Catechin loaded PLGA submicron-sized fibers reduce levels of reactive oxygen species induced by MWCNT in vitro

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Abstract

Reactive oxygen species (ROS) are common products of normal aerobic cellular metabolism, but high levels of ROS lead to oxidative stress and cellular damage. Therefore, effective antioxidant therapies are needed to prevent ROS overproduction. This study reports the development of poly(l-lactide-co-glycolide) (PLGA) bicomponent fibers loaded with selected amounts of the natural polyphenolic antioxidant catechin. Thereby a novel route based on emulsion electrospinning is investigated to obtain tailored and sustained release rates for chatechin. The activity of the released catechin was assessed for its influence on multi-walled carbon nanotube (MWCNT) induced formation of reactive oxygen species (ROS) in human alveolar epithelial cells of the cell line A549.

Homogenous fiber morphologies were obtained at specified ranges of PLGA concentrations within the emulsions including the formation of a core – sheath structure localizing the drug within the fiber core. *In vitro* measurements of the delivery showed moderate burst release kinetics in a first phase followed by a linear and smooth release at long term. In combination with polymer degradation studies a mostly diffusion controlled release mechanism was revealed exhibiting only marginal degradation of the polymer during the time span of the drug delivery. The activity of released catechin in A549 cells stimulated with MWCNTs revealed a high reduction of ROS production in a dose dependent manner. This effect diminishes over time indicating a depletion of catechin.

Key words

catechin; nanofiber; PLGA; drug delivery; reactive oxygen species.

1 Background

Reactive oxygen species (ROS) play an important role in numerous physiological processes but at high levels they can lead to oxidative stress [1, 2]. They are formed as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis [1]. An overproduction of ROS species caused e.g. by environmental stresses such as exposure to nanoparticles or ultraviolet light irradiation, may initiate damage of intracellular components such as nucleic acids, lipids or proteins which may ultimately lead to cell death [3]. Nature has endowed us with protective antioxidant mechanisms - superoxide dismutase, catalase, glutathione, glutathione peroxidases and reductase, vitamin E etc. As a supporting measure, the administration of antioxidants at the site of action (e.g. on wounds) with tailored release kinetics is a promising route for enhanced healing efficiencies [4].

Polyphenols are a structural class of mainly natural, but also synthetic, organic molecules characterized by multiple active phenol structural units important with respect to biological activity [5]. Catechin is a non-toxic polyphenolic compound that belongs to the flavonoid family and is considered to be beneficial for the human organism owing to its antioxidant, antiviral, anti-inflammatory, anti-carcinogenic and anti-aging properties [6-10]. The bioactive compound, catechin, is widely found in many dietary sources especially in green tea as well as in skins and seeds of fruits like grapes and apples [11]. The stability of catechin is influenced both by environmental pH and temperature conditions as well as exposure to UV light [12, 13] which makes it a predestinated candidate for stabilisation by encapsulation to obtain enhanced and prolonged bioactivitity.

Electrospinning is an efficient technique for producing continuous nanofibers with diameters ranging from a few micro- to several nano-meters in the form of porous membranes [14, 15]. Due to many attractive properties such as large surface - to - volume ratio,

nanoporous structure, interconnectivity of pores and the possibility to safely encapsulate active compounds, nanofibers have received great attention in the last years. The encapsulation of therapeutic substances within e-spun fibers allows for adapted and controlled release kinetics including application at the site of action. These unique properties propose them as candidates for a variety of applications, especially as drug delivery systems and scaffolds for tissue engineering [16-18].

Recently, electrospun fibers containing polyphenols such as green tea polyphenols (GTP), epigallocatechin-3-*O*-gallate (EGCG), have been shown to successfully inhibit the proliferation of bacteria and cancer cells when applied in wound dressing with the purpose to enhance e.g. wound healing processes [19, 20] [21, 22]. Ghitescu et al. encapsulated synthetic as well as natural polyphenols from spruce bark extracts into swellable and non-toxic poly(2-hydroxyethyl methacrylate (pHEMA) leading to high release rates in a first phase followed by a smooth and sustained release at long term. The encapsulated polyphenols retained their antioxidant activity after release revealing a highly delayed degradation to be induced by environmental factors [23].

A wide range of synthetic and natural polymers have been used to produce nanofibers by e-spinning procedures. Poly(lactic-co glycolic acid) (PLGA) is a synthetic, non-toxic, biodegradable and cytocompatible polymer with high potential in biomedical applications such as for drug delivery or tissue engineering [24]. Furthermore, PLGA has tunable properties by controlling relevant parameters such as polymer molecular weight and the ratio of lactide to glycolide, influencing polymer degradation rate and wettability and thus drug release kinetics. All these properties make PLGA an ideal choice particularly for drug delivery [25].

The required release kinetics of the bioactive agent depend strongly on the applications. With respect to sustained release, the successful treatment of infections requires antimicrobial

therapies at long term [26]. Catechin loaded microspheres revealed sustained release kinetics and showed high in vitro anticancer activity using human epidermoid cancer cells (HEP2) and are thus potential candidates to sensitize tumor cells in anticancer therapies [27]. Within the food sector, antioxidant treatments are highly demanded, e.g. by use of drug delivery systems acting at long term conditions [28].

Recently, electrospinning of emulsions has attracted interest because of the capability to produce fibers with e.g. encapsulated bioactive agents followed by controlled delivery without loss of their bioactivity [29, 30]. Water-in-oil emulsions usually contain a continuous organic phase incorporating the dissolved matrix polymer, and a water phase incorporating the bioactive therapeutic component [31]. The properties of the nanofibers, including fiber diameters, pore sizes as well as fiber structure can be tailored by carefully selecting polymers, solvents, and electrospinning processing conditions [32]. Collagen-like protein has been encapsulated within PLGA fibers, in the form of core-sheath fibrous structures by emulsion electrospinning. The release profiles of encapsulated protein from the nanofibers and the cytocompatibility to the fibroblast cell line L929 were investigated revealing its potential application as a drug release device as well as a tissue engineering scaffold [33] Norouzi et al. prepared PLGA / gelatin core-sheath fibers incorporating recombinant human epidermal growth factor by use of emulsion electrospinning. The sustained release allowed for hemostasis giving rise to potential application in skin tissue engineering as a wound dressing [34].

The administration of the therapeutic encapsulated agents from e-spun non-wovens strongly depends on the type of disease and the site of delivery. To this end, typical routes of administrations from such fibrous materials are topical applications (e.g. transdermal [35] or gastro-retentive [36]) to deliver locally, or into the systemic circulation system [26].

The objective of this study was to investigate the determining e-spinning parameters to obtain homogeneous catechin loaded PLGA fibers by use of stabilized water-in-oil emulsions. To obtain information about the release mechanism, the polymer degradation behavior of the fibers as well as the *in vitro* release characteristics of catechin was investigated. Finally, the antioxidant effect of catechin released from the fibers on the human alveolar epithelial cell line A549 was examined.

2 Methods

2.1 Materials

PLGA (weight-average molecular weight M_w=76.000-115.000 g/mol 75:25), chloroform (purity >97%), SPAN 80, phosphate-buffered saline (PBS) and catechin hydrate were purchased from Sigma-Aldrich (Switzerland). All reagents were used as received.

2.2 Preparation of electrospinning polymer emulsion

To obtain spinnable emulsions, catechin was first dissolved in distilled water to obtain 0.1 w/v %; 0.25 w/v % and 0.5 w/v % aqueous solutions. PLGA was dissolved in chloroform to obtain concentrations of 10 w/v %, 15 w/v %, 17 w/v % and 20 w/v %, followed by addition of 0.2% w/v of the surfactant SPAN 80 to form the oily phase. Subsequently, the aqueous solution of catechin was added drop wise while stirring at 500 rpm in order to obtain homogenous and stable emulsions with water to oil phase volume ratio of 1:8 (Fig. 1a).

