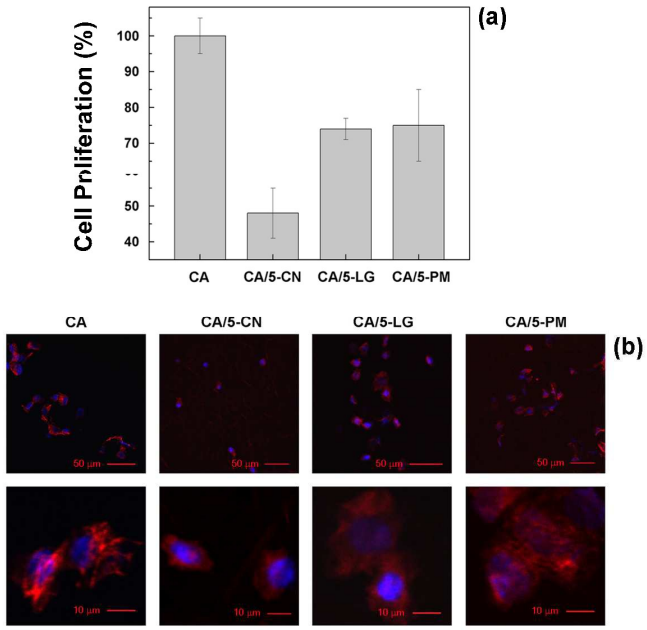

Fig. 4 Viability of fibroblast cells on the different types of electrospun fibres (CA, CA/5-CN, CA/5-LG, CA/5-PM) after 24 h: (a) results of MTT assay, (b) fluorescence micrographs of DAPI (nucleus) and phalloidin (actin filaments) at different magnifications.

temperature and air) before being tested against *E. coli*. We observed that they still retained the capability to stop the growth of these bacteria (Fig. S5).

The biocompatibility of the fibrous scaffolds was evaluated using two *in-vitro* models: immortalized fibroblasts and normal human keratinocytes. We selected cell lines that are directly involved in the wound healing process and in the regeneration of cutaneous tissue.[26] The analysis was conducted for fibres electrospun from a CA solution containing a high amount of EOs (5 v/v%), in order to prove the non-cytotoxicity of the fabricated scaffolds even at those concentrations. Figure 4a shows the viability of fibroblast cells on the produced fibrous substrates after 24 hours. As control samples, we used pristine CA fibres without EOs. The CA mats exhibited high cell compatibility and no cytotoxicity, and the morphological analysis in Figure 4b reveals that the cells could attach and spread onto the fibres' surface. However, we observed that cell proliferation and migration was strongly affected by the size of the fibres and by the presence of large pores, as documented in literature.[27-29] A reduction of cell viability (from 25 to 50%) was instead noticed for CA fibres containing essential oils. This result was expected since the anti-proliferative effect of essential oils for eukaryotic cells has been already reported in literature.[29-30]

A similar behaviour was observed for the proliferation of normal human keratinocytes (Figure 5). In all cases (CA fibres with and without essential oils), HaCaT cells exhibit a good tolerance to the fibrous scaffolds also for long term incubation, even if a time dependent reduction of the cell viability was detectable for CA/EOs substrates. After 96 hours, the WST-8 assay revealed around 75% of viable cells for CA/5-CN, 90% for CA/5-LG and 80% for CA/5-PM. In the first two cases we noted a time dependent viability reduction that it was instead less appreciable for CA/5-PM. For these fibres, in fact, the cell growth was of around 80% already after 24 hours of incubation, likely due to the presence of chemical compounds in PM oil that affect keratinocytes proliferation.[31, 32]

