EMULSION ELECTROSPINNING – METHOD TO INTRODUCE PROTEINS FOR BIOMEDICAL APPLICATIONS

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Abstract

The aim of this work was to obtain polymer fibers by the emulsion electrospinning. For this purpose, polycaprolactone (PCL) was used, which was modified before the electrospinning stage with micelles obtained by the oil-in-water (O/W) emulsion method. Micelles were obtained by combining the non-ionic surfactant Tween 80 or Triton X-100 used at different concentrations with the amino acid alanine. The obtained fibrous substrates had a typical unimodal fiber size distribution and their average size was in the range of 590-800 nm. The effectiveness of the emulsion electrospinning process was confirmed by Fourier Transform Infrared Spectroscopy - Attenuated Total Reflectance (FTIR-ATR) showing the presence of surfactants. The addition of micelles to the polymer solution significantly reduces the contact angle of nonwoven fabrics: from 120° (for PCL) to ~20-30° for surfactant-loaded nonwovens, and the micellar form allows tracking the release of alanine into the solution (UV-Vis). The combination of the core-shell--morphology of the emulsion electrospun fibers allows comparable amino acid release times. There were no significant differences in both the amount of alanine released and the rate of its release between PCL/Tween80/alanine and PCL/Triton X-100/alanine fibers, which were characterized by a similar fiber size.

Keywords: *emulsion electrospinning, alanine, polycaprolactone, Tween 80, Triton X-100, micelle*

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Introduction

The possibility to produce nanofiber scaffolds that mimic the microstructure of the extracellular matrix (ECM) is an advantage of electrospinning techniques used in tissue engineering and regenerative medicine. Modification of electrospinning parameters: environmental, apparatus as well as the possibility of modifying the solution used to produce nanofibers gives new opportunities to prepare scaffolds with the desired behavior in a living organism. This is especially important when tissue regeneration requires a special approach i.e. targeted drug delivery or release of bioactive agents. Promoting cell adhesion, proliferation, migration and ingrowth are one of the main requirements of tissue engineering. There are many methods to stimulate an appropriate response of cells to the scaffold, including growth factors, adhesive peptides, ECM proteins, cytokines, hormones, or genes [1,2].

The main role of incorporating these molecules is to provide binding sites for cells, and such an approach allows to mimic not only the microstructure of the extracellular matrix but also its chemistry and signaling, thus ensuring a better similarity to the natural tissue microenvironment [1,3]. Peptide-based scaffolds are gaining more and more interest as an alternative to proteins due to their structural and biological similarity. In addition, these biomolecules are easier to introduce and more resistant to environmental conditions than larger proteins. Amino acids are modifiers that also have a beneficial effect on cells, and as the basic building blocks of proteins, they influence processes such as cell growth, differentiation, and metabolism. Research shows that amino acids, including methionine, leucine, and glutamine, act as activators of growth pathways [4]. Another amino acid that plays an important role in cell growth and T-cells activation is alanine, and its deprivation leads to impairment of cell growth, proliferation, and function [5]. These advantages make it an attractive biomodifier to stimulate an appropriate cellular response to the implant surface. There are many methods to incorporate bioactive molecules into fibers, which include their external immobilization and encapsulation within them. Due to their controlled release kinetics, core-shell nanofibers are gaining tremendous interest as a substrate for cell proliferation. Moreover, the encapsulation of proteins and pharmaceuticals in electrospun fibers is one of the strategies to overcome the barriers associated with maintaining the stability and effectiveness of the active ingredient during the formulation process [6]. The methods of producing core-shell fibers mainly include blend electrospinning or coaxial electrospinning. The first technique, which consists in dissolving both components in a suitable solvent, has the fastest loading rate compared to the other methods. However, due to the use of an organic solvent, there is a risk of denaturing bioactive molecules. Furthermore, diffusion of the encapsulated particles to the peripheral parts of the fibers contributes to the burst release effect [7,8]. Contrary to this technique, coaxial electrospinning, due to separate solution delivery systems, allows for the use of a wider range of polymers for the core part and the shell, as well as for the diversified distribution of biomolecules in the fibers and their release kinetics [9]. However, there is still the possibility of diffusion of the bioagent into the shell part during the formation of the Taylor cone, which leads to a non-linear release kinetics [8]. Emulsion electrospinning is another modification that is used to produce nanofibers that release biologically active compounds or drugs. This simple method is based on the use of an oil-in-water (O/W) or water-in-oil emulsion (W/O) as the electrospinning solution, and a surfactant (which acts as an emulsifier) is used to stabilize the emulsion.