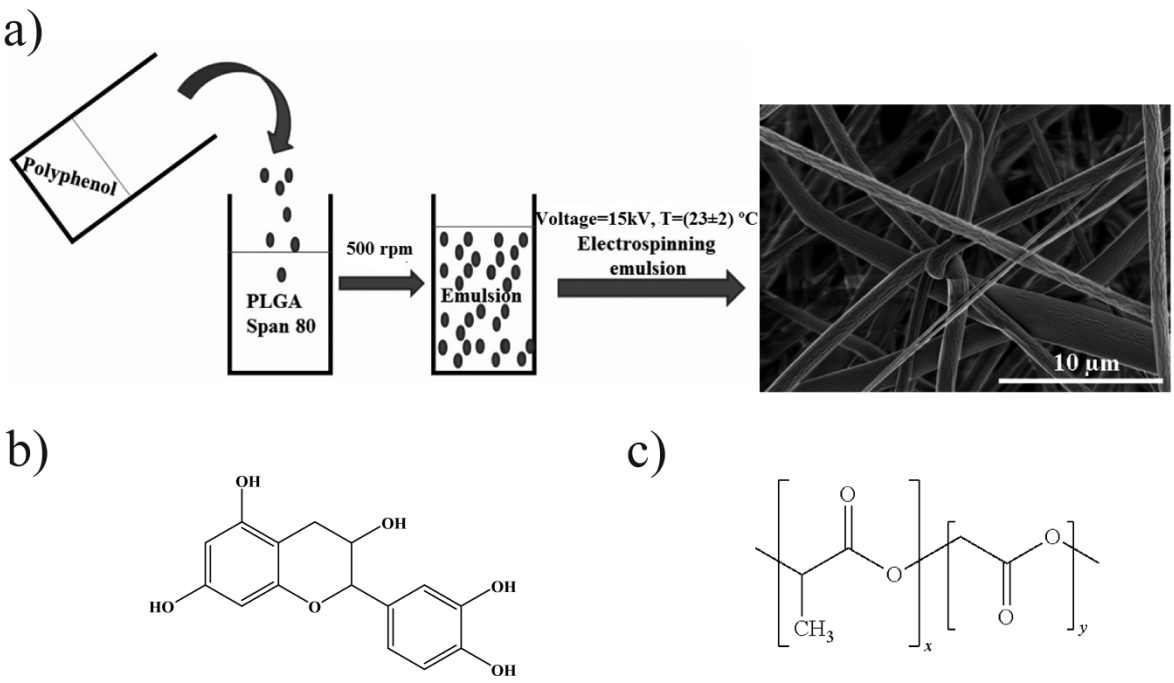

Figure 1 a) Schematic process description for the preparation of emulsions consisting of PLGA, catechin and Span 80, and respective spinning conditions, b) molecular structure of catechin and c) PLGA polymer

2.3 Electrospinning of PLGA nanofibers

The electrospinning setup was operated in a horizontal mode which included a medical infusion pump (KD scientific, USA), two high voltage power supplies (aip Wild AG, Switzerland) connected to the needle (positive potential) and an aluminium collector (negative potential). Emulsions were delivered from 1 mL syringes fitted to a stainless steel needle of 0.8 mm inner diameter. The distance between the needle and the collector plate was fixed at a distance of 15 cm. A voltage of +15 kV / -2 kV was applied between the needle (anode) and the collector plate (cathode). The volume flow rate of the emulsion spinning dope was set to 10 µL/min. The whole equipment was placed in a grounded Faraday cage within a chemical hood. The spun samples were air dried for at least 24 h before characterization. All

experiments were carried out at room temperature (23±2) ºC and below a relative humidity of 45%.

2.4 Emulsions and fibers characterizations

To investigate morphological features of the spinning emulsions, 20 µL of each was placed onto a glass slide and covered by a cover slip to image the aqueous droplets by optical microscopy (Keyence, VHX-1000, Belgium) using identical time intervals between measurements for all emulsion types. The droplet size distribution was determined by Image J software (http://imagej.nih.gov/ij/) using the particle size analyzer tool.

A rheometer (Anton Paar Physica MCR 301, Austria) equipped with a plate - cone geometry was used to study the rheological properties of the solutions and emulsions. To exclude aging of emulsions, a pre-shearing of 50 s^{-1} was applied for 30 s at 20 °C before measurements. Flow curves with shear rates varying from 0.01 to 500 s^{-1} were recorded at 25 °C in triplicates.

The morphologies of pure and catechin - loaded PLGA nanofibers were examined by scanning electron microscopy (SEM) on a Hitachi S4800 (USA & Canada) by use of an accelerating voltage of 2 kV. Prior to imaging by SEM, all samples were coated with 5 nm gold using a plasma coating unit from Polaron Equipment Ltd (E5100, Switzerland). The average diameter of the nanofibers was determined by Image J software by evaluation of at least 50 independent measurements. The scanning transmission electron microscopic measurements (STEM) were recorded using 30 kV accelerating voltage and 10 mA current flow (Hitachi S4800, USA & Canada). The fibers were spun directly onto 200 mesh carbon coated copper grids (Plano, Germany).

FTIR spectra of the fiber membranes were recorded by using a BioRad “FTS 135” FTIR (USA) spectrometer equipped with a Specac “Golden Gate” ATR accessory. The scans (32 scans) were recorded between 4000 and 500 cm^{-1} at 4 cm^{-1} resolution.

Wide angle X-ray scattering (WAXS) measurements were performed using a Bruker Nanostar diffractometer with a sealed tube Cu Kα (1.5418 Å) operated at 600 μA and 50 kV and a 2D Vantec detector with an active area of 14 x 14 cm^2 and with a pixel size of 68μm x 68μm. The sample to detector distance was 132 mm and the exposure time was one hour. All measurements have been performed at room temperature under vacuum. The acquired two dimensional scattering patterns were integrated into a one-dimensional scattering function I(q) using BRUKER DIFFRAC.EVA V. 4.1 software.

2.5 Catechin release from PLGA nanofibers under *in vitro* conditions

The release profiles of catechin from PLGA fibers were recorded by UV-vis absorption measurements in phosphate buffered solution (PBS). During the studies, about 0.02 g electrospun nanofiber membranes were immersed in 2 mL PBS (pH=7.4) and incubated at 37ºC under agitation at 100 rpm. At predetermined time points 200 μL aliquots were withdrawn from the test tube for analysis and the same volume of fresh PBS was added to maintain a constant volume. At the end of each predetermined incubation period, the absorbance was measured at 276 nm by use of an ultraviolet spectrophotometer (BioTek SynergyMx multi-mode microplate reader, USA). Concentrations of catechin released from PLGA membranes were calculated by use of a standard calibration curve of catechin solutions with the regression equation: $y = 0.0068x + 0.0149$ with a correlation coefficient $R^2 = 0.9985$ (Figure S1 supplement). No interference of the signal and no bias effect was found with respect to possibly released SPAN80, which was confirmed by measuring different concentration ratios for catechin and SPAN80 at constant catechin concentration.

2.6 In vitro degradation of PLGA - catechin nanofiber membranes

Membranes were incubated in PBS at 37 °C and pH 7.4 at specified time intervals (2 weeks and one month), followed by rinsing with distilled water and drying under vacuum at room temperature prior to the determination of the molecular weights and their distributions by gel-permeation chromatography (GPC). Thereby, a Viscotek GPCmax (Viscotek, Houston, USA) equipped with a refractive index (RI) detector and three consecutive columns (PSS SDV 10^3 Å, PSS SDV 10^5 Å and PSS SDV 10^7 Å) (PSS, Germany) was used. For the measurements, PLGA was dissolved in tetrahydrofuran (THF) (4 mg/mL) at ambient temperature. The solutions were passed through a 0.45 µm syringe filter to remove non-dissolved material and dust, and THF was used as the mobile phase. The average molecular weight was calculated using polystyrene standards (PS) and the universal calibration method. The molecular weight loss of PLGA nanofibers was calculated employing the formula:

Mass loss (%) = (Initial M_w of sample –M_w of degraded sample) / Initial M_w of sample x 100

2.7 Cell culture

The human alveolar epithelial cell line A549 (ATCC: CCL-185) was obtained from the American Type Culture Collection and cultivated in RPMI-1640 cell culture medium (Sigma, Buchs, Switzerland) supplemented with 10% fetal calf serum (FCS, Lonza, Switzerland), 2 mM L-glutamine (Gibco, Invitrogen, Switzerland) and 1% penicillin-streptomycin-neomycin (PSN) (Gibco)). Cells were grown in tissue culture flasks at 37 °C in 5% CO_2 humidified atmosphere and were sub-cultured at approximately 80%–90% confluency using 0.5% Trypsin - EDTA (Sigma-Aldrich, Switzerland).

