Inducing the formation of new blood vessels (angiogenesis) is an essential requirement for successful tissue engineering. Approaches have been proposed to enhance angiogenesis using growth factors and other biomolecules; however, these approaches present drawbacks in terms of high cost and patient safety. Copper is known to effectively regulate angiogenesis and can offer a more cost-effective alternative than the direct use of growth factors. With this study, a strategy to incorporate copper in electrospun fibrous scaffolds with pro-angiogenic properties is presented. Polycaprolactone (PCL) and copper(II)-chitosan are electrospun using benign solvents. The morphological and physicochemical properties of the fiber mats are investigated through scanning electron microscopy (SEM), static contact angle measurements, energy dispersive X-ray, and Fourier-transform infrared spectroscopies. Scaffold stability in phosphate buffered saline at 37 °C is monitored over 1 week. A bone marrow stromal cell line (ST-2) is cultured for 7 days and its behavior is evaluated using SEM, fluorescence microscopy and a tetrazolium salt-based colorimetric assay. Results confirm that PCL/copper(II)-chitosan is suitable for electrosprining. The fiber mats are biocompatible and favor cell colonization and infiltration. Most notably, the angiogenic potential of PCL/copper(II)-chitosan blends is confirmed by a three-fold increase in VEGF secretion by ST-2 cells in the presence of copper(II)-chitosan.

1. Introduction

In the past decades, angiogenesis has emerged as a key challenge for tissue engineering.[1] There is a general consensus that to achieve successful tissue regeneration research must further investigate this phenomenon and find ways to guide and regulate angiogenesis.[2–4] Established technologies are not yet effective in vascularizing newly formed tissues, both soft and hard, and the lack of vessels often results in accumulation of metabolites and degradation products, formation of fibrotic tissue, and even necrosis.[5] Emerging technologies have shown promising results in terms of effective control of angiogenesis during the regeneration of various targeted tissues (e.g., skin, cardiac tissue, nerves).[6–8] Suggesting that their application could increase both the size of the treated defect and the rate of tissue regeneration.[9] For example, the effective control of angiogenesis could be important for the fabrication of small vessel grafts (inner diameter <6mm).[10] The current golden standard in the replacement of damaged small vessels is the use of autografts harvested directly from the patient (e.g., saphenous or arm veins, mammalian, or radial arteries). However, this type of procedure has consistent drawbacks, especially limited availability in terms of harvesting sites.[10] On the other hand, synthetic polyethylene terephthalate (PET) or expanded polytetrafluoroethylene (ePTFE) grafts are characterized by several complications, mainly correlated to the regeneration of nonfunctional endothelium (aneurysm, intimal hyperplasia, calcification, thrombosis, and infection).[10] Vascular tissue engineering is a promising approach to resolve the issue. In this context, pro-angiogenic biomaterials that improve the formation of new endothelium without significantly altering the activated partial thromboplastin time (APTT) should be sought. The main current approach in biomaterials science to improve angiogenesis and vessel infiltration in tissue engineering scaffolds is through chemotaxis.[2,3] The structural biomaterial is modified in order to stimulate cell growth and neovascularization either ex vivo, prior to implant, or directly in vivo. This stimulation is achieved either by surface modification with extracellular matrix (ECM) proteins or ECM-derived
peptides,[11–13] or by addition of signaling biomolecules, namely growth factors (vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF-β)).[14,15] The results achieved with biomolecules are very encouraging. However, there are several issues concerning their clinical use, such as very high costs, questions about patient safety (e.g., immunogenicity, carcinogenicity), and difficulties in regulation and scale-up.[16,17] To date, the commercialization of growth factors and similar biomolecules for biomedical applications still appears prohibitively expensive and non-viable on an industrial scale.[14] Due to these significant drawbacks, research has focused on the identification of possible alternatives. Among others, therapeutic metallic ions (TMIs) are an interesting and cost-effective alternative to growth factors.[18] TMIs are a family of biologically active metal ions. They interact with several biological pathways in cells and can specifically influence and regulate their activity, with different effects depending on the ion.[18] Research on the topic is flourishing.[19,20] Many recent studies looked into the incorporation of ions into inorganic matrices to improve tissue regeneration, and angiogenesis in particular,[21] with highly promising results. For example, if its concentration is carefully controlled, copper can be an essential therapeutic ion with antibacterial, pro-angiogenic, and proliferative effects.[22–25] For concentrations above 10 ppm, the metal is a broad spectrum antibacterial agent with strong activity against both Gram-positive and Gram-negative bacteria.[26] At lower dosages, in the range of 1–4 ppm,[27] there is evidence that copper can promote eukaryotic cell proliferation and expression of pro-angiogenic factors. In addition, the price of copper salts (for instance $\approx 5$ per gram for CuCl₂, source: Sigma-Aldrich) is very competitive compared to the cost of angiogenesis-related growth factors. These properties make TMIs, and copper in particular, very promising candidates for a new generation of tissue engineering scaffolds based on bioinorganic-based chemotactic stimuli.[18,28,29]