Moreover, the type (ionic, non-ionic) and concentration of the surfactant affect the surface tension and conductivity of the solution, which ultimately affects the morphology and internal architecture of the fibers [10]. The advantage of emulsion electrospinning is control of the kinetics of the release of the active ingredient or biomolecule and high-efficiency encapsulation. Due to the use of surfactant, the bioagents are separated from the organic solvent, which reduces the risk of its denaturation and enables the combination of hydrophobic polymers with hydrophilic biomolecules [7,11]. The advantage of emulsion electrospinning over traditional coaxial electrospinning is that it does not require additional equipment (special spinneret, separate delivery systems) for the production of core-shell fibers. Emulsion electrospinning uses a standard single-nozzle system, which makes it a more convenient and cost-effective method [12]. Due to the above-mentioned advantages, this method is often used for the encapsulation of biomolecules into synthetic polymers to increase their biocompatibility.

Polycaprolactone (PCL) due to its relatively good biocompatibility, low toxicity, and good mechanical properties, is one of the most widely used synthetic polymer in the biomedical field. However, its high hydrophobicity and low bioregulatory activity impair appropriate cellular adhesion and proliferation. Therefore, emulsion electrospinning is used to incorporate various biomolecules, such as peptides or growth factors, to increase PCL hydrophilicity and biological activity without the risk of damaging them [13]. Baskapan et al. used this method to encapsulate laminin in PCL fibers for kidney regeneration. As a result they obtained a nonwoven fabric with higher elastic properties and hydrophilicity, which resulted in better renal cell adhesion [14]. In turn, Basar et al. obtained binary system PCL/gelatin loaded with Ketoprofen mats with more sustained release kinetics in comparison to single PCL mat. The use of emulsion electrospinning enabled more sustained drug release up to 4 days and significantly inhibited burst release [13]. However, the possibility of encapsulating low molecular weight biomolecules still needs to be explored.

In our research, we focused on the possibility of loading alanine into nanofibers using emulsion electrospinning, which creates core-shell nanofibers from an emulsion consisting of an organic phase (polymer) with a surfactant and an aqueous phase (surfactant with alanine). To prevent the negative interaction of the model biomolecule (alanine) suspended in the aqueous phase with the polymer organic solvent during the electrospinning process, commercial surfactants were used (Tween 80, Triton X-100). The next step was to optimize electrospinning conditions to obtain fibers enriched with encapsulated amino acids. Specifically, we investigated the solution and emulsion process parameters for electrospinning to achieve high nanofiber biomolecule loading, prolonged formulation stability, and controlled protein release for future biomedical applications.

Materials and Methods

Preparation of the emulsion

A water-in-oil (W/O) emulsion was prepared for an electrospinning solution, with the oil and water phases being prepared separately. Polycaprolactone 10% (w/v) (PCL, Mw = 80 kDa, Sigma-Aldrich, Germany) and surfactants (Tween 80 and Triton X-100, Sigma-Aldrich) at various concentrations (0.5% v/v, 1% v/v and 5% v/v for Tween 80 and 0.5% v/v, 1% v/v and 3% v/v for Triton X-100) were dissolved in dichloromethane (DCM, Chemland SA) and dimethylformamide (DMF, Chemland SA) (volume ratio of 7:3 v/v), while alanine (L-alanine, Sigma-Aldrich, Germany) was dissolved in distilled water to prepare the water phase. The alanine solution (5% by weight of dry PCL) was then added dropwise to the polymer solution and stirred in an ice bath. The emulsions were used for electrospinning immediately after preparation. The selection of a safe amount of surfactant was based on data from the literature. The applied amount of surfactant should not adversely affect the cellular response [15].