2.8 Measurement of ROS species

To induce ROS formation in A549 cells MWCNTs from Bayer Technologies Service (Baytubes®, Leverkusen, Germany) were used. A thorough characterization of the used

MWCNT material (Thurnherr et al., 2009) as well as a protocol for their dispersion was described by Roesslein et al. [37].

The influence of pure catechin as well as catechin released from PLGA fibers on ROS production in cells was analyzed using the dichlorofluorescein assay (DCF). For this pure catechin, pure PLGA fibers as well as fibers containing either 1.1 or 2.3 wt.% of catechin were immersed in HBSS (Hank's balanced salt solution) for 3 days. The resulting supernatant was composed of HBSS and pure or released catechin, respectively, and is termed conditioned buffer. Seven serial 1:2 dilution steps of the conditioned buffer in HBSS were performed. Each of the resulting concentrations, from 100% (undiluted) down to 0.78%, was mixed with 20 µg/mL MWCNTs shortly before cell treatment. A549 cells were seeded in 96-well plates at a density of 2×10^4 cells / well in a volume of 200 µL complete cell culture medium. After 24 h, cells were incubated for 60 min in 50 µM 2′,7′-Dichlorodihydrofluorescein diacetate H_2DCF-DA (Molecular Probes, Invitrogen, Switzerland) in HBSS at 37 °C and 5% CO_2. Afterwards, cells were washed twice with pre-warmed HBSS and treated with 100 µL of conditioned buffer containing 20µg/mL MWCNT or selected concentrations of catechin in HBSS, also in the presence of 20 µg/mL MWCNT, at 37 °C and 5% CO_2. Fluorescence intensity was measured at 10, 30, 60 and 120 minutes using a FLX800 fluorescence microplate reader (BioTEK Instruments, Winooski, VT, USA) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm.

3 Results and discussion

3.1 Morphology of electrospun fibers from pure PLGA solutions

Firstly, in order to obtain smooth and uniform fibers, selected concentrations of pure PLGA solutions in chloroform, which acted later as the continuous phase within the emulsions, were

investigated for spinning applications. Molecular structures for catechin and PLGA are presented in Figure 1a and b.

Rheological measurements of PLGA solutions (polymer concentrations of 10% w/v, 15% w/v, 17% w/v and 20% w/v) revealed Newtonian flow behavior at low shear rate conditions, whereas at higher shear stresses a slight shear thinning behavior was determined (Figure 2a). The solution viscosity was directly proportional to the polymer concentration (Figure 2b) being accompanied with an increase of fiber diameter with increasing PLGA solution concentration (Figure 2b). The fiber diameter increased with increasing polymer concentration according to a power law relationship as pointed by Huang et al. [38] (Figure 2b).

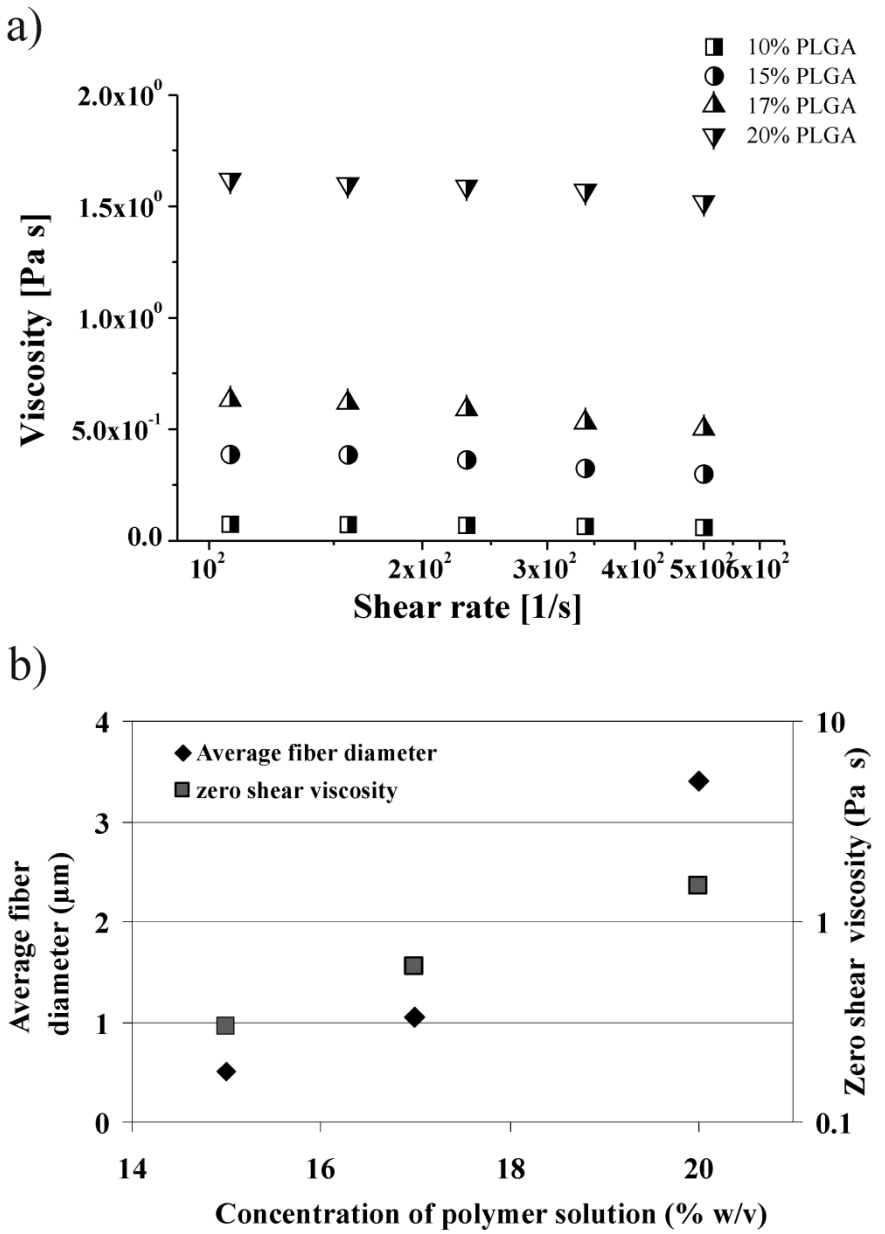

Figure 2 a) Flow curves of PLGA solutions at different concentrations of the polymer, b) Relationship between fiber diameter, viscosity and concentration of polymer solution

Using a 10% w/v polymer solution concentration bead formation was observed due to low viscosity and thus low concentration of polymer chain entanglements leading to jet ruptures (Figure 3a). Increasing the polymer concentration to 15% w/v beaded fiber formation was seen with mean fiber diameters of 0.5±0.1 µm (Figure 3b). Finally, pure fiber formation was obtained at higher polymer concentrations with increasing fiber homogeneity,

incorporating fiber diameters of 1.04±0.11 µm and 3.40 ±0.29 µm for polymer solution concentrations of 17 % w/v and 20 % w/v, respectively. To this end, an optimum polymer concentration of 20 % w/v was defined and used for further emulsion spinnings (Figure 3d).

