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A Novel Design of a PVA Electrospun Nanofibrous Scaffold Incorporating Liposomes as Drug Delivery Carriers for Tissue Engineering

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Abstract

Electrospinning is an emerging technology for the production of fibrous biodegradable polymeric scaffolds. Liposomes have received widespread attention as carriers of therapeutically active compounds due to their unique characteristics. The combination of liposomes with electrospun nanofibers may result in a scaffold with high efficiency in tissue regeneration as the controlled degradation and high surface-to-volume ratio of nanofibers, make them excellent carriers for therapeutic or tissue regeneration agents. The aim of this work was to develop a novel scaffold for Tissue Engineering (TE) applications, incorporating a drug delivery system based on PVA nanofibers enriched with drug-loaded liposomes. Two liposomal compositions were considered, i.e. -1,2-Distearoyl-sn-glycero-3-phosphocholine: Cholesterol-[DSPC/Chol] and Phosphatidylcholine: [PC]. We showed that blend electro spinning of PVA leads to successful incorporation of liposomes into the fibers for both liposomal compositions. Morphology of the fibers and incorporation of liposomes into the fibers was examined by SEM and Confocal Laser Scanning Microscopy. The integrity of the liposomes embedded on the electrospun fibers, was determined fluorometrically by measuring the calcein retention in liposomes (Latency). It was proven that between the two types of embedded liposomes, DSPC/Chol liposomes exhibited a high retention of the encapsulated calcein in contrast with the PC liposomes which presented a rather low one. Given the combined properties of liposomes and nanofibers, the above system could serve as a convenient delivery vehicle for a number of biologically active compounds in tissue engineering and regenerative medicine.

Keywords: Drug delivery; Electrospinning; Liposomes; Polymeric scaffolds; PVA

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Introduction

Engineered scaffolds produced by electrospinning of biodegradable polymers offer 3D, nanofibrous environment with controllable structural, chemical, and mechanical properties that mimic the extracellular matrix of native tissues and have shown promise for several tissue engineering applications [1-5]. The fabrication versatility and the unique properties of electrospun nanofibrous membranes, as well as their controlled degradation, and high surface-to-volume ratios, make them excellent carriers for therapeutic agents, such as antibiotics, anticancer agents, proteins and DNA [6]. Drugs can be attached by using post spinning modifications such as drug adsorption or layer-by-layer assembly. Alternatively, polymer/drug blend electrospinning can be used to incorporate the drug molecules throughout the fiber interior [7]. In a more sophisticated variation of this method, coaxial electrospinning has been implemented to incorporate the drug molecules into a drug/polymer core within a shell of a different polymer composition [8].

Through the outstanding characteristics of nanofibers, many studies have been conducted on various types of polymers as carriers for drug delivery applications [9-13]. PVA has been identified as a suitable candidate that can be used for releasing biological and medical materials in a controlled way [9]. It has unique properties such as good chemical and mechanical resistance, thermal stability, biocompatibility and is also hydrophilic and non-toxic, thereby it is suitable to be electrospun as a polymeric drug carrier [14,15].

Over the past few decades, liposomes have received widespread attention as a carrier system for active therapeutic compounds, due to their unique characteristics such as capability to incorporate and entrap hydrophilic and hydrophobic drugs, good biocompatibility, low toxicity, lack of immune

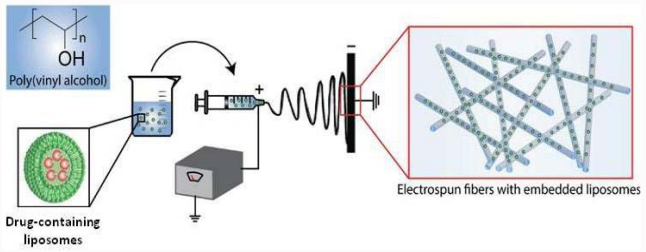

Figure 1: Schematic representation of the electrospinning process.

system activation, and targeted delivery of bioactive compounds to the site of action [16-19]. However, their short half-life, low stability, and poor control of drug release over prolonged periods limit their use for long-term drug delivery applications [20]. One approach to overcome these drawbacks and extend their use in biomedical engineering involves the encapsulation of drug-loaded liposomes within polymer-based systems such as polymeric scaffolds [21-23].

Calcein is a water soluble, fluorescent and self-quenching probe that is extensively used in studies of cell viability, while its release kinetic have been utilized as an effective index to evaluate drug stability in a variety of conditions [24,25]. It can be incorporated into liposomes, used as a model of hydrophilic drug for the investigation of drug/liposome interactions and determination of the encapsulated efficiency.