Preparation of the fibrous membrane by electrospinning

The electrospinning process was carried out at an ambient temperature (~25°C) and relative humidity in the range of 40-50%, and the emulsion was fed at a rate of 0.7 ml/h. The fibers were collected on 10 cm aluminium foil from the spinneret to the collector and a voltage of 18 kV was applied.

Electrospun mats characterization

Physicochemical properties

The microstructure of the nanofibers was observed with a scanning electron microscope (NOVA NANO SEM 200) and the samples were coated with a 10 nm gold layer prior to observation. The diameter of 100 random fibers in each sample was measured with ImageJ software, and the fibers size distribution was obtained.

The effectiveness of the modification of polymer fibers by micelles and the presence of surfactants were investigated by Fourier Transform Infrared Spectroscopy in the ATR mode (Bruker Tensor 27 FTIR). Infrared spectra were acquired using 64 scans in the range of 4000-400 cm^{-1} and spectra resolution of 4 cm^{-1}.

To determine the wettability of the scaffold, the contact angle was measured by a sessile drop test using a Kruss DSA 25 goniometer. Measurements were made by placing a drop (1 µL) of deionized water on the sample surface. The contact angle value was determined as the average of 10 measurements for each sample. The test was carried out at room temperature.

All prepared samples with the same weight of ~15 mg (to normalize the thickness differences) were incubated in NaCl solution for one month, and the concentration of alanine in the solution was assessed weekly by spectrophotometry (UV-Vis, Cecil 2520). A reaction with ninhydrin was performed to determine the amount of alanine released. Briefly, ninhydrin was dissolved in ethanol to obtain a 1% (w/v) solution. Then, 1 mL of incubation solution was added to 1 mL of ninhydrin solution and heated for 1 min (until colour turned violet) and placed in the spectrophotometer chamber. The determination of alanine concentration was performed at a wavelength of 570 nm and was determined from a standard curve derived from solutions of known concentration. To obtain the standard curve, the solutions with an increasing concentration of alanine were prepared (0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL, 1 mg/mL) and reaction with ninhydrin was performed. Then, based on the obtained data, a regression curve was determined, which was used to determine the concentration of released alanine. Loading efficiency (LE) was calculated using the equation:

$$\%LE = \frac{\text{mass of alanine in fibers}}{\text{mass of fibers}} \cdot 100\% \qquad (1)$$

Statistical analysis

All results are presented as mean ± standard deviation. To determine the significance level for the biological study, statistical analysis was conducted with one-way ANOVA followed by Tukey's post hoc analysis. Probability values less than 0.05 were considered statistically significant. The ANOVA analysis was performed using Origin Pro 2021 software.

Results and Discussion

To assess the impact of the amount of the surfactant on the fibers diameter and morphology, different concentrations of the surfactants were used. It was found that the morphology of the fibers obtained from emulsion electrospinning strongly depended on the amount of surfactant in the solution (FIG. 1). For higher concentration, defects and pores on the fibers surface were observed (FIG. 1A and 1D), while thin, smooth and uniform fibers were obtained at 1% v/v and below (FIG. 1B-C and 1E-F). The histograms presenting the fiber diameter distribution (FIG. 2A-C) indicate that the use of emulsion as a spinning solution affects the diameter of the PCL fibers. The PCL fibers diameter was in the range of 450-1100 nm for pure PCL (FIG. 2A) and the distribution was unimodal with a maximum at 400-800 nm for PCL/Tween80/alanine (FIG. 2B) and PCL/Triton X-100/alanine (FIG. 2C). However, emulsion electrospinning contributed to the formation of fibers that mostly had diameters smaller than PCL (below 480 nm). In addition to nanometer-range fibers, fibers significantly larger than PCL (over micrometers) were also observed. The difference was also confirmed by the ANOVA test, which showed significant differences between the entire population means. However, there were no significant differences between the fibers obtained from emulsion with Tween 80 independently on the concentration (ANOVA, $p = 0.615$) which indicates the fact that the use of Tween 80 results in obtaining more uniform fibers. Moreover, depending on the size of the micelles (for PCL/Tween80/alanine $ø_{mean}$ = 22 μm with polydispersity index PI = 0.45 and for PCL/Triton X-100/alanine was $ø_{mean}$ = 31 μm and PI = 0.97), the fiber microstructure was different, and the fibers were either completely internally hollow or exhibited an intrinsic hierarchical porosity (FIG. 1G-H).