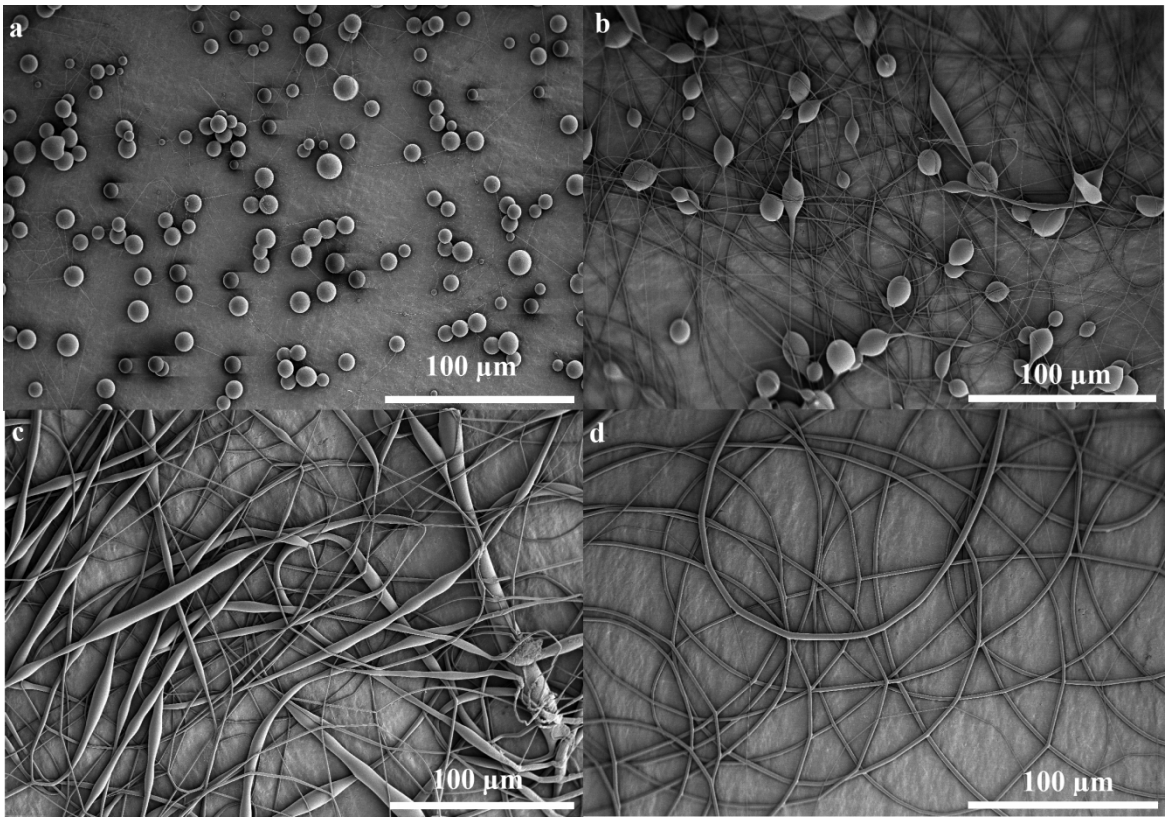

Figure 3 SEM micrographs of pure PLGA spun by use of the following solution concentrations: a) 10% w/v b) 15% w/v c) 17% w/v d) 20% w/v

Compared to pure PLGA fibers prepared by solution electrospinning, the use of dissolved catechin in the form of water-in-oil emulsions induced reduction of the fiber diameters by a factor of 4 to 5 as well as the formation of roughened fiber surfaces (Figure 4). Due to the higher dielectric constant of H_2O (ε = 80.4) compared to $CHCl_3$ (ε = 4.8), higher drawing ratios are provoked during fiber formation leading to smaller diameters for emulsion spun fibers.

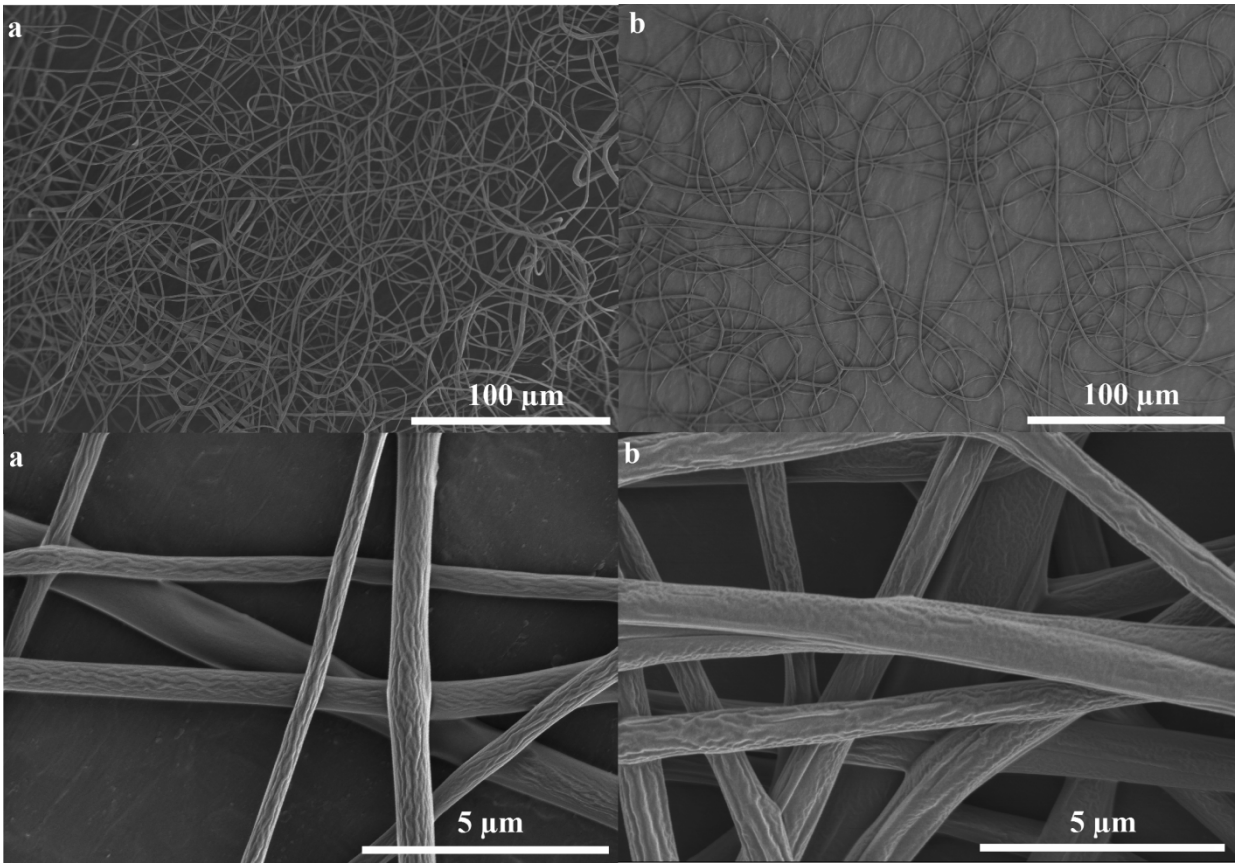

Figure 4 SEM micrographs of e-spun PLGA fibers incorporating catechin: a) PLGA fibers incorporating 0.9 wt% catechin (from 0.1 w/v % aqueous solution), fiber diameters 634 ±203 nm, b) PLGA fibers incorporating 2.3 wt% catechin (from 0.25 w/v % aqueous solution), fiber diameters 708 ±367 nm.

3.2 Morphological and structural properties of PLGA emulsions and respective electrospun fibers

To study the effect of catechin concentrations with respect to emulsion morphology, optical microscopy was performed to assess droplet sizes and droplet size distributions within the emulsions. As shown by Li et al. [39] for water-in-oil emulsions (poly(lactic acid) / cellulose nanocrystals) the droplet size can have an impact on morphology of e-spun fibers, and only optimized emulsions give raise to pure core-shell structures.

A linear increase of mean droplet size was found starting from 0.6 +/- 0.3 µm for 0.1 % w/v, 0.9 +/- 0.2 µm for 0.25 % w/v to 1.3 +/- 0.5 µm for 0.5 % w/v catechin emulsions measured in 20 % w/v PLGA concentrations (Figure 5). The variation of catechin concentration in the water phase and within the concentration range analyzed, produced emulsions with narrow droplet size distribution for every concentration, one important parameter for spinning of homogenous fibers. Increasing the concentration of catechin within the emulsion was accompanied by an increase of droplet size. Our results are in concordance with results for a system based on tea polyphenols within water in oil emulsion [40].