In this context, our previous studies[30] showed how chitosan, a widespread linear polysaccharide of natural origin,[31] can be used as an efficient carrier for copper thanks to an intrinsic ability to chelate metal ions.[32] The copper ions are successfully incorporated in the polysaccharide matrix and their release can be finely tailored by suitably changing the formulation of the material, as reported in a previous publication.[33] Copper(II)-chitosan has been effectively used as antibiotic-free antibacterial material against both Gram positive and negative bacteria without significant cytotoxicity against fibroblasts.[30] Further on, we hypothesized that copper(II)-chitosan could be used not only as an antibacterial, but also as a component of tissue engineering scaffolds. In fact, the release of copper ions from the material could upregulate the expression of pro-angiogenic growth factors and stimulate angiogenesis.[22] Copper(II)-chitosan was thus used to fabricate electrospun fibers to test our hypothesis. The electrospinning of chitosan is known to be very challenging, especially when avoiding the use of highly toxic organofluorine solvents (e.g., trifluoroacetic acid).[34] The most common strategy to overcome the problem is electrospinning the polymer in combination with synthetic polymers, such as poly(caprolactone) (PCL),[34,35] poly(vinyl alcohol) (PVA)[36] or poly(ethylene oxide) (PEO).[37] Among other candidates, PCL was chosen in this study due to its interesting properties, including biocompatibility, biodegradability, availability from renewable sources, FDA approval for several clinical applications, and considering the large body of research available about PCL processing by electrospinning.[38,39] The fabrication of PCL electrospun fibers has been widely reported using chloroform, dichloromethane, dimethylformamide, and methanol or a mixture of the above.[39] Most interestingly, positive results have been also reported using acetic acid, formic acid, and acetone.[40] According to ICH guidelines,[41] these solvents belong to Class 3 and are deemed benign at a residual concentration <0.5% (higher amounts may also be acceptable with a proper justification), as also previously described.[42] The use of such class of solvents in electrospinning has been recently proposed as an interesting processing method for biomedical polymers.[43] The use of benign solvents for electrospinning is an emerging concept that aims at the development of electrospinning protocols based on low-toxicity solvents, avoiding typically used halogenated solvents, such as chloroform. This in turn results in better lab worker safety and simpler waste management.[40] It also opens up to a wider range of possibilities for drug loading, since benign solvents will reduce the risk of denaturation/neutralization of biomolecules/biopolymers/drugs during processing and post-processing.[44]

In this work, we successfully fabricated fibers of PCL blended with copper(II)-chitosan by electrospinning with benign solvents.[44] The fibers were characterized morphologically by scanning electron microscopy (SEM) and chemically by energy dispersive X-ray (EDX) and Fourier-transform infrared (FTIR) spectroscopies. Possible variations in contact angle as a consequence of copper(II)-chitosan loading were also assessed. The stability of the blend was studied in phosphate-buffered saline (PBS), pH, water uptake, and variations in dry weight were measured over 1 week. Bone marrow stromal cell line (ST-2) was seeded on fiber mats and cultured for 7 days. Their morphology, adhesion, viability, and proliferation were evaluated respectively using SEM, a tetrazolium salt-based colorimetric assay (WST-8), and rhodamine phalloidin/DAPI fluorescent staining. Finally, in order to verify the angiogenic potential of the PCL/copper(II)-chitosan blend, free VEGF in the culture medium of cells cultured on both fiber mats containing copper(II)-chitosan and neat PCL fiber mats used as control was measured after 7 days of culture.

2. Experimental Section

2.1. Materials

Polycaprolactone (v-PCL, nominal molecular weight = 80 kDa) was purchased from Ashland Specialties Ireland Ltd. (Dublin, Ireland) and was guaranteed negligible solvent contamination of the final product thanks to its proprietary manufacturing process. Copper(II)-chitosan was prepared according to a previously published protocol[30] modifying medium molecular weight chitosan ($M_w = 190-310$ kDa, DDA = 75–85%, Sigma-Aldrich, Germany). The amount of copper loaded in the polysaccharide matrix corresponded to a saturation of 12% of the amino groups of chitosan (CuChi12).[30] Benign (low-toxicity) solvents (glacial acetic acid >99.85% and formic acid >94.5%; VWR, Germany) were of high purity analytical level and used
without further purification. The solutions were prepared for electrospinning adapting a previously published protocol for the preparation of blended polycaprolactone/chitosan fiber mats.\textsuperscript{[14]} Initial solubility tests and electrospinning trials were performed in order to identify the optimal concentration. In a typical preparation, acetic acid (AA) and formic acid (FA) were first mixed at an AA/FA ratio of 60/40. Then, PCL was added at a concentration of 20\% w/v. After complete dissolution of the polyester, CuChi12 was added at a concentration of 0.5\% w/v calculated on the original volume of AA/FA mixture used. With this procedure, it was possible to obtain two spinnable solutions: a neat v-PCL and a blended v-PCL/CuChi12 solution. The overall content of copper in the final material was 1 mg of copper per gram of PCL/CuChi12 (=0.1\% w/w).

2.2. Molecular Weight Distribution of PCL

The molecular weight distribution and polydispersity (PD) of v-PCL were measured by gel permeation chromatography (GPC). Approximately 40 mg of sample was dissolved in 10 mL of chromatographic-grade chloroform and syringe filtered through 0.45 $\mu$m filter membranes. Alongside the samples, a set of Agilent EasyVial calibration standards (364–435 kDa polystyrene) was prepared in 2 mL of chloroform. Aliquots of samples and calibration standards were then transferred to 2 mL autosampler vials for analysis. The analysis was performed at 35 °C and 1.0 mL min\textsuperscript{−1} flowrate by an Agilent 1260 GPC system equipped with two Agilent mixed-C columns (300 mm $\times$ 7.1 mm) with 5 $\mu$m particles. Data were recorded and computed by Agilent GPC software. PCL (80 kDa) supplied by Sigma-Aldrich (Germany) was used as control (labeled s-PCL). The recorded parameters were: peak molecular weight ($M_n$), number average molecular weight ($M_n$), mass-average molecular weight based on average weight ($M_w$), Z-average molecular weight ($M_z$), and polydispersity (PD = $M_w$/[$M_n$]).

2.3. Rheology of PCL and Blended Solutions

The rheological properties of a solution can highly influence its spinnability. Therefore, the rheological behavior of the two optimized v-PCL and blended v-PCL/CuChi12 solutions was investigated ($n = 3$). A shear rate ramp mode test with a 0.1–200 s\textsuperscript{−1} range at 25 °C was performed using a Discovery Series Hybrid Rheometer HR-2 (TA Instruments, United Kingdom). The temperature was set according to the electrospinning conditions (i.e., room temperature, 25 °C). A solution of 20 \% w/v s-PCL in 60/40 AA/FA was used as control. Results were plotted as shear stress versus shear rate. Assuming the hypothesis of laminar flow of a Newtonian fluid in a cylindrical pipe, the Newtonian viscosity of the solutions was calculated as the coefficient of the regression line interpolating the Newtonian portion of the shear stress/rate curve (i.e., low shear rates <30 s\textsuperscript{−1}).