The aim of the present work was to develop an electrospun polymeric scaffold for tissue engineering incorporating a drug delivery system based on liposome-enriched nanofibers. Two liposomal compositions were considered therefore, both containing calcein to study their integrity during the electrospinning process. Blend electrospinning was selected for the incorporation of calcein-loaded liposomes into nanofibers. The morphology of the nanofibers and the incorporation of intact liposomes were examined by use of Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM). The integrity of the liposomes embedded on the electrospun fibers, was determined fluorometrically by measuring the calcein retention (Latency) in liposomes before and after the addition of Triton X-100. The results presented in the current study exhibit the potential of the above drug delivery system for use in various fields of tissue engineering and regenerative medicine.

Materials and Methods

Liposomes preparation and physicochemical characterization

Materials: Two different lipid compositions were considered, i.e. 1,2-distearoyl-sn-glycero-3-phosphocholine [DSPC] and Phosphatidylcholine [PC], purchased from Lipoid Gmbh, (Ludwigshafen, Germany). Calcein, Triton X-100 and Cholesterol (99%) [Chol] were purchased from Sigma-Aldrich Hellas (Athens, Greece). All solvents used were of analytical grade and were purchased from Merck, Germany.

Preparation process: Multilamellar vesicles (MLV) were prepared by the thin film hydration method [26]. To this end, a specific weight of lipid was dissolved in a Chloroform/Methanol (2:1 v/v) mixture and subsequently evaporated under vacuum (in a round bottomed flask connected to a rotor evaporator) until a thin lipid film was formed. Any residual organic solvents were removed from the flask by applying a nitrogen flow. Afterwards, the lipids' film was hydrated with a volume of a 100 mm water solution of calcein, the osmotic pressure of which was adjusted with NaCl to be equal to that of PBS used in next step [300 mOsm/L] to protect liposome integrity. Upon completion of hydration, associated with spontaneous formation of liposomes, the dispersion was placed in a bath sonicator (Bransonic 2510) for 30 min, to obtain smaller liposomes and break existing aggregates. Sonicated Unilamellar (SUV) liposomes were prepared by subjecting the MLV dispersions (as described above) to titanium probe sonication (Sonics, Vibra Cell) for one or two 10 min cycles until the dispersions were completely clear. After this, the SUV dispersions were centrifuged at 13,000 RPM (Labofuge 200, HERAEUS) for 10 min, to precipitate any titanium fragments released from the sonication probe because of the high intensity of sonication. Finally the liposome dispersions were incubated for 1 hour to 2 hours at a temperature above the lipid transition temperature for annealing structural defects of their membranes. Separation of liposomes from non-encapsulated calcein was achieved by use of size-exclusion chromatography on 1 cm × 30 cm Sephadex G-50 column eluted with Phosphate Buffered Saline (PBS). Liposomes were stored at 4°C before use.

Measurement of liposome size distribution, surface charge and lipid concentration: The size distribution (mean diameter and Polydispersity Index [PI]) as well as ζ-potential of the prepared liposome dispersions, were measured. Size distribution was measured by means of dynamic light scattering (DLS) on a Nano-ZS (Malvern Nano-ZS, Malvern Instruments), which enabled the calculation of the mass distribution of particle size. Measurements were made at 25°C with a fixed angle of 173°. Sizes quoted are the *z*-average mean (dz) for the liposomal hydrodynamic diameter (nm). For ζ-potential measurements, the same liposome dispersions are used to measure their electrophoretic mobility at 25°C (Zetasizer Nano, Malvern Instruments, UK). Zeta potentials of the dispersions were calculated (by the instrument) from the Smoluchowski equation. The lipid (phospholipid) concentration was measured by the Stewart technique [27].

Measurement of Liposome Integrity: Since calcein-loaded vesicles could be distinguished from free calcein, we assessed calcein latency in the electro spun scaffold incubation medium to provide information about the liposome integrity. Calcein latency was measured after liposomes were separated from non-encapsulated calcein and before they were used for the preparation of liposome-trapping scaffolds [28], to be sure that separation was complete or that calcein did not leak out of the vesicles during storage. In cases where calcein latency was lower than 95 percent, the liposomes were re-centrifuged before being used. Additionally, calcein latency was used as a method to evaluate the percent of intact liposomes incorporated in the polymeric scaffolds by calculating the latency of the calcein extracted from the fibers after their preparation. For calcein latency calculation, 20 μl of the liposome dispersions were diluted with 4 ml buffer, pH 7.40 and Fluorescence Intensity (FI) was measured (EM 470 nm, EX 520 nm) before and after addition of Triton X-100 at a final concentration of 1% v/v (that ensures liposome disruption and release of all encapsulated calcein). In the liposome integrity experiments the media was measured without dilution. Percent latency was calculated from Equation (1):

$$\%\ \text{Latency} = \frac{1.1 \times \left(F_{AT} - F_{BT}\right)}{1.1 \times F_{BT}} \times 100$$

Where: F_{BT} and F_{AT} are calcein fluorescence intensities before and after the addition of Triton X-100, respectively.