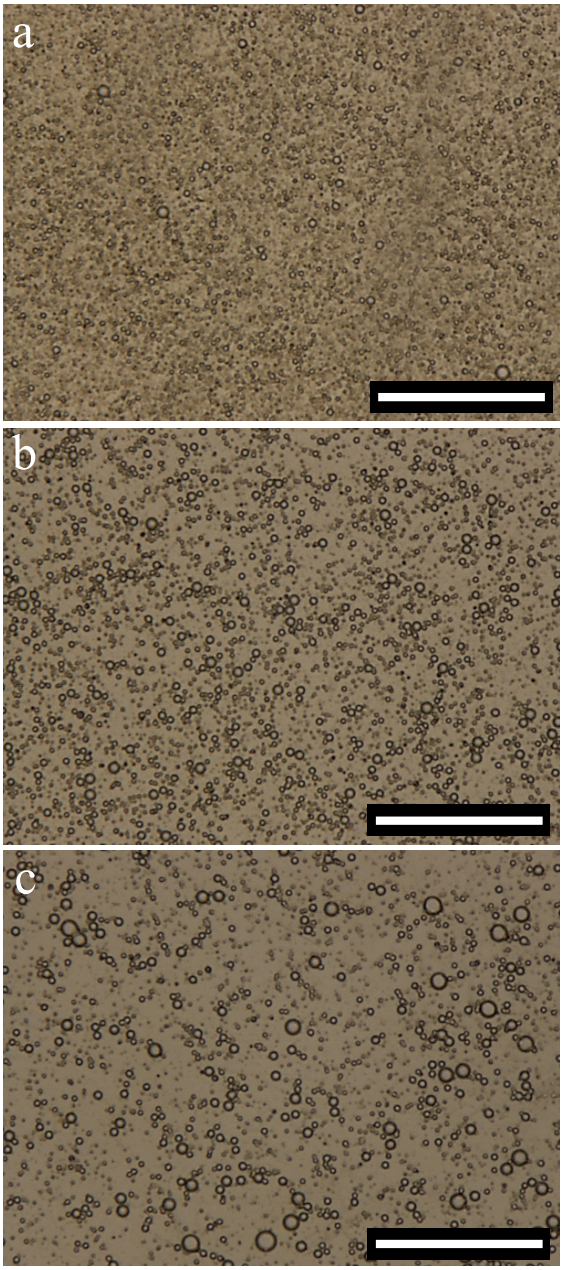

Figure 5 Optical micrographs of PLGA emulsions (20 % w/v) containing a) 0.1 % w/v, b) 0.25 % w/v and c) 0.5 % w/v of catechin, including 0.2 % w/v Span80 as the surfactant (scale bars denote 100 µm).

In order to get in depth insight into the spinning characteristics of the emulsions and respective fiber morphologies, STEM analysis was performed (Figure 6a). The micrograph depicts nanoscaled bicomponent fibers with a suggested core enriched with catechin (brighter

area) and PLGA as the main sheath material (darker area). The use of dispersions and emulsions revealed similar core-sheath structured fibers, as found for cellulose nanocrystals encapsulated within PLGA fiber sheaths, Polyvinylalcohol encaspulated within Polycaprolactone, or Poly(ethylene oxide) encapsulated within a Poly(ethylene glycol)-poly(L-lactic acid) diblock copolymer [41-43]. Reasons of a core-sheath formation are based on two factors, which are the drawing of the emulsion droplets and the different solvent evaporation rates of the solvents. The movement of the water droplets within the needle and the jet (which solidifies within tens of milliseconds [44]) and respective shear stresses turns them into elliptical shape in fiber direction. A further fact is that chloroform evaporates faster than water due to high differences in vapour pressure. So, the viscosity of the $CHCl_3$/PLGA oily phase increases faster compared to the H_2O/catechin phase. This viscosity gradient from the outer layer to the inner layer results in an inward movement of the elliptical aqueous droplets, and results in their mergence, which was investigated thoroughly by Xu et al. [45] for a water-in-oil emulsion based on a diblock copolymer (within the oil phase) and a fluorescently labelled poly(ethylene oxide) (within the water phase) [45].

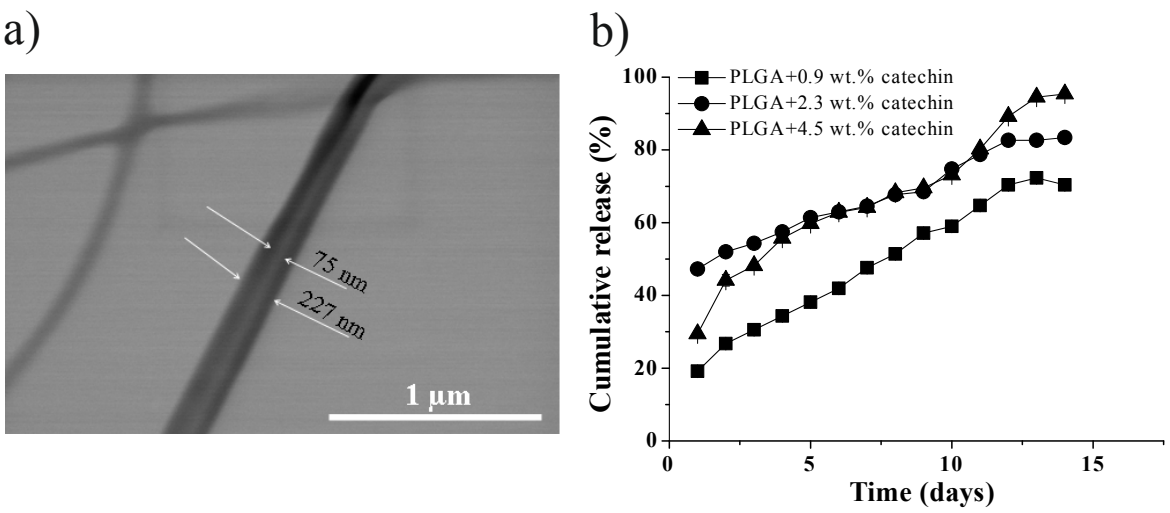

Figure 6 a) STEM micrograph of a PLGA fiber loaded 2.3 wt.% catechin prepared by emulsion electrospinning, b) cumulative release of catechin from PLGA e-spun fibers in function of time.

WAXS data measured on PLGA fibers spun from solution (as in Fig. 3d, 20 wt.% PLGA) and PLGA fibers from emulsion with (4.5 wt.%) and without incorporated catechin showed amorphous structural characteristics for all fiber samples (Figure S3 within the Appendix). Catechin had no influence on the structural nature of the polymer and consequently did not act as a nucleation center.

3.3 *In vitro* catechin release and its relation to fiber properties

Release of catechin from fibers was studied in PBS at 37°C and pH 7.4 as a function of drug concentration and time (Figure 6b). All fiber membranes exhibited initial burst release after immersion, followed by a smooth and linear release behavior. This initial rapid release might be attributed to residual catechin in the superficial zone being adsorbed near the fiber surface [46]. It is suggested that not a pure core-sheath structure is formed during fiber formation, but a core enriched with catechin including minor amounts near the fiber surface.

Compared to *in vitro* release measurement results determined by Wei et al. [33] and Norouzi et al. [34] for protein / PLGA core-sheath e-spun fibers similar trends are found as for the presented PLGA / catechin fiber system despite the much smaller geometrical dimension of the catechin molecule. However, differences need to be stated with respect to fiber diameters and thus diffusion pathways as well as different chemical affinities of the active agents towards the polymers, leading to changed release rates. Compared to the polyphenol epigallocatechin-3-gallate encapsulated PLGA nanoparticles [47, 48] a more sustained release is found for our fibrillic membranes due to longer diffusion pathways deriving from the relatively large fiber diameters.

The degradability and the morphological behavior of the electrospun composite nanofibrous membranes were investigated by controlled immersion in PBS at 37°C at a pH = 7.4.

In contrast to the homogenous fiber morphologies with relatively smooth surfaces before incubation, visible changes of the morphologies were observed after immersion (Figure 7). Although fiber morphology was still present after 14 days of incubation, a roughened surface was formed with larger fiber diameters (1.93 ± 0.56 µm) due to swelling of the polymer. After one month of incubation time, a partial loss of fiber morphologies needs to be stated, although a high degree of membranes porosity was retained.