2.4. Electrospinning of PCL and Blended Fibers

Electrospinning was performed using a commercially available setup (Starter Kit 40 KV Web, Linari srl, Italy). Optimized electrospinning parameters for both the solution of v-PCL and v-PCL/CuChi12 prepared as previously described were identified through a series of initial trial-and-error experiments that led to the following fabrication parameters, summarized in Table 1: needle diameter, needle-to-target distance, and flow rate were kept constant at 21G, 11 cm, and 0.4 mL h\textsuperscript{−1}, respectively; voltage and time were 15 kV and 30 min for v-PCL and 20 kV and 45 min for v-PCL/CuChi12, in order to obtain fiber mats of comparable thickness. The increase in both time and voltage counteracts the increase in viscosity after the addition of the second polymer. Environmental parameters, temperature (T) and relative humidity (RH), were monitored and held in the range T: 24–27 °C and RH: 30–45%.

2.5. Morphological and Physicochemical Characterization

2.5.1. SEM

Sample morphology was assessed by SEM (LEO 435 VP, LEO Electron Microscopy Ltd., UK and Ultra Plus, Zeiss, Germany). Fiber average diameters were calculated using ImageJ (NIH, Bethesda, MD, USA)\textsuperscript{[45]} performing 120 fiber measurements per sample on three separate samples ($n = 3$). In parallel, EDX was performed in order to qualitatively investigate sample composition, confirm the presence of copper, and rule out the possibility of contamination. A Silicon Drift Detector (SDD, X-Max\textsuperscript{3}, Oxford Instruments, United Kingdom) was used ($V_{\text{max}} = 20$ keV). Prior to investigation, the samples were sputter coated using a Q150T S equipped with a gold target (Quorum Technologies, United Kingdom). In EDX spectra, the peak of gold (related to the sputtering process) was removed during post-processing.

2.5.2. FTIR

Infrared spectra of electrospun mats were obtained in attenuated total reflectance mode (ATR-FTIR) using a Shimadzu IRAffinity-1S (Shimadzu Corp, Japan) equipped with a Quest ATR GS10801-B single bounce diamond accessory (Specac Ltd., England). Data were collected in the mid-IR region (4000–400 cm\textsuperscript{−1}) at 40 scans and a resolution of 4 cm\textsuperscript{−1} ($n = 3$). Processing was performed using the LabSolution IR software by Shimadzu.

Table 1. Summary of electrospinning parameters for the tested solutions.

|          | PCL [\% w/v] | CuChi12 [\% w/v] | Voltage [kV] | Distance [cm] | Flow rate [mL h\textsuperscript{−1}] | Time [min] | Needle diameter |
|----------|--------------|------------------|--------------|---------------|---------------------------------------|------------|----------------|
| v-PCL    | 20           | –                | 15           | 11            | 0.4                                   | 30         | 21G            |
| v-PCL/CuChi12 | 20       | 0.5              | 20           | 11            | 0.4                                   | 45         | 21G            |
2.5.3. Static Contact Angle

The contact-angle of v-PCL and v-PCL/CuChi12 electrospun fibers was measured to identify possible variations in hydrophilicity following the blending with chitosan. Contact angle was performed at room temperature using a Krüss DSA30 Drop Shape Analysis System (Krüss GmbH, Germany). Briefly, a deionized water droplet of 3 µL was deposited on the sample, after 15 s an image of the drop was acquired, and the angles between the droplet and the substrate (both left and right) were measured six consecutive times within 3 s by DSA4 software (Krüss GmbH, Germany). The analysis was replicated on three different samples per type.

2.5.4. Degradation Behavior in PBS

The effect of a saline solution on the fiber mats was also investigated. Samples (n = 5) were immersed in PBS solution (Amresco LLC, USA) using suitable scaffold holders (Cell-Crown 24, Scaffdex, Sigma-Aldrich, Germany). They were kept in an incubator-shaker for 1 week at 37 °C to simulate in vivo conditions. The variations in pH of the solution and sample weight were monitored at four time points (0, 1, 3, and 7 days). Samples after degradation were also characterized by SEM-EDX and FTIR, as previously described.

2.6. Biological Characterization

2.6.1. Cell Seeding and Culture

Cell culture studies assessing cell viability, proliferation, and morphology were performed using a bone murine stromal cell line (ST-2, Leibniz-Institut DSMZ—German Collection of Microorganisms and Cell Cultures GmbH, Germany) isolated from bone marrow of BC8 mice. Prior to testing, ST-2 cells were cultured in polystyrene flasks using Roswell Park Memorial Institute medium (RPMI 1640) supplemented of Microorganisms and Cell Cultures GmbH, Germany) isolated from bone marrow of BC8 mice. Prior to testing, ST-2 cells were cultured in polystyrene flasks using Roswell Park Memorial Institute medium (RPMI 1640) supplemented with rhodamine phalloidin and DAPI (4′,6-diamidino-2-phenylindole, dilactate), both purchased from ThermoFisher Scientific, Germany. Briefly, samples were fixed using a fixative solution prepared using 1,4-piperazinediethanesulfonic acid buffer, ethylene glycol tetraacetic acid, polyethylene glycol, parafomaldehyde, PBS, and sodium hydroxide (all reagents by Sigma-Aldrich, Germany). An 8 µL mL−1 rhodamine phalloidin solution and a 1 µL mL−1 DAPI solution were used for staining. Images of phalloidin-DAPI stained cells were taken with a fluorescence microscope (FM) (Axio Observer D1, Carl Zeiss Microimaging GmbH, Germany).

