Initial Study on Fibers and Coatings for the Fabrication of Bioscaffolds

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Scaffolds composed of a mixture of poly (L-lactic) acid (PLLA) and polyethylene glycol (PEG) biodegradable polymers were prepared by electrospinning. Three-dimensional scaffolds of highly porous non-woven fibers were produced for biomedical applications and coated with calcium phosphate for bone tissue engineering. A mixture (80/20) of PLLA/PEG was dissolved at 5.7%, 7%, 8% and 9% blend solution concentrations. The structure and morphology of the scaffolds were investigated by scanning electron microscopy. Average fiber diameters ranging from 600 nm to 800 nm were obtained as result of the change in viscosity. The low polymer concentration fibers were found to be flat with fused junctions between fibers. For high polymer concentrations fibers they were cylindrical with fibers overlaying each other. For samples deposited at 9% concentration, individual fibers contained pores on their surface with nanometric dimensions. In addition, thin films of calcium deficient hydroxyapatite were prepared by rf magnetron sputtering on silicon substrates heated to temperatures between 300-600°C. These results suggest that it is feasible to fabricate biopolymer scaffolds using methods combining electrospinning and sputtering techniques.

Key words: Biodegradable polymers, Scaffolds, Electrospinning, Hydroxyapatite, Sputtering

The natural scaffold for most tissues is the extracellular matrix (ECM), whose structure and morphology contribute greatly to the properties and function of each organ. The EMC contributes to the rigidity and tensile strength of bone, the resilience of cartilage, the flexibility and hydrostatic strength of blood vessels, and the elasticity of skin (1). ECM is composed of two main groups of macromolecules, proteoglycans and collagen that together form a composite-like structure. Fibrous collagens embedded in proteoglycans maintain structural and mechanical properties. The collagen is organized in a three dimensional network composed of collagen fibers that form hierarchical structures from nanometer scale multifibrils to macroscopic tissue architecture (2-3). The ideal dimensions of the building blocks of the scaffold should be on a similar scale than of those of natural ECM. Hence, a nano-structured porous scaffold with interconnective pores, a wide pore size distribution and large surface area is needed for cell in-growth in a three dimensional fashion. The structure of these scaffolds is expected to promote cell adhesion, maintain cell functions and organize their growth. For bone generation, for example, pore sizes between 100 and 300 μm and porosities of more than 90% are preferred (4). The ideal scaffold should also have a sufficient mass of seeded cells, and these cells should be uniformly distributed throughout the entire scaffold. Other transport issues, including nutrient delivery, waste removal, and exclusion of material or cells, and protein transport are also affected by the pore size distribution and hydrophobicity of the scaffold. In addition, scaffolds should have the mechanical supportive properties while at the same time guiding cell differentiation and function.

A simple method to produce scaffolds formed by nanostructures in the shape of nano-micro fibers that are exceptionally long in length and uniform in diameter is electrospinning. This technique is based on the uniaxial stretching of a viscoelastic polymer solution through the action of electrostatic forces acting on charges in the solution when exposed to an external electric field. Under the action of this external electric field, the polymer-based jet continuously stretches and reduces its diameter due to the repulsion between the charges and the evaporation of the solvent. A fiber is formed with long length, and uniform diameter. Attracted to a grounded collector the charged fibers are usually deposited as a randomly oriented, non-woven mat. The morphology and diameter of the electrospun fibers are dependent on a number of

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parameters such as the properties of the polymer and solvent, the strength of the applied electric field, the distance between the spinneret and the collector, and the feeding rate of the polymer solution (5). Scaffolds produced by electrospinning of polymer solutions also allow for the control of specific cell binding interactions, control of degradation rate, the release of cell growth factors included in the solution and the use of polymers that can respond to environmental cues (6).

A number of natural and synthetic polymers are currently being employed as tissue scaffolds. Most synthetic biodegradable material such as polyglycolides (PGA) and polylactides (PLLA) can provide the necessary strength for structural stability. PLLA is one of the most widely used biomedical polymers owing to its biodegradability and biocompatibility. However, its application has been limited because of its stiffness and hydrophobicity. One method to improve the hydrophilicity of PLLA is to modify its bulk properties by adding Poly (ethylene glycol) (PEG) to the electrospinning solution. In a recent work, Bhattarai et.al., was able to demonstrate that adding a small fraction of low molecular weight PEG to the PLLA electrospinning solution improves the hydrophilicity of the scaffolds. A mixture of PLLA/PEG (80/20) was the best candidate amongst different ratios tested (7).

In the case of scaffolds for bone growth, coating the polymers with hydroxyapatite (HA) is expected to improve the scaffold's osteoconductive or bone formation ability. Hydroxyapatite (HA), the main constituent of bone and teeth, is a calcium phosphate (CaP) material in the apatite crystalline phase and shows the best bioactivity among the CaP ceramics (8). The crystalline phase hydroxyapatite shows excellent biointegration with no fibrous connective tissue formation, low tendency to evoke cytotoxic responses, and good osteoconductivity (9-10). The most important biomedical applications of HA are its use as a bone graft substitute in dental and orthopedic applications and as