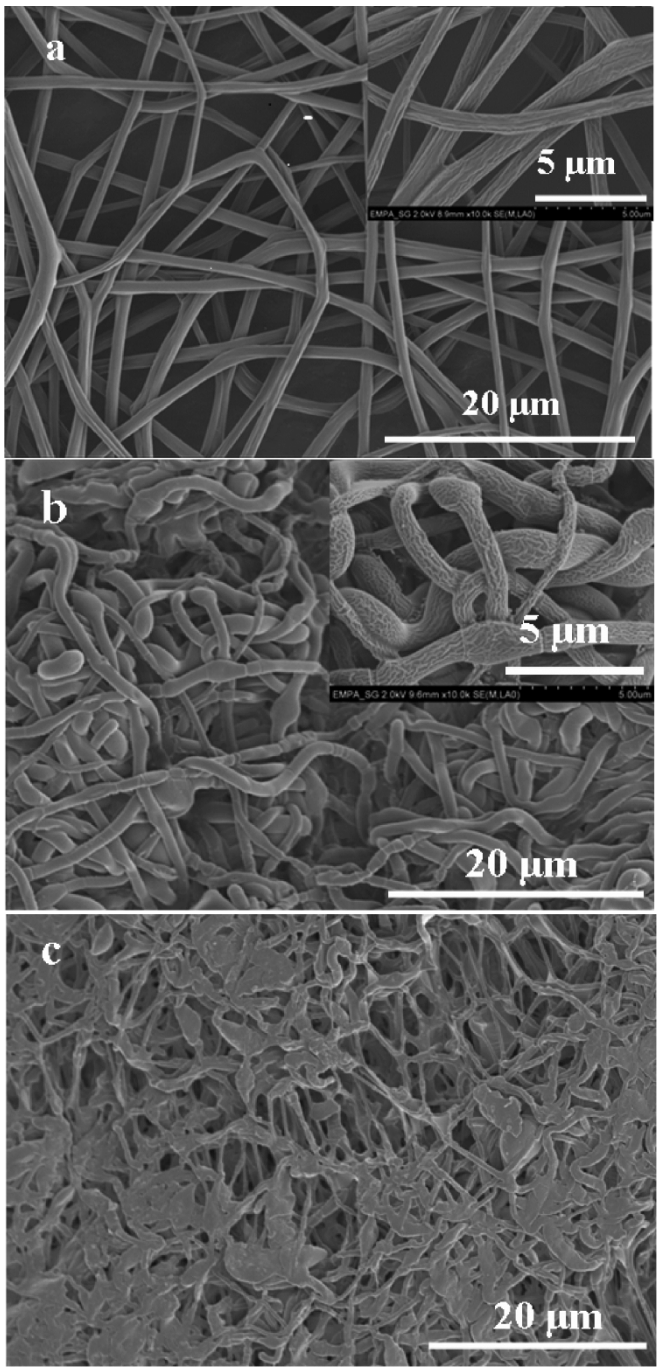

Figure 7 SEM micrographs showing representative morphologies of the nanofibrous membranes electrospun from PLGA / catechin emulsions before and after the incubation studies where a) fibers before immersion in PBS, b) fibers after 14 days of immersion in PBS, c) fibers after one month in PBS

Polymer PLGA, composition L/G: 75:25	M_w	M_n	M_w/M_n	Weight loss ΔW (%)
Pure PLGA as received	85.2	49.0	1.74	-
PLGA / 0.5 wt.% catechin- after one month in PBS	74.6	42.8	1.74	12.5
PLGA / 1.1 wt.% catechin- after one month in PBS	75.7	41.9	1.81	11.1
PLGA / 2.3 wt.% catechin- after one month in PBS	76.9	44.4	1.73	9.7

M_w: weight average molecular weight), M_n: number average molecular weight, M_w/M_n: Polydispersity,

Weight loss ΔW (%) = $(W_{initial\ sample} - W_{degraded\ sample})/ W_{initial\ W\ of\ sample}$ x100

Table 1 The average molecular weights before and after the degradation of PLGA loaded catechin in phosphate buffer (pH 7.4, 37ºC)

After one month of incubation average molecular weights diminished by 10 to 15 % compared to the original value (Table 1). It shows that chain degradation of PLGA with ratio lactic/glycolic 75/25 was present. Weight losses of the membranes, likely being caused by chain scission processes, are presented in Table 1.

The FTIR spectra show peaks at 1749 cm^{-1} (carbonyl stretching), 1093-1454 cm^{-1} (C-O stretching) and 2851 and 2948 cm^{-1} (C–H bending of the $–CH_2$ and $–CH_3$ group) (Figure S2, supporting information). Similar peaks have been recorded for PLGA by Fouad and Elsarnagawy [49] and Stevanovic et al. [50]. By comparing spectra of bulk and pure fiber PLGA as well as fibers incubated for 14 days, no significant change in band spectra was observed, at least within the uncertainty limits of the FTIR measurements. In conclusion, the

results from FTIR together with GPC analysis confirm that the degradability of PLGA fibers after 14 days was not pronounced.

The mechanism of the catechin release from the PLGA fibers was analyzed by use of the cumulative release profiles for zero-order kinetics, first-order kinetics, the Higuchi and the Hixon-Crowell kinetic models [51]. The regression coefficients are shown in the supplementary material, Table S1. The catechin release profiles could be fitted by a bimodal distribution. Thereby, the Higuchi model was the best fit for the first 5 days revealing a regression coefficient $R^2 = 0.9747$. Thereby, the cumulative percentage of the drug release (Q) was plotted against the square root of time ($t^{1/2}$), i.e. $Q = K \times t^{1/2}$, where K depicts the Higuchi rate constant. This result indicates a diffusion process within the PLGA fiber matrix, where the release is not dominated by polymer degradation. The fitting of the catechin release profile from 5 to 14 days was best for the zero-order kinetic model with $R^2 = 0.9913$. Here, the cumulative percentage of drug release is linear, i.e. $Q = K_0 \times t$, where K_0 depicts the zero-order rate constant. This result is associated with the degradation of PLGA fibers by scission of ester bonds by hydrolysis [52]. This suggestion is underpinned by our PLGA / catechin fiber degradation studies giving a reduction of the polymer molecular weight M_w of 10 to 15% % and a mean sample weight loss of ΔW of 11 % after 1 month of immersion (Table 1). A comparable bimodal distribution and thus time dependent difference in release mechanism was found by Guimarães et al. for the system PLGA / daunorubicin [53]. Thereby, in a first phase of the drug release the Higuchi model yielded the best fit, followed by a zero-order model in a second phase, thus same release mechanisms as found in our observations.

3.4 Reactive Oxygen Species (ROS)

To investigate catechin's antioxidant activity, MWCNT induced ROS formation in A549 cells was assessed using the DCF assay. MWCNTs have been shown to induce elevated

ROS levels in A549 without influencing cell viability [37] and thus served as the positive control in the study. Untreated cells in contrast indicate the base level of ROS under normal cell culture conditions and therefore represent the negative control. All values given are normalized to values of MWCNT treated samples set to 100%.

First the activity of pure catechin at concentrations ranging from 0.008 to 1 mg/mL was analyzed. 20 µg/ml MWCNT and respective catechin amounts were added simultaneously to dye loaded A549 cells and the ROS production was measured after 10, 30, 60 and 120 min. As shown in Figure 8a catechin reduces ROS production in a dose dependent manner, thus confirming its reported ROS inhibiting properties [54]. The observation that ROS levels increase again over time indicate the depletion of active catechin molecules during this period of time through reaction with the ROS molecules. Thermal or pH induced degradation of catechin can be excluded under our conditions as reviewed by Ananingsih et al. [55].

In a next step, we investigated whether the pure PLGA fibers itself influences ROS production, e.g. to study influences caused by PLGA degradation components or leached surfactant molecules. Therefore PLGA fiber membranes without catechin were incubated for 3 days at 37°C in HBSS. A549 cells were treated with the resulting supernatant (in the following termed conditioned buffer) in different concentrations ranging from 100% (undiluted conditioned buffer) down to 0.78% (serially diluted in HBSS). All samples simultaneously received 20 µg/mL MWCNT to induce ROS production. Potential effects were monitored over time after 10, 30, 60 and 120 min. As shown in Figure 8b pure PLGA fibers without catechin neither increase nor decrease MWCNT induced ROS levels at all concentrations and time points tested. Thus no influence of the polymer membrane with respect to ROS production was observed.