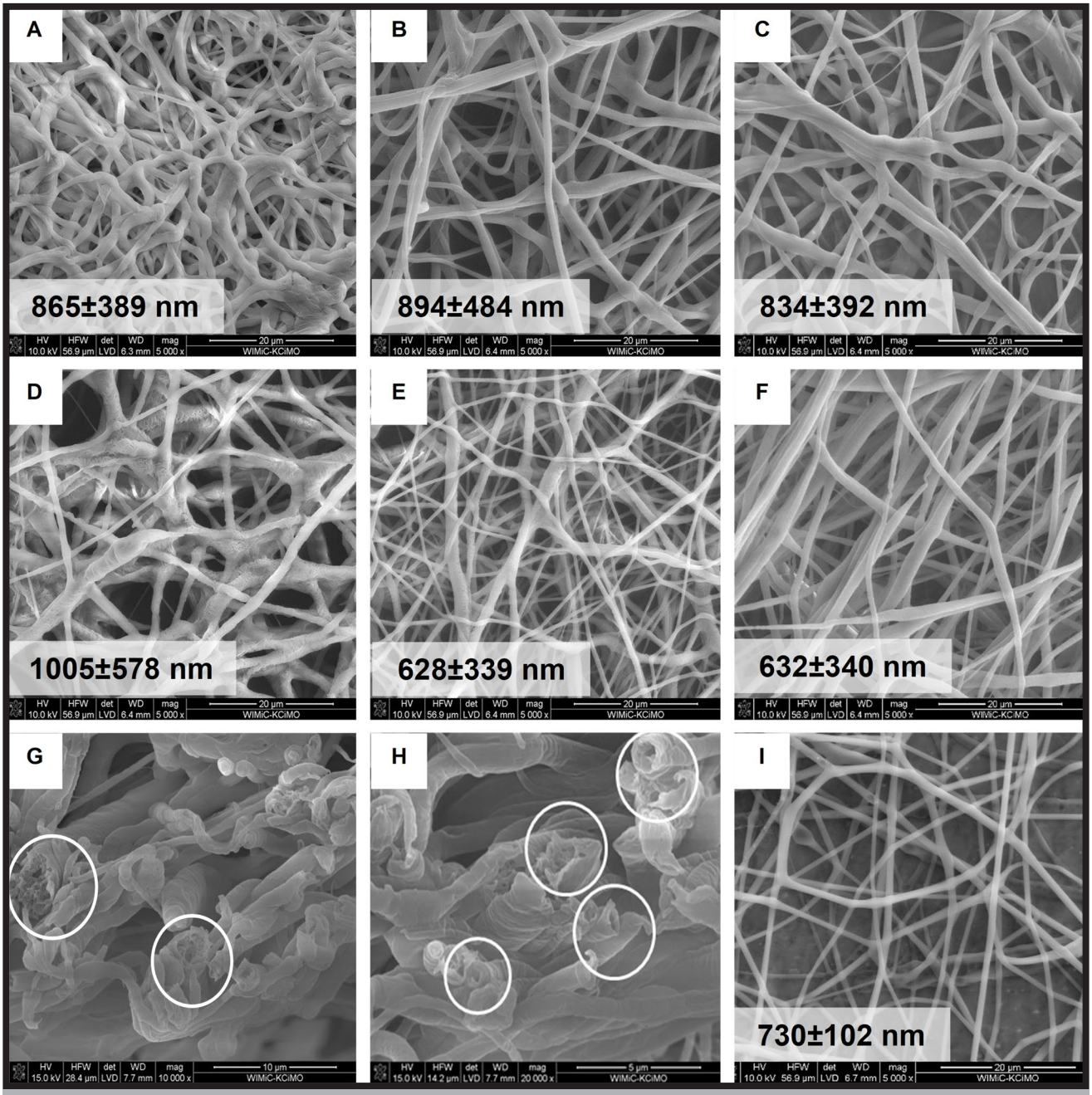

FIG. 1. SEM images and mean fiber diameter ± standard deviation of PCL/alanine nanofibers prepared using Tween 80 at concentrations: (A) 5% v/v, (B) 1% v/v, (C) 0.5% v/v and Triton X-100 at concentrations: (D) 3% v/v, (E) 1% v/v, (F) 0.5% v/v. Cross-sections of the PCL/alanine nanofibers prepared using: (G) 1% v/v Tween 80, (H) 1% v/v Triton X-100. Image (I) presents neat PCL fibers.