2.6.2. Cell Adhesion and Morphology (SEM)

At the selected time points (1, 3, and 7 days), cell culture samples were prepared for SEM observation. ST-2 cells were fixed by immersion in a proper fixation buffer composed of glutaraldehyde, parafomaldehyde, sucrose, and sodium cacodylate trihydrate (Sigma Aldrich, Munich, Germany) for 1 h. Then, samples were ethanol dehydrated with progressive series of water/ethanol solutions ranging from 30% to 98% in ethanol fraction. Samples were then air dried and gold coated (Q150T sputter coater, Quorum Technologies, Darmstadt, Germany).

2.6.3. Cell Viability (WST)

A CCK-8 assay (Sigma-Aldrich, Germany) was performed. The test is based on the reduction of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) to formazan. The reaction is proportional to the metabolic activity of cells and allows the colorimetric determination of cell viability. The test was performed on all the samples in triplicates at 1, 3, and 7 days after the seeding, according to the supplier guidelines. After incubation with the agent, variations in absorbance of the medium were assessed by a microplate reader (PHOmo Autobio, Labtec Instruments Co. Ltd. China) at 450 nm. Cell viability (V) can be calculated as the ratio between the absorbance of a given specimen (A), and the absorbance of a relevant neat PCL fiber mat positive control (A0). ANOVA one-way analysis was performed to evaluate the results of cell viability. Significance was set at α = 0.05.

2.6.4. Cell Fluorescent Staining

Cell morphology was assessed for all time points by staining with rhodamine phalloidin and DAPI (4′,6-diamidino-2-phenylindole, dilactate), both purchased from ThermoFisher Scientific, Germany. Briefly, samples were fixed using a fixative solution prepared using 1,4-piperazinediethanesulfonic acid buffer, ethylene glycol tetraacetic acid, polyethylene glycol, parafomaldehyde, PBS, and sodium hydroxide (all reagents by Sigma-Aldrich, Germany). Then, the samples were rinsed with PBS and immersed in a permeabilization buffer containing Triton X-100, sucrose, and PBS (Sigma-Aldrich, Germany). An 8 µL mL−1 rhodamine phalloidin solution and a 1 µL mL−1 DAPI solution were used for staining. Images of phalloidin-DAPI stained cells were taken with a fluorescence microscope (FM) (Axio Observer D1, Carl Zeiss Microimaging GmbH, Germany).

2.6.5. VEGF Expression

The amount of VEGF released into the culture medium by ST-2 cells cultured for 7 days on v-PCL/CuChi12 and v-PCL (as control) was measured by a RayBio Human VEGF ELISA (Enzyme-Linked Immunosorbent Assay) kit. This assay is specifically designed for the colorimetric quantification of mouse VEGF in cell culture supernatants, by means of an
antibody specific for the growth factor. Changes in color of the liquid sample from blue to yellow can be detected and measured by spectrophotometry at 450 nm (spectrophotometer as previously described). The assay was performed according to the instructions manual provided by the supplier.

3. Results and Discussion

3.1. Polymer and Electrospinning Solution Characterization

3.1.1. Molecular Weight Distribution

Prior to electrospinning, v-PCL was compared in terms of molecular weight ($M_w$) distribution to another well-known similar polymer (nominal 80 kDa polycaprolactone by Sigma-Aldrich, s-PCL), which was studied in-depth in previous work on benign solvents for electrospinning published by the same authors.[19,34,46,47] The results of GPC analysis are reported in Table 2. According to our results, the $M_w$ of s-PCL is much higher than the nominal value reported by the supplier (i.e., more than twofold). This incongruence has already been mentioned by other authors, who reported similarly high $M_w$ distribution values[48] and highlights the need for careful characterization of the properties of a material every time it is used. In this study, the key element was confirming that s-PCL and v-PCL have similar molecular weight distributions. GPC analysis confirmed that. Only slight differences were assessed: compared to s-PCL, v-PCL is characterized by a small decrease in $M_n$ and increase in $M_w$, indicating that the molecular weight distribution of this polymer is slightly wider than s-PCL. This result is also confirmed by an increase in PD (i.e., the $M_w/M_n$ ratio). Overall, both PCL polymers appear to be very similar in terms of molecular weight. Furthermore, exception made for a small PEG moiety used as initiator, the polymers are also expected to be chemically very similar. This behavior confirmed that previously published protocols[47] optimized for s-PCL could be used without substantial modifications and that v-PCL could be an ideal candidate for the fabrication of electrospun mats.[39]

Table 2. Molecular weight distributions for PCL by Ashland (v-PCL); PCL by Sigma-Aldrich (s-PCL with a nominal $M_n = 80$ kDa) was used as control.

| Polymer  | $M_p$ [kDa] | $M_n$ [kDa] | $M_w$ [kDa] | $M_z$ [kDa] | PD   |
|----------|-------------|-------------|-------------|-------------|------|
| s-PCL    | 180 780     | 129 946     | 211 511     | 305 506     | 1.628|
| v-PCL    | 179 087     | 107 451     | 213 461     | 341 370     | 1.987|

3.1.2. Rheological Properties

As suggested by Nezarati et al.,[49] the rheological properties of s-PCL, v-PCL, and v-PCL/CuChi12 solutions were monitored. The authors propose that, in order to investigate and increase the reproducibility of the electrospinning process, solution viscosity rather than concentration is a more useful parameter to investigate. In particular, in this study, the viscosity of the selected v-PCL solution was compared to a literature standard (s-PCL). In parallel, the effect of the addition of CuChi12 was also investigated. All tests were performed in flow sweep mode across a range of shear rates from 0.1 to 200 s$^{-1}$. In Figure 1, typical results for all three samples are reported as shear stress ($\tau$) versus shear rate ($\gamma$). The viscosity, as a function of shear rate, is also represented.