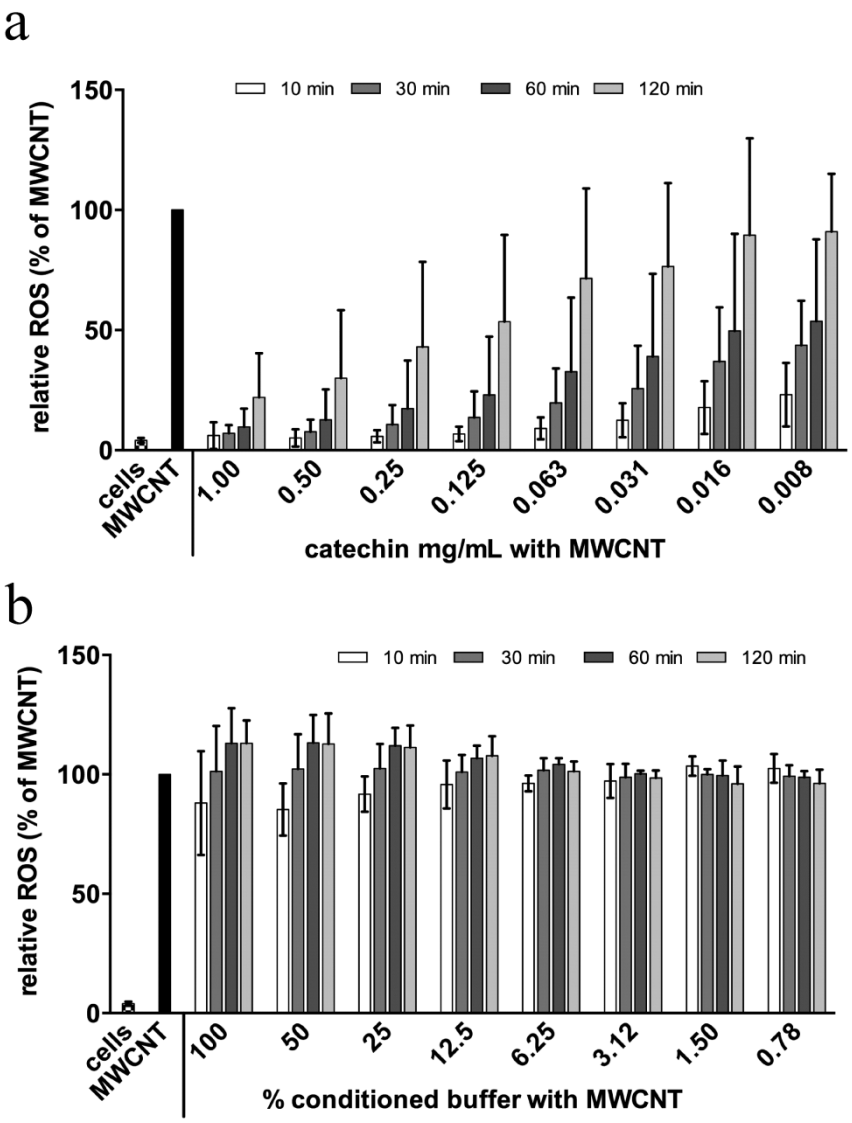

Figure 8 a) Influence of pure catechin on intracellular ROS formation, b**)** Influence of pure PLGA fiber membranes on intracellular ROS formation. Untreated cells ("cells") serve as the negative control, MWCNT treated cells ("MWCNT") as the positive control. Results are given normalized to MWCNT treated samples and represent the mean ±SD from three independent experiments.

To assess whether catechin released from electrospun fibers retains its antioxidant activity over the spinning / membrane production process we made use of the same cellular setup for the *in vitro* assay. We investigated again time as well as concentration dependent effects. Therefore, conditioned buffer from PLGA fibers incorporating 1.1 wt.% and 2.3 wt.% of

catechin (corresponds to 0.0076 mg and 0.0094 mg catechin in buffer, respectively) was produced and diluted as described above. The effects of pure catechin at a concentration of 0.011 mg/ml and 100% conditioned buffer retrieved from PLGA fibers containing 1.1 wt.% or 2.3 wt.% catechin are most prominent 10 min after administration (Figure 9). While pure catechin on average reduces the MWCNT induced ROS signal down to 23%, undiluted conditioned buffer, from fibers containing 1.1 wt.% and 2.3 wt.% catechin results in a reduction to 53% and 42%, respectively. Comparable to pure catechin (Figure 8a) this effect diminishes over time resulting in only marginal reduction of the signal after 120 min in all catechin containing samples. This indicates that catechin released from PLGA fibers retains its antioxidant activity.

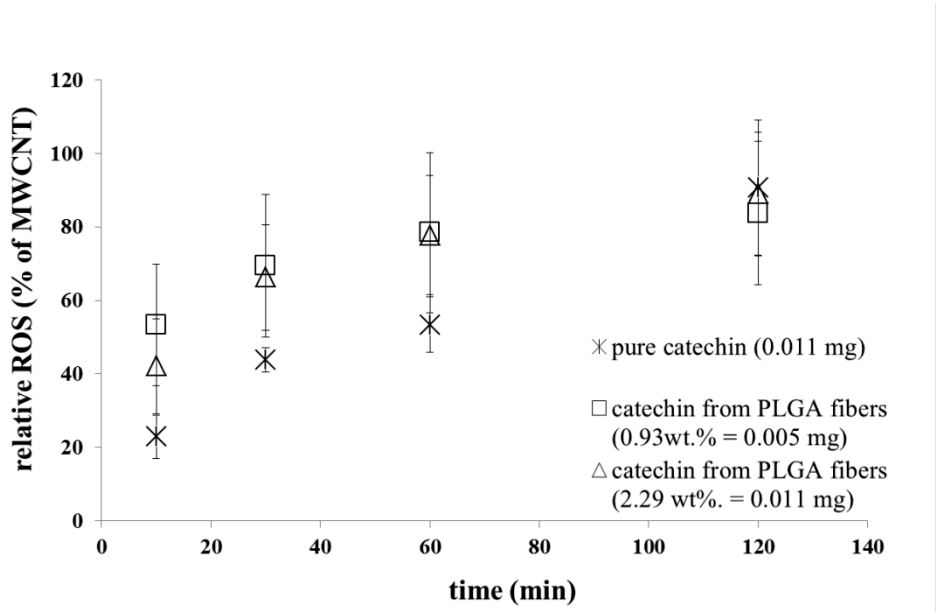

Figure 9 Catechin released from PLGA fibers reduces ROS in a concentration and time dependent manner. Untreated cells served as the negative control, MWCNT treated cells as the positive control. Values given are normalized to MWCNT treated samples (100%) and represent the mean +/- SEM from three independent experiments.

4 Conclusions

Stabilized water in oil emulsions were prepared by use of PLGA and the water soluble polyphenol catechin in selected concentration ranges for fiber and membrane development. The electrostatic spinning process revealed the formation of submicron-sized bicomponent fibers incorporating catechin within the core and PLGA within the fiber sheath. *In vitro* catechin release studies showed a burst release at the starting phase followed by a linear delivery at long term being driven by a diffusion controlled mechanism. Antioxidant activities of the pre-released polyphenol in A549 cells gave high reduction values with respect to the production of reactive oxygen species, being induced by MWCNT. These results depict electrostatic spinning procedures as an effective tool for encapsulation and controlled release of functional active substances at the site of action, and allows for localized and sustained applications in the fields of tissue engineering and wound healing.

Acknowledgements

The authors wish to thank the Scientific Exchange Programme SCIEX NMS-CH - Crus Switzerland (Project Code 5211.00770) for financial support. The authors also acknowledge the support by Dr. Gelu-Marius Rotaru from the Center of X-ray Analytics, Empa for the Wide Angle X-ray Scattering measurements.