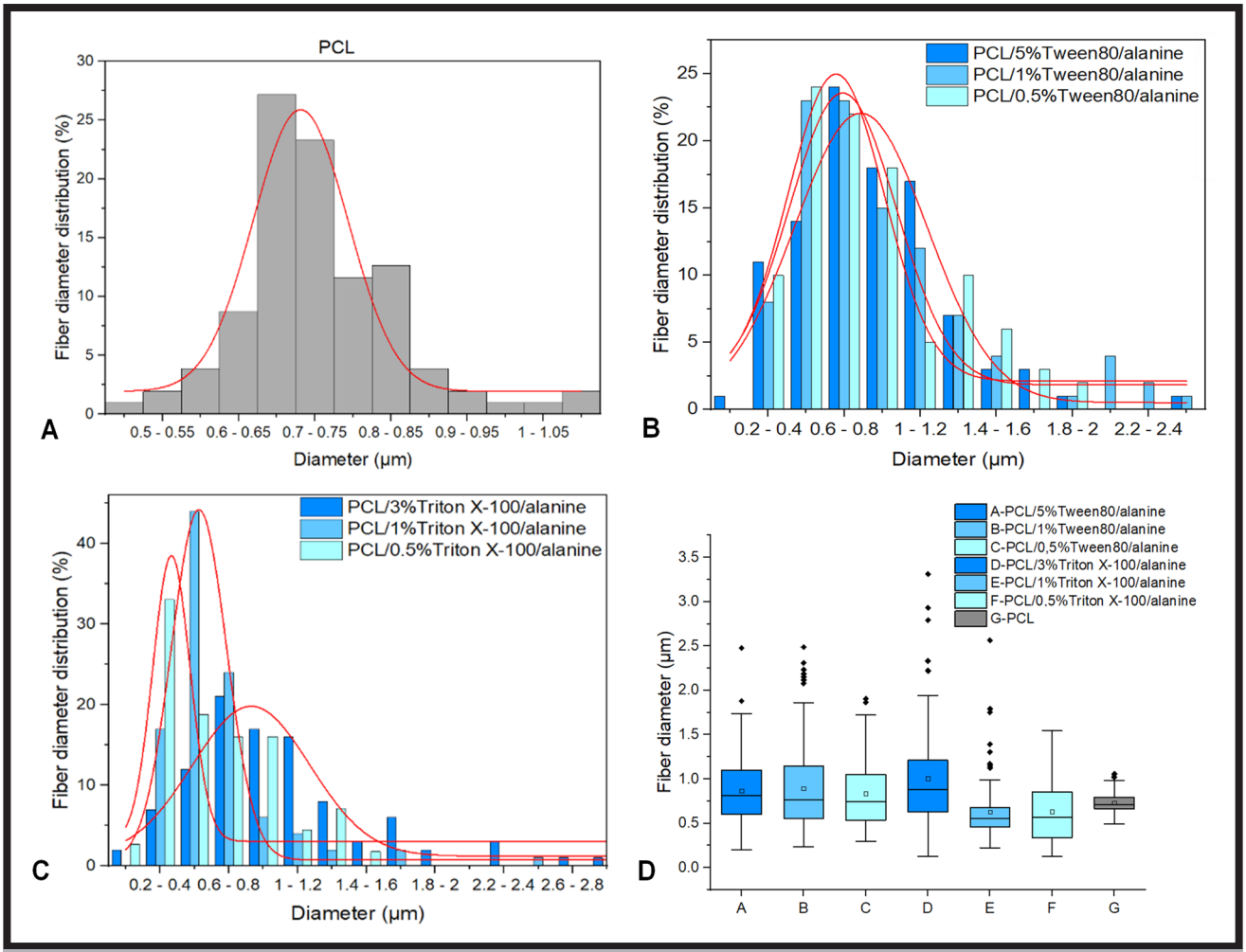

FIG. 2. Diameter distribution of (A) neat PCL, (B) PCL/Tween80/alanine, (C) PCL /Triton X-100/alanine fibers and (D) mean diameters of the fibers. Based on n = 100 measurements.

A similar internal microstructure was obtained by Johnson et al. only by addition of the surfactant [16]. This may indicate that some of the surfactants not bound to alanine created closed pores inside the fibers.

The FTIR-ATR spectra of emulsion electrospun membranes are presented in FIG. 3. Since the same FTIR spectra for all samples were acquired and the high concentration of surfactant results in the defected microstructure, samples with a surfactant concentration of 1% v/v were selected for FTIR studies. All samples exhibit characteristic peaks of the pristine PCL spectra, including bands such as 2940 cm^{-1} and 2865 cm^{-1} (asymmetric and symmetric CH_2 stretching, respectively), 1722 cm^{-1} (C=O stretching), 1294 cm^{-1} (C–C and C–O stretching), and 1240 cm^{-1} (asymmetric C–O–C stretching) [11]. Moreover, the analysis of the chemical structure confirmed the presence of Triton X-100 in the material, as evidenced by the carbonyl band in the range of 1609 cm^{-1}, C-O-C band at 1113 cm^{-1}, C-H and C-C stretching in an aromatic ring at 1510 cm^{-1} and band at 837 cm^{-1} typical for the structure of Triton X-100 [17]. On the other hand, a change in the ratio of the 1190–1160 cm^{-1} bands was observed in materials where Triton X-100/alanine emulsions were introduced into the fibers during the electrospinning process; however, none of these characteristic peaks were detected for Tween 80. This could be due to either overlapping characteristic