Figure 1. Shear stress and viscosity plotted as a function of shear rate for solutions of s-PCL (20% w/v), v-PCL (20% w/v), and v-PCL (20% w/v)/CuChi12 (0.5% w/v) ($n = 3$).
Results indicated that all solutions are shear thinning: viscosity decreases as shear rate increases. In particular, v-PCL is characterized by a reduction from 5000 cP for low shear rates down to ~1500 cP at 200 s⁻¹, very similar to the 4500 to 1200 cP decrease of s-PCL. In fact, s-PCL was generally characterized by slightly lower, however not significantly (p < 0.05), shear stress and viscosity compared to v-PCL. This difference could be ascribed to the small variations in molecular weight between the two polymers (Table 2); viscosity is known to increase proportionally to $M_w$ and PD, among other parameters.\(^{[50]}\) When 0.5% w/v CuChi12 is added, both stress and viscosity of v-PCL follow the same trend, but with significant increases (p < 0.05). In particular, viscosity ranges between ~8000 and 4300 cP. At first approximation, the fluid dynamic behavior of the electrospinning solution in the needle can be modeled as a laminar flow inside a circular pipe, the shear rate ($\dot{\gamma}$) can be calculated from the flow rate ($Q = 0.4 \text{ mL h}^{-1}$), and needle radius ($r = 0.362 \text{ mm}$) according to Equation (1):

$$\dot{\gamma} = \frac{4Q}{\pi r^4} \approx 3 \text{ s}^{-1} \quad (1)$$

The calculation confirms that in the case of the electrospinning performed in this study, the shear rate (~3 s⁻¹) sits within the Newtonian portion of the curve (i.e., with constant viscosity, below 30 s⁻¹). As such, the calculation hypotheses are met. Therefore, the three solutions can be compared in terms of Newtonian viscosity ($\eta_0$). The value was computed for the three solutions by linear regression of the shear stress/rate curves for shear rates below 30 s⁻¹. The values of $\eta_0$ for s-PCL, v-PCL, and v-PCL/CuChi12 are 4300 ± 140, 5000 ± 120, and 7700 ± 260 cP, respectively. In particular, the difference between v-PCL before and after the addition of CuChi12 is statistically significant (p < 0.05). The addition of copper(II)-chitosan determines a consistent increase in Newtonian viscosity. Compared to other reports of PCL/chitosan solvent blends in acetic and formic acid,\(^{[51]}\) the results of viscosity obtained in this work are higher. However, this increase is somewhat expected and can be explained as an effect of the higher molecular weight of the polymers used in this work compared to previous reports.\(^{[51]}\) The addition of copper could also have a part in changing the viscosity of the system\(^{[30]}\); however, this hypothesis was not further investigated.

### 3.2. Successful Fabrication of Fiber Mats

#### 3.2.1. Fiber Morphology and Hydrophilicity

The use of solvent mixtures of formic and acetic acids as benign solvent for electrospinning has already been reported for both neat PCL and its blend with chitosan.\(^{[34,47,52]}\) The adaptation of previously developed protocols for PCL and chitosan dissolved in benign solvents\(^{[46]}\) was successful for the preparation of fiber mats based on blends of copper(II)-chitosan with PCL, effectively adding the therapeutic ion to the structure of the scaffold. SEM micrographs confirm that defect-free fiber mats without bead formation were obtainable both in the case of v-PCL and of v-PCL/CuChi12 (Figure 2A,B). Furthermore, formation of copper salt crystals was not observed, confirming that copper remains complexed with chitosan after processing, as desired. Additional work will be also necessary in the future in order to characterize possible differences in mechanical properties due to the chosen solvent system as well as to the addition of copper(II)-chitosan.

The fiber size and morphology of pristine v-PCL mats was found to be consistent with previous work on similar systems using acetic/formic acid mixtures.\(^{[34]}\) In particular, as reported by Bongiovanni et al.,\(^{[48]}\) the use of formic acid seems to allow the fabrication of fibers in the submicron range (in this work 240 ± 10 nm) compared to acetic acid alone. In this work, a dual solvent system was chosen primarily to allow the dissolution of chitosan, which would not dissolve in glacial acetic acid,\(^{[53]}\) but in parallel it confirmed to be an ideal solvent for the polymers. The addition of CuChi influences fiber distribution, increasing the average diameter and broadening the range of fiber diameters, as shown in Figure 2B. When considering the minimum and maximum of the fiber size distribution, the variations appear to be mainly focused on the upper part of the fiber size range, while the smallest fiber diameters remain essentially unaltered (i.e., there is a local maximum between 0.2 and 0.3 μm, see Figure 2C,D). A hypothesis could be that the presence of copper determines an increase in electrical conductivity, which in turn can influence the draw of the polymer from the Taylor’s cone thus influencing fiber morphology.\(^{[54]}\) However, this phenomenon would most likely cause a decrease in fiber size, as previously described.\(^{[54]}\) The reasons of this variation in fiber size, drifting from a nano/submicrometric toward a micrometric scale, should be instead sought in the increase in viscosity caused to the electrospinning solution by the addition of chitosan.\(^{[51]}\) This would also explain why time and voltage had to be increased in order to obtain satisfactory v-PCL/CuChi12 (see Table 1): stronger drawing forces were necessary to pull fibers, owing to the increased shear stress caused by the higher viscosity.\(^{[49,55]}\) This demonstrates once again the high versatility of the electrospinning of PCL with benign solvents\(^{[47]}\) a process that could be easily adapted for the successful production of fibers with novel therapeutic agents.\(^{[30]}\) If necessary, the parameters used in this study could be further adapted to reduce fiber size range of v-PCL/CuChi12 mats (i.e., by reducing the concentration of PCL\(^{[55]}\) or by increasing voltage or target distance\(^{[56,57]}\)). Nevertheless, seeking a reduction in fiber size was not necessary: while nanofeatures can guide cells, giving them topographical cues that resemble the ECM, micrometric fibers are associated with wider pores that enhance cell infiltration.\(^{[58]}\) As mentioned above, electrospun v-PCL/CuChi12 fiber mats exhibit submicrometric fibers similar to v-PCL, only more widely dispersed. This feature could be very useful to increase the penetration of cells within the scaffold. Together with surface topography and porosity, hydrophilicity is also a key variable to take into consideration when engineering a surface for cell attachment and proliferation. Since the addition of CuChi12 could cause variations in the native hydrophobicity of PCL, wettability in terms of static contact angle was measured (Figure 2C,D). PCL was characterized by a typical hydrophobic behavior,
with high contact angles values (>120°), in accordance with results of other studies in literature. After the addition of CuChi12, no statistically significant variation in contact angle was detected. Chitosan did not reduce hydrophobicity, as suggested in previous studies, most likely because of the small amount of copper(II)-chitosan introduced. The increase of fiber size seems to have increased the dispersion of contact angle values, which resulted in higher standard deviations for v-PCL/CuChi12 compared to neat v-PCL. However fiber size did not influence hydrophobicity. Although this behavior had previously been reported, recent studies on the topic highlighted how fiber diameter is not necessarily a sufficient parameter to estimate contact angle, since the hydrophilic behavior depends on surface roughness and the fiber to pore size ratio more than fiber diameter. Our results confirm this assumption and highlight the need, although not within the scope of the present work, of further investigation to clarify the relation between fiber morphology and hydrophilicity.