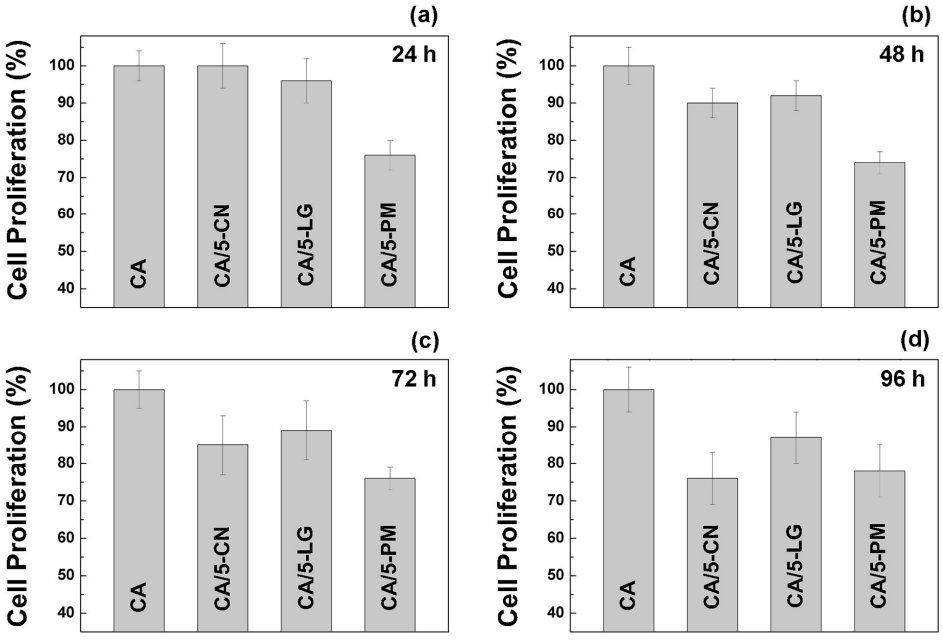

Fig. 5 WST-8 assay for HaCaT cells proliferated on the different electrospun scaffolds (CA, CA/5-CN, CA/5-LG, CA/5-PM) after (a) 24, (b) 48, (c) 72 and (d) 96 h of incubation.

Conclusions

We exploited the antimicrobial properties of essential oils of cinnamon, lemongrass and peppermint for the production of fibrous dressings for wound care. We created composite fibrous scaffolds, consisting of cellulose acetate and EOs, by the electrospinning technique. We analysed the effective encapsulation of the EOs into the fibres by Raman spectroscopy, demonstrating that the active principles, responsible for the antiseptic activity of the oils, were contained inside them. In fact, the Raman spectrum of CA/5-CN fibres showed peaks at 1625 and 1673 cm^{-1} that are characteristics of cinnamaldehyde and eugenol; Peaks at 1630 and 1670 cm^{-1} were detected for CA/5-LG fibres, corresponding to neral and geranial; Bands at 769 and 2870 cm^{-1} were observed for CA/5-PM fibres that were ascribed to the presence of menthone and menthol. The efficacy of the CA/EOs fibres was tested against *E. coli*, resulting in a complete inhibition of bacteria growth. We observed that fibrous mats encapsulating even low amount of essential oils (6.2 w/w %) were able to inhibit the formation of *E. coli* biofilms. Moreover, biocompatibility assays on two different cell lines (fibroblasts and human keratinocytes) revealed that the non-cytotoxicity of the scaffolds, which can be therefore advantageously used to prevent wound infections.
We envisage that the here proposed antibacterial mats can find application as innovative patches or gauzes for wound care. To this end and in view of a real clinical application, the recently developed portable electrohydrodynamic guns are a practical and attractive alternative to the conventional electrospinning process.[33-34]

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Notes and references

[a] Smart Materials, Nanophysics, Istituto Italiano di Tecnologia (IIT), via Morego 30, 16163 Genoa, Italy.
ioannis.liakos@iit.it; athanassia.athanassiou@iit.it; elisa.mele@iit.it.
[b] Center for Biomolecular Nanotechnologies, Istituto Italiano di Tecnologia @UniLe, via Barsanti, 73010 Arnesano, Lecce, Italy.
[c] Present address: Department of Chemistry, and Centre for Molecular and Medical Virology, University College London (UCL), 20 Gordon Street, WC1H 0AJ, London, United Kingdom.
[d] DIBRIS, University of Genoa, Via Opera Pia 13, 16145, Genoa, Italy.

† Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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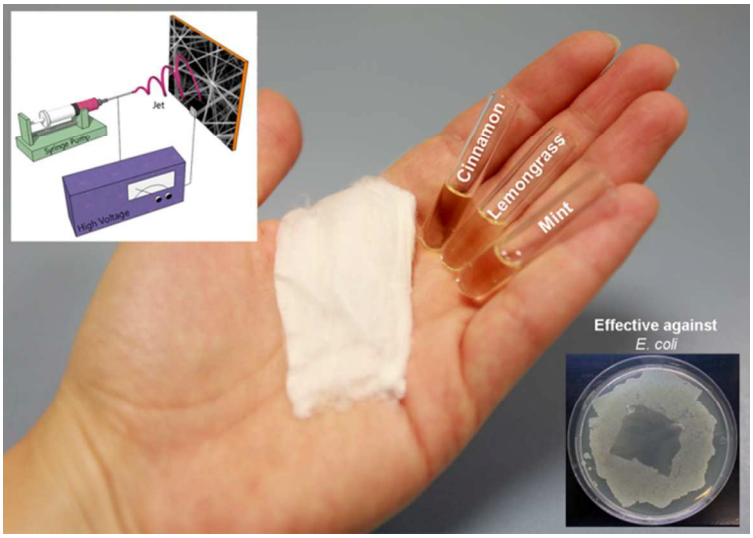

Essential oils with high antibiotic activity were incorporated into cellulose acetate natural polymer. By using the electrospinning technique nanofibrous matrices were prepared to be used as effective antimicrobial wound dressings.