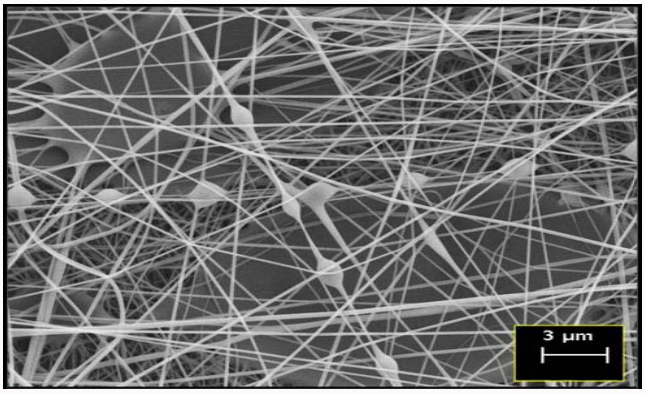

Figure 2: SEM image of the liposome-containing [DSPC/Chol] electrospun nanofibers. Similar images are obtained for the [PC] liposome type.

All measurements of sample fluorescence intensity were performed with a Shimadzu RF-5301 Spectrofluorometer at 37 ± 0.1°C. It is worth noting that the measurement of calcein retention in the fibers was done on portions of the produced fibers having comparable amounts (mg) of incorporated lipid.

Production and characterization of polymeric nanofiber membranous scaffolds

Materials: Polyvinyl Alcohol (Mw 85,000 to 124,000, 87% to 89% hydrolyzed) in the form of crystals provided by Sigma-Aldrich was electrospun into nanofibrous membranes. PVA with high molecular weight is useful in preparing gel which possesses both high strength and modulus. Hence, it is used to produce fibers, films or gels. The specific hydrolysis grade (87% to 89%) was chosen, as in that range, optimum water solubility occurs.

Blend electrospinning of nanofibers: After preliminary experiments an optimum polymer blend preparation and electrospinning setup was followed. According to this, PVA (10% w/v) was dissolved in distilled water at 85°C to 90°C for 3 hours. Afterwards, the solution was cooled to room temperature and stayed overnight under constant stirring. Prior to electrospinning, the calcein-containing liposomes were mixed with the PVA solution (300 μl/ 5 ml). Lipid concentration was 1.93 mg for the [DSPC/Chol] liposomes and 1.75 mg for the [PC] liposomes. The electrospinning process was carried out on a custom made electrospinning apparatus. The PVA mixture was loaded into a syringe equipped with a stainless steel 20 G blunt tip needle and the solution feed was controlled, at a feeding rate of 0.25 ml/h, using a syringe pump (Model: NE-1000, New Era Pump Systems Inc). A voltage of 14 kV DC from a high-voltage source (Spellman SL 300 DC power supply unit) was applied between the positive electrode connected needle tip and a vertical aluminum plate, served as the grounded collector. The needle to collector distance was set at 12 cm. The electrospun polymer solution was guided horizontal from the needle tip due to the repulsion forces applied from the high electrical field and after separation in nano fibers into a Taylor's cone was deposited on the grounded collector as a nano fiber membrane. A schematic representation of the electrospinning of drug-containing nanofibers as well as the electrospinning apparatus utilized is depicted in Figure 1.

Morphology characterization of liposome-enriched nanofibers: After some hours for the completion of drying the nano fiber membranes were gently removed from the collector surface. Samples from the PVA scaffolds were cut in rectangular form, sputter-coated with gold and observed in a Scanning Electron Microscope (JEOL 6300, JEOL) at the Laboratory of Electron Microscopy and Microanalysis (LEMM) of University of Patras. The mean diameter of the fibers was measured using the scientific software Image J (version 1.51 n, National Institutes of Health, USA), and their average value was reported along with the standard deviation.

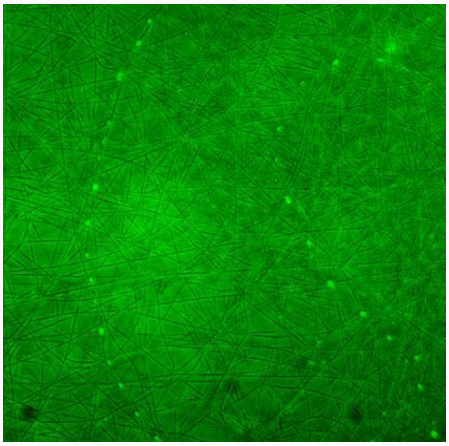

Figure 3: Visualization of the liposome-containing [DSPC/Chol] nanofibers using Confocal Laser Scanning Microscopy. A similar view is obtained for the [PC] liposome type.