### 3.2.2. Chemical Characterization of Blended Polycaprolactone/Copper(II)-Chitosan

The presence of copper(II)-chitosan was confirmed by EDX analysis performed in conjunction with SEM. The analysis also established the absence of significant contaminations (Figure 3). Two peaks for carbon and oxygen corresponding to the structure of PCL can be found in v-PCL fibers. Once CuChi12 is added, a peak in correspondence of the L-line of copper appears in the spectrum. The K-line of copper, on the other hand, could not be detected, probably as a consequence of the low concentration of the element in the material. A small peak for nitrogen can be also spotted, probably due to the presence of amines in chitosan. Since nitrogen is known to be difficult to detect with EDX analysis, this observation can be considered only an assumption at this stage. This result, combined with the absence of secondary crystalline phases, confirms the presence of copper(II)-chitosan and its successful blending with v-PCL. Compared to other similar non-woven systems for ion release, we were successful in preparing fiber mats that contain the therapeutic agent (i.e., copper) within the polymeric matrix. This leads to increased homogeneity both in the loading and release of the agent. Furthermore, considering the versatility of chelating chitosan derivatives, this protocol could be rather straightforwardly adapted for the delivery of other divalent therapeutic ions of interest (e.g., zinc, calcium, iron).

ATR-FTIR analysis was used to investigate the chemical composition of electrospun mats and to evaluate possible variations due to the presence of copper(II)-chitosan (Figure 4). The spectrum of v-PCL exhibits all the characteristic bands of polycaprolactone. In particular, two peaks centered around 2943 and 2866 cm⁻¹ due to asymmetric and symmetric carbon–hydrogen stretching (νC-H), an intense peak at...
1724 cm\(^{-1}\) that can be ascribed to carbonyl stretching (\(\nu_{C=O}\)), a group of peaks going from 1471 to 1365 cm\(^{-1}\) which are related to the bending of carbon-hydrogen bonds (\(\delta_{C-H}\)), and finally three peaks at 1294, 1240, and 1170 cm\(^{-1}\), due to stretching of the carbon–oxygen bond within the ester moieties (\(\nu_{C-O}\)).\(^{[64]}\) The same peaks are found also in v-PCL/CuChi12 samples. The spectra of v-PCL and v-PCL/CuChi12 are essentially equivalent. The only difference is a small change in peak shape at the 1170 cm\(^{-1}\) spectral band: a possible sign of glycosidic stretching happening at the very high end of the characteristic wavenumbers for this type of bond.\(^{[30,63,65]}\) However, since other expected changes after the addition of chitosan,\(^{[66]}\) such as the appearance of the fingerprint of the glycosidic bond in the 1000–1100 cm\(^{-1}\) range or the increase in intensity in correspondence of amine and amide bond vibrations (\(\nu_{N-H}, \delta_{N-H}, \omega_{N-H}\) at 3400, 1600, and 700 cm\(^{-1}\), respectively)\(^{[30,67]}\) did not occur, it is unlikely that the change in shape at 1170 cm\(^{-1}\) is due to chitosan. Most probably, the variation is clueing to the occurrence of minor acid catalyzed hydrolysis of polycaprolactone during the dissolution of the polymer in acetic and formic acid.\(^{[68]}\) Future investigations should focus on the quantification of this phenomenon by measuring possible variations in \(M_w\) after the electrospinning process. This phenomenon was already described for a similar experimental setup\(^{[48]}\) and it is an intrinsic limit of using acids as benign solvents for electrospinning.\(^{[40,48]}\) However, degradation can be easily reduced by minimizing the time PCL remains in solution. The lack of signal corresponding to hydroxyl stretching (\(\nu_{OH}\)) at about 3400 cm\(^{-1}\) confirms that v-PCL retained its high molecular weight and no significant degradation occurred, as \(\nu_{O-H}\) can be related to the presence of PCL low-molecular-weight moieties.\(^{[68]}\)

### 3.3. Degradation in PBS

Before proceeding to cell culture test, the stability and possible initial degradation of fiber mats in a biologically relevant saline solution (PBS) and the possible differences occurring as a consequence of the addition of copper(II)-chitosan were investigated (Figures 5 and 6). The behavior of the fiber mats once in contact with PBS was quantified in terms of water uptake, pH value of PBS, variation in dry weight, morphology (SEM), and chemical changes (FTIR).