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Supporting information

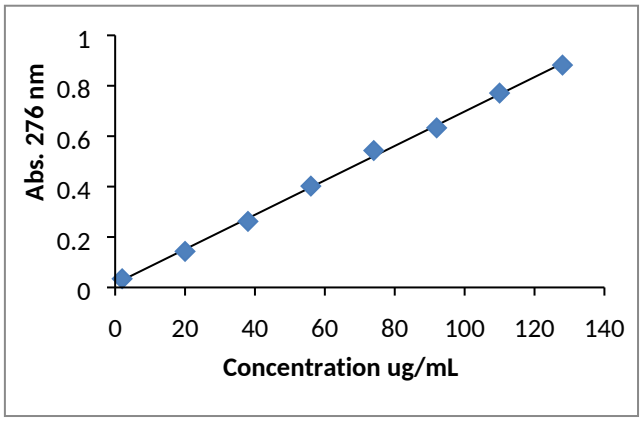


Figure S1 Calibration curve of catechin for the determination of the release kinetics from e-spun PLGA / catechin fibers

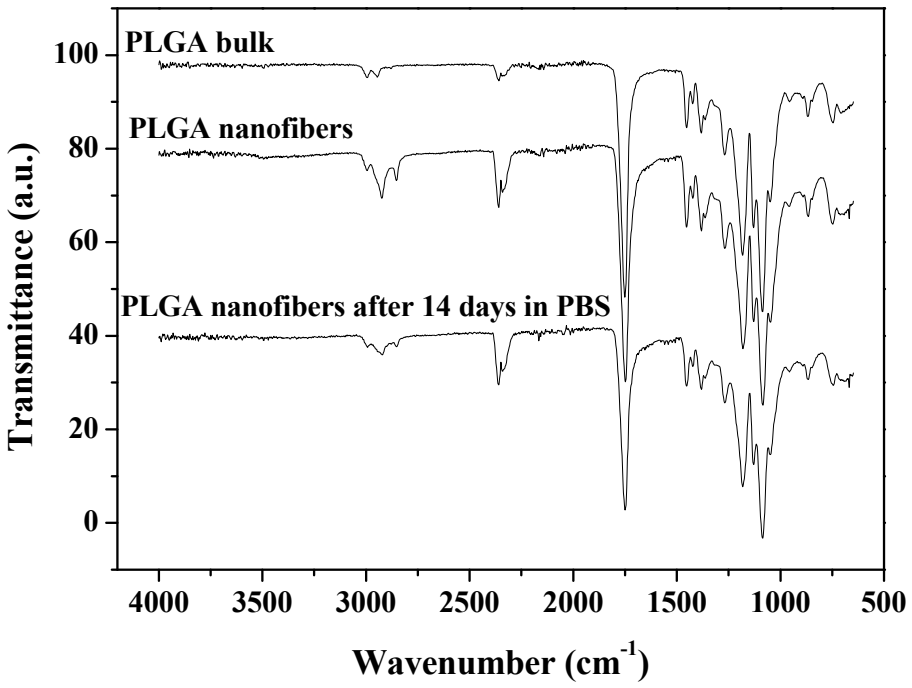


Figure S2 FTIR spectra of bulk PLGA polymer and PLGA fibers

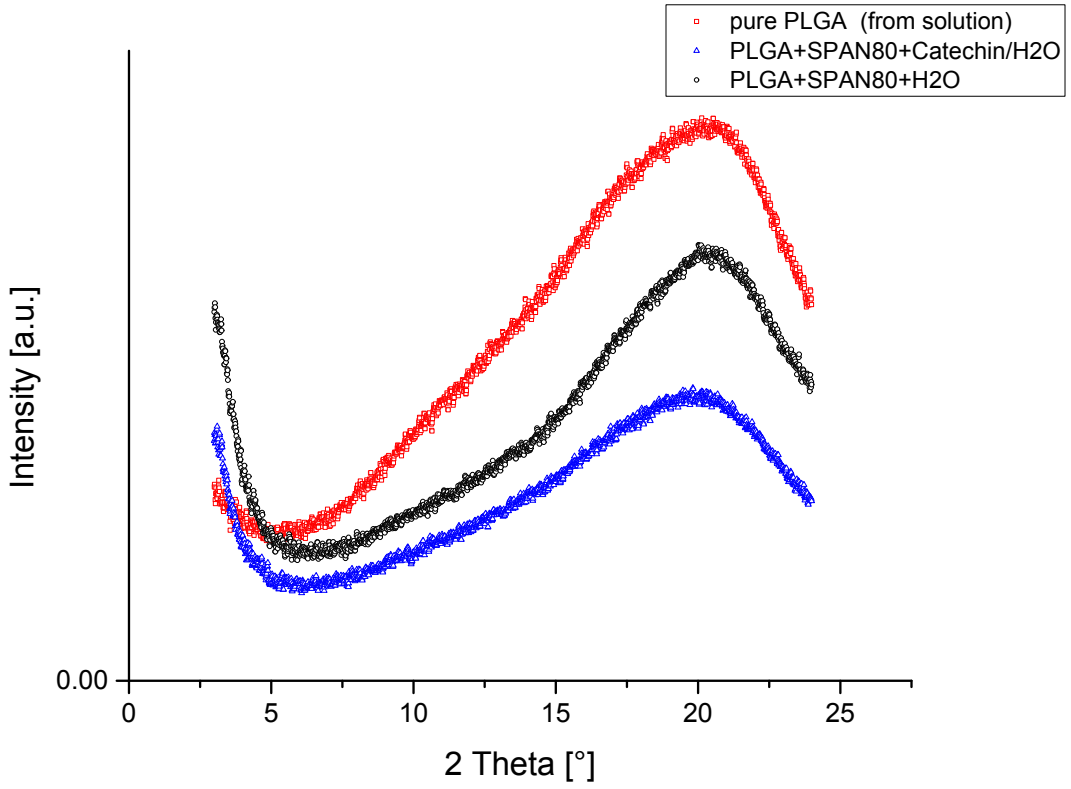
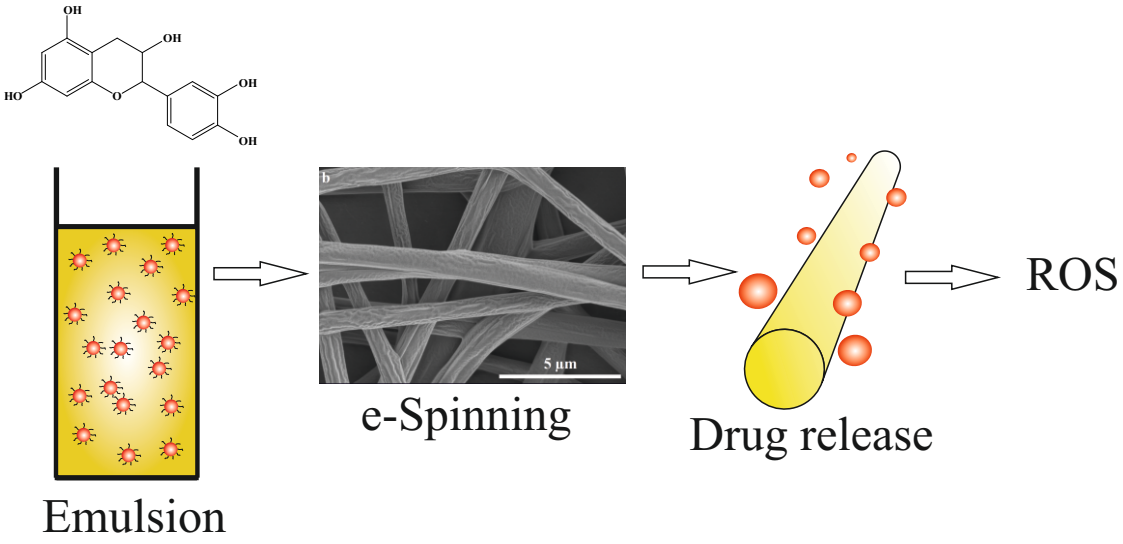


Figure S3: WAXS diffractograms for PLGA spun from solution (see also Fig. 3d, 20 wt.%), PLGA spun from emulsion with and without catechin

Mathematical model	(R^2) for first 5 days	(R^2) 5 to 14days
Zero-order	0.9382	**0.9913**
First-order	0.8820	0.9638
Higuchi	**0.9747**	0.9638
Hixon-Crowell	0.9607	0.9462

Table S1. Calculated regression coefficients (R^2) using mathematical models for the catechin release from PLGA - (4.5 wt.%) catechin e-spun fibers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Drug release