bands of Tween 80 and PCL or too small amount of surfactant to be detected. Protein macromolecules encapsulated in micelles do not yield bands characteristic of the proteins used, and the fact that they cannot be detected on the surface indicates that they are trapped inside the fibers. The outcomes are consistent with the results obtained by Jue Hu et al. where the core-shell fibers also showed the structure of a pristine PCL, without a trace of encapsulated biomolecules and surfactants [10]. The fact that in this study there are characteristic bands of Triton X-100 present in the FTIR spectra may result from the much higher concentration of the surfactant used in this experiment.

TABLE 1 presents the results of the contact angle measurements for the prepared membranes, which confirm that the addition of surfactants strongly affects the physicochemical properties of the fiber surface. The presence of surfactants, regardless of concentration, completely changed the wettability of PCL samples from hydrophobic (~130°) to highly hydrophilic (~20-35°). However, as the concentration of the surfactant increases, the contact angle decreases slightly. This could be attributed to the presence of a surfactant on the surface of the fibers. The effect was enhanced by the high humidity of the process (~50%) and a small amount of the water phase, which contributed to a more uniform distribution of the surfactant between oil-air and oil-water interfaces, since both were thermodynamically favourable for surfactant location. The same sharp decrease in the contact angle was also observed by Johnson et al. during their studies on emulsion electrospun PCL/Span 80/water fibrous mats [16].

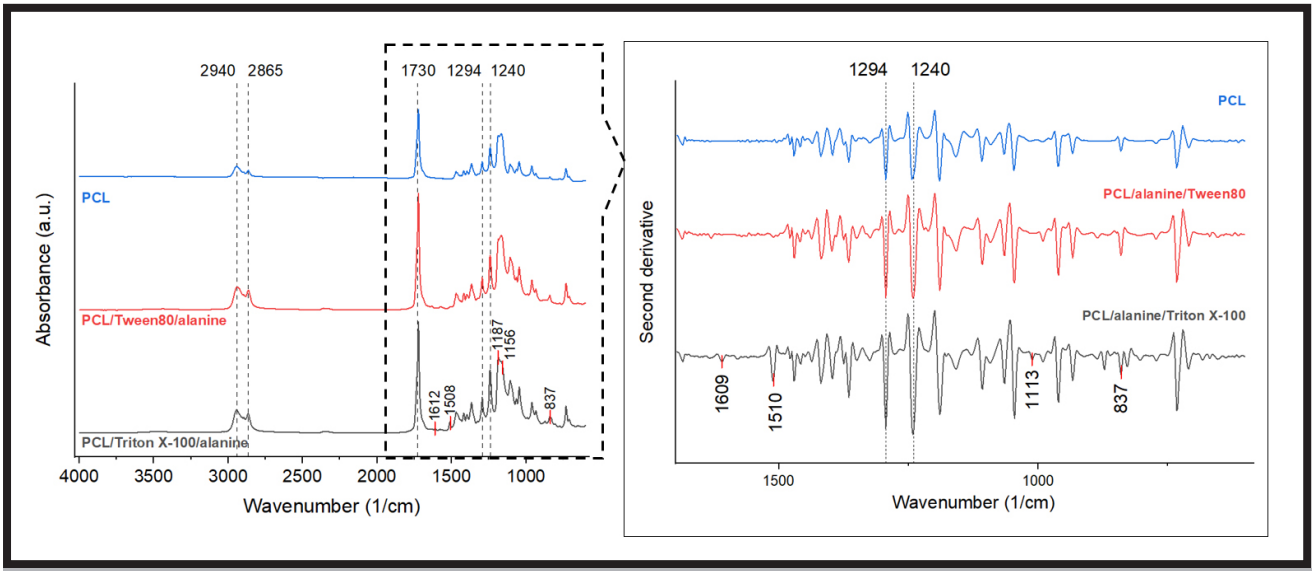

FIG. 3. FTIR spectra of the PCL, PCL/Tween80/alanine and PCL/Triton X-100/alanine using 1% Tween 80 and 1% Triton X-100.

TABLE 1. Contact angle of the electrospun fibers using emulsions with different content of the surfactants and loading efficiency of alanine.

Sample	Concentration of surfactant (%)	Loading efficiency of alanine (%)	Average contact angle (°)
PCL/Triton X-100/ alanine	0.5	4.95	29.5 ± 3.7
	1	2.21	21.5 ± 2.7
	3	1.29	20.5 ± 4.8
PCL/ Tween80/ alanine	0.5	6.84	35.3 ± 5.2
	1	2.10	24.0 ± 3.9
	5	1.35	22.0 ± 2.5
PCL	-	-	128.8 ± 2.2

The analysis of spectrophotometric results (UV-Vis) confirms the continuous release of a small amount of alanine (up to ~0.7 mg) from the fibers over a period of 1 month (FIG. 4). Since the highest content of the surfactant resulted in a defected microstructure, the fibers obtained by emulsion electrospinning with the use of surfactants at a concentration of 1% were subjected to release studies. There were also no significant differences in the amount of alanine released or the release rate between PCL/Tween80/alanine and PCL/Triton X-100/alanine fibers, having similar fiber size. This means that the combination of core-shell morphology and uniform fiber size distribution influenced the release time of amino acid (alanine) and prevented the burst release phenomenon. Such a character of the curves indicates the mechanism of diffusion – erosion release of biomolecules. After the first day of incubation, an immediate increase in alanine concentration was observed due to the diffusion of water into the exposed pores containing the biomolecule. The subsequent constant increase in the concentration of alanine is associated with the slow erosion of the matrix, which led to the formation of small channels in the fibers leading to the migration of the active ingredient to the incubation solution [18].

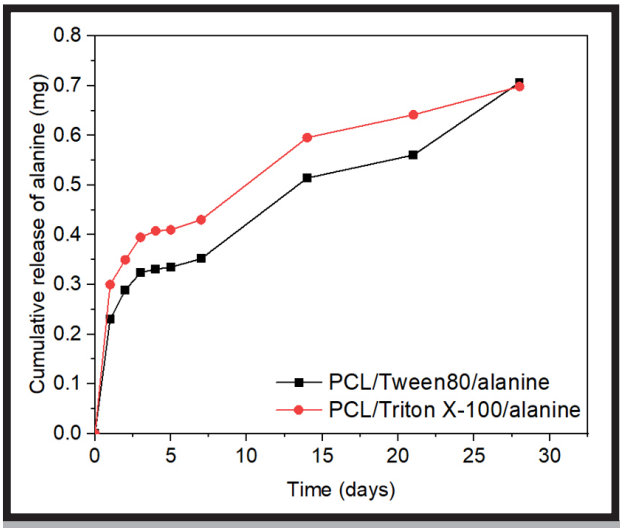

FIG. 4. Cumulative curve of alanine release from fibrous meshes prepared from emulsions PCL/ Tween80/alanine and PCL/Triton X-100/alanine using 1%Tween 80 and 1%Triton X-100.

The observed release curves in FIG. 4 are difficult to clearly attribute to the mathematical models of Highuchi or Pappas-Kosmayer describing the kinetics of drug release (in DDS systems). In the case of fibers or microspheres, these limitations are mainly due to the form of the introduced drug (the need to use a drug in saturated form), which cannot always be used in this form due to the formulation process of the carrier [19]. Thus, in *in vitro* conditions, we have a combination of different mathematical models that allow us to describe the drug release profile. The most common drug release profile from polymeric drug delivery systems is the three-phase profile, which combines both effects related to drug diffusion and carrier erosion [20]. The work of Yin et al. showed that the diffusion-erosion mechanism is common to both fibers and microspheres [21]. Such a mechanism allows for the correlation of the drug release rate with degradation of the polymer matrix and to preserve the geometric shape of the fiber, and thus its role as a scaffold. However, due to the slow degradation rate of PCL, further studies of the release kinetics are required.

Conclusions

The emulsion electrospinning process allows to obtain fibers modified with micelles with biological compounds. By varying the concentration of surfactants, it was possible to obtain fibers with different morphology and diameter; however, excess amount of surfactant adversely affected both the microstructure and the surface of the fibers (increase in fiber diameter and surface roughness). In turn, the use of a lower concentration of surfactant resulted in obtaining more uniform and defect-free fibers. Furthermore, the addition of surfactants with encapsulated peptide increased the hydrophilicity of the fibers, which should be important for cell adhesion. The presence of the external polymer layer (shell part), as well as the differentiation of the internal porosity of the fibers, allowed for better control of the biomolecule release kinetics. The diffusion-erosion mechanism of release of biomolecules resulted in a prolonged release time and prevented burst release. The research results indicate that emulsion electrospinning is a promising method of encapsulating biological molecules, which can be used to stimulate tissue regeneration processes that require a specific approach, e.g. soft tissue. To confirm the potential of the obtained substrates as the vehicles for the biomolecule in further research, we will focus on optimizing the surfactant concentration, which will provide high cell viability.

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