![Figure 5](https://example.com/figure5.png)  
**Figure 5.** Parameters of water uptake and degradation of fiber mats over 1 week in PBS (\(n = 5\)). From left to right: water uptake (%), pH, and dry weight variation (%). The fiber mats proved themselves to be stable in PBS over the testing time. Chitosan impacts the swelling (*: \(p < 0.05\), #: \(p < 0.001\)).
Mats of pure v-PCL reach a very low plateau of water uptake at only 5% of the specimen dry mass, a value that can be considered statistically equal to zero. On the other hand, v-PCL/CuChi12 settles at ≈20% swelling. This difference, although minor, is statistically significant (p < 0.001 at 7 days) and indicates that water uptake increases after the addition of chitosan.

No relevant variation either in the pH of PBS, the dry weight of the mats, or the FTIR spectra was detected, indicating that no hydrolysis of v-PCL occurred. As a matter of fact, the hydrolytic degradation of PCL is known to have very slow kinetics (i.e., the polymer is stable for years in saline solution and even in vivo).[69] Specimens of v-PCL/CuChi12 showed similar trends, indicating that the solvent blending of the two polymers was successful and resulted in a stable polymer blend even after electrospinning. A peak corresponding to copper was observed in all the EDX spectra of v-PCL/CuChi12 for all time points selected, confirming that the metal does not undergo burst release and is still embedded in the matrix after 1 week. In addition, the morphological analysis performed in parallel to the degradation assay showed that the fiber morphology is maintained after the immersion in PBS. Minor compaction of the mats occurs (i.e., reduction in pores between fibers), but the fibers maintain their shape and integrity. This compaction happened especially for v-PCL, probably as a consequence of the smaller fiber diameter of this mat type, which makes them more prone to collapse. Both for v-PCL and v-PCL/CuChi12, the results of swelling/degradation testing confirm that the mats are stable in physiological saline water solutions and are suitable for cell testing and, more broadly, for applications in contact with body fluids.

3.4. Cell Adhesion and Proliferation

The ST-2 mouse bone marrow stromal cell line was selected owing to its reported ability to differentiate into several phenotypes, components of both hard and soft tissue (osteoblasts,[70] chondrocytes,[71] and adipocytes[71,72]). This makes ST-2 a very versatile and suitable cell line for preliminary assessment of cell viability and proliferation of v-PCL/CuChi12 for various tissue engineering applications. Furthermore, they are known to be sensitive to variations in ionic concentration of the culture medium and to be able to modulate the gene regulation of several paracrine signals (among them VEGF) accordingly.[71,73] As such, the ST-2 cell line is a good candidate to assess the therapeutic effect of copper. Before investigating the role of copper, however, the electrospun mats were evaluated in terms of cytocompatibility, monitoring ST-2 adhesion, and proliferation over 7 days of culture with colorimetric assay based on WST-8 tetrazolium salt (Figure 7). The absorbance increased steadily over 7 days, showing that both for v-PCL and v-PCL/CuChi12 are characterized by similar growing curves without statistically significant differences. These values of absorbance are similar to previously reported results[74] and are in line with the well-documented cytocompatibility of polycaprolactone.[42,52,74–76] Most importantly, they confirm that the addition of copper(II)-chitosan does not cause cytotoxicity effects, a problem that might have risen due to copper ions and their possible toxicity.[30] This result joins a growing research trend that aims to demonstrate that through careful dosage it is indeed possible to tune the amount of metal ion present and use its therapeutic effects without significant drawbacks.[30,77,78] The relative rate of growth (ΔG) per each sample at each time point was calculated.
normalizing every measurement of absorbance ($A_i$) with respect to the respective absorbance at day 1 ($A_1$).

$$\Delta G = \frac{A_i}{A_1} \times 100$$  \hspace{1cm} (2)

Considering the results of this calculation (see Figure 7 bottom right inset), it can be observed how the growth curves of the two formulations do not differ significantly.

Fluorescence staining performed in conjunction with cell viability measurements confirmed the results obtained by WST-8 colorimetric assays (Figure 8). Bone marrow stromal cells rapidly colonize the samples at day 1. Numerous cells with a high number of filopodia were found on both mats, with a higher density toward the border of the cell inserts (i.e., Scaffdex), probably as a consequence of medium flow upon inoculation. Cell morphology was similar for both sets of samples and for all the time tested, confirming that copper does not have detrimental effects neither on cell adhesion, nor on spreading or proliferation. Cells were healthy and successfully spread throughout the substrate, probably aided and directed.

**Figure 7.** WST-8 absorbance (450 nm) graph for both v-PCL and v-PCL/CuChi12 samples at 1, 3, and 7 days after seeding. No significant difference was measured between the two trends ($p \gg 0.05$, $n = 3$). On the bottom right, the values of absorbance at day 3 and 7 normalized to day 1 are reported.

**Figure 8.** Fluorescence images showing actin filaments (red) and cell nuclei (blue) of cells cultured on v-PCL (left) and v-PCL/CuChi12 (right) fiber mats. Three time points corresponding to cell viability measurements are displayed (1, 3, and 7 days after seeding).
by the random fiber pattern, a phenomenon that goes under the name of contact guidance.[79]

Generally, one difference observed between v-PCL and v-PCL/CuChi12 was that cells were easier to focus in the case of neat polycaprolactone compared to blended fiber mats. This could be due to the fact that cells penetrated deeper in v-PCL/CuChi12 compared to v-PCL. As mentioned above, the fiber size distribution of v-PCL/CuChi12 differs from that of v-PCL: fibers are larger and the pores between them are wider. Also, as we anticipated, smaller fibers are not necessarily a feature to be sought. According to our results, the wider fiber texture of v-PCL/CuChi12 allowed better colonization of the scaffold in the third dimension compared to v-PCL. The explanation for this behavior should be sought in the difference in fiber morphology, given that the physicochemical characterization showed that the differences between v-PCL and v-PCL blended with copper(II)-chitosan were minimal in terms of chemical structure, wettability, and degradation behavior.

This hypothesis of better 3D infiltration is supported and confirmed by post-culture SEM analysis, in which several portions of the cell colony in v-PCL/CuChi12 were found deep under the surface of the sample, well entangled within the fibers. On the other hand, cells on v-PCL proliferated mostly on the specimen surface, in a 2D fashion. In the left and right insets of Figure 9, two relevant examples of this behavior are shown, highlighting the different growth of cells above the fibers (in the case of v-PCL) and within the fibers (in the case of v-PCL/CuChi12).

3.5. Vascular Endothelial Growth Factor Upregulation

At the end of the cell culture period (7 days after cell seeding), the vascular endothelial growth factor (VEGF) release from ST-2 cells was measured. The quantification of VEGF, among several other parameters that could be monitored to characterize the angiogenic potential of copper, is generally considered the golden standard for the assessment of this property in biomaterials.[16,73,80–82] In addition, ST-2 cells are known to provide reliable results in terms of pro-angiogenic behavior. However, they are of mouse origin. Future investigations should also aim at reproducing the results of this study using human cells. Populations seeded on both v-PCL and v-PCL/CuChi12 electrospun mats were evaluated. The results reveal a statistically significant threefold increase in VEGF expression for cells cultured on PCL/copper(II)-chitosan specimens. The VEGF concentration in the culture medium spikes from 374 pg mL$^{-1}$ to a remarkable 1.27 ng mL$^{-1}$ (Figure 10), indicating that the copper(II)-chitosan blended in the PCL fibers can strongly influence cell behavior and growth factor expression. In fact, copper is known to be able to interact, once uptaken by cells, with several cytoplasmic components of cell physiological pathways.[22–24,83] These interactions produce a cascade of events that ultimately lead to enhanced proliferation and angiogenesis, but also to cell damage if copper concentration happens to be too high.[84] For instance, copper can impact on both the bioactivity and the production of several growth factors including VEGF, interleukin 1$\alpha$ (IL-1$\alpha$), basic fibroblast growth factor (bFGF), and angiogenin.[82] The molecular biology of the proangiogenic effect of copper is rather complex and still not completely understood. However, it seems to be mainly linked to increased availability of the hypoxia-inducible factor 1 (HIF-1), a transcriptional factor for the VEGF gene. In turn, this excess of HIF-1 works as a transcription promoter and enhances VEGF synthesis and secretion.[22] The growth factor, once released extracellularly, can promote endothelial cell activity and angiogenesis. The VEGF concentration obtained with PCL/CuChi12 reaches very promising values at the lower end of the therapeutic window of the growth factor.[85]
This could allow its use in successful electrospun scaffolds without raising significant safety concerns as regards the possible dangers of using VEGF.[24,86]

Compared to other existing technologies, v-PCL/CuChi12 induces VEGF secretion at levels that are competitive against the ones that can be achieved using bioactive glasses (BGs), a key material platform for therapeutic ions controlled release. In particular, the VEGF overexpression was comparable with the one obtained by treating cells with particle suspensions of S53P4 BC[82] and copper-doped BG.[81] When compared with composite biomaterials based on aliphatic polyesters, v-PCL/CuChi12 performed better than PCL/boron-containing BC[16] and PLGA/collagen/64S BG composite films, both with and without Mg and Co doping.[80] Possibly, this is, among other factors, also a consequence of the better proliferation substrate provided by electrospun fiber mats compared to a film.

The very substantial increase in VEGF secretion, together with the fact that the cell culture tests on ST-2 cells demonstrated that the quantity of copper loaded into the fiber mats is not significantly toxic, confirms that cells seeded on copper(II)-chitosan-containing scaffolds can benefit from the therapeutic effects of copper without being hindered by it. The upregulation of VEGF in ST-2 cells can be observed on the graph in Figure 10, and it is clear evidence of the strong angiogenic potential of PCL/CuChi12 electrospun fiber mats. This result opens up to a plethora of investigations aiming at deepening the understanding of the angiogenic properties of copper in biomaterials and scoping the potential of PCL/CuChi12 electrospun mats in targeting different tissue engineering challenges. Future work will include, for instance, gathering information regarding the capillary network formation ability of the scaffolds as well as investigating the possibility to influence cell behavior on a longer term (i.e., weeks), as a consequence of both the sustained release of copper and the onset of the degradation of the polymers, which does not occur within the currently tested timeframe.

4. Concluding Remarks

By means of a versatile and relatively cost-effective fabrication method based on benign solvents for electrospinning fibrous scaffolds with ideal morphology for cell adhesion, infiltration, and proliferation were successfully fabricated. At the same time, the obtained scaffolds resulted in upregulation of media tors that are fundamental for the formation of new blood vessels. The electrospun scaffolds were made of a novel stable and homogeneous blend of polycaprolactone and copper(II)-chitosan that can be manipulated with the sole use of benign solvents for electrospinning. In addition, owing to the use of naturally sourced chitosan and polycaprolactone with negligible solvent contamination (guaranteed by the supplier), the present green approach is environmentally friendly, an ever increasing important responsibility for industries worldwide. Polycaprolactone/copper(II)-chitosan electrospun mats were not only prepared trying to reduce the environmental impact of their production, but they also attained outstanding biological results. Cell culture performed on the mats using ST-2 bone marrow stromal cells revealed that cells can adhere, proliferate, and infiltrate well within the fibers, colonizing the whole scaffold. The presence of copper within the blend was also correlated with a significant increase in the secretion of VEGF, a growth factor that can have high impact on endothelial cells, guiding angiogenesis ex vivo and in vivo. Possible applications of the obtained fibrous scaffolds could be related to the field of vascular tissue engineering, especially for small diameter vessels, which cannot be successfully replaced with synthetic grafts. Furthermore, it is well known that to date angiogenesis remains a major bottleneck for the regeneration of most tissues. In this work, a possible solution based on therapeutic ions was presented: thanks to its simplicity and relatively low cost, the present Cu-containing fibrous scaffolds could offer a successful solution for diverse applications in regenerative medicine.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

angiogenesis, benign solvents, chitosan, copper, electrospinning, polycaprolactone
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