Visualization of the drug-containing nanofibers was also conducted, by use of Confocal Laser Scanning Microscopy (CLSM) (Nikon Eclipse TE2000-U Inverted Microscope coupled with Nikon D-Eclipse C1 Lab Bench top Laser Scanning Confocal Microscope System). Calcein, which was encapsulated into the liposomes, acted as a fluorescent probe and its presence confirmed that the liposomes had been successfully incorporated into the polymeric scaffolds.

Results and Discussion

Liposome size distribution and surface charge

The mean diameter (along with the Polydispersity Index (PI)), as well as the ζ-potential of the liposome types used in the present study are shown in Table 1. The vesicle mean diameter for the DSPC/Chol liposomes is almost twice the size of the PC liposomes, which is probably owed to the addition of Cholesterol in the DSPC vesicles. The Polydispersity Index (PI) (which ranges from 0-1) was calculated at 0.196 for the DSPC/Chol liposomes and 0.244 for the PC liposomes, respectively. Generally, these values increased with polydispersity and is an indication of the homogeneity of the vesicle size, thus, we can assume that homogeneity of the liposomes size has been achieved.

SEM images depict thin (Figure 2), homogeneous and continuous fibers without visible defects, in which the presence of ellipsoidal structures (possibly liposomes or liposomes agglomerations) is evident, for both liposome types considered. The average diameter of the fibers was found to be 265 nm ± 48 nm (n=50).

CLSM micrographs showed the punctuated signals of the fluorescent probe (calcein) (Figure 3), suggesting the existence of individual or agglomerations of liposomes (for both liposomal types examined) incorporated into the electrospun nano-fibers.

Liposome integrity measurements

Calcein latency (%) was used as a method to evaluate the presence of intact liposomes in the polymeric scaffolds, and thereby their stability during the electrospinning procedure. To this end,

Table 1: Mean diameter and ζ-potential values of liposomes.

Lipid Composition	Diameter (nm) (Mean ± SDEV, n=3)	ζ-Potential (mV) (Mean ± SDEV, n=3)
DSPC/Chol	120.2 ± 2.397 [PI: 0.196]	-3.15 ± 1.38
PC	67.76 ± 1.639 [PI: 0.244]	-3.11 ± 0.686

Table 2: Calcein latency (%) measurements from liposomes (initial) and liposome-containing scaffolds.

PVA	[DSPC/Chol]	[DSPC/Chol] + PVA	[PC]	[PC] + PVA
1.60 ± 0.28	87.70 ± 0.65	82.86 ± 4.15	83.86 ± 0.87	31.88 ± 3.45

we calculated the latency of the calcein released in the media from scaffolds incorporating both types of liposomes studied herein. Measurements from liposomes, prior to being electrospun from the PVA solution, were also made for comparative reasons as well as from pure PVA scaffolds (to be sure that there is no auto fluorescence in the background). A number of 3 samples were considered for each case. The results can be seen in Table 2.

The high calcein latency calculated for both liposome types prior to electrospinning indicates that most of the calcein present in the solution to be processed for the formation of the scaffolds is in the form of intact liposomes. Measurements from the [DSPC/Chol] liposome-containing scaffolds exhibited also a high latency value (82.86%), confirmed that a great percent of the present liposomes remained stable when incorporated into the nanofibers and that their integrity was not substantially affected by the procedure. This is something logical, as the addition of Chol in the DSPC liposomes results in substantial increase of the vesicle integrity; liposomes are more stable when Chol is included in their membrane [29]. However, this is not the case for the [PC] liposome-containing scaffolds where a low calcein latency value (31.88%) was observed. The physical instability of the specific liposome type seems to have been further affected by the electrospinning process and thus, the encapsulated calcein has been released from the vast majority of these liposomes.

Conclusion

The scope of the present work was to develop a tissue engineering polymeric scaffold based on PVA nanofibers, encapsulating liposomes as a drug delivery system. In this context PVA/Liposomes blend electro spinning was implemented and a successful incorporation of the liposomes into the nanofibers was achieved for both liposomal compositions considered. Liposome incorporation was confirmed by use of Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy. The stability of the liposomes embedded on the electrospun fibers, was determined fluorometrically by measuring the calcein retention in liposomes (Latency) before and after the addition of Triton X-100. The calcein latency for the DSPC/Chol liposomes indicated that drug-containing liposomes remained stable in the electrospun membranes, while for the PC liposomes the latency measured was significantly lower, which can be attributed to the physical instability of the specific lipid composition resulting in their substantial disruption due to the electrospinning procedure applied.

The results presented in the current study exhibit the potential of the above drug delivery system for use in various fields of tissue engineering and regenerative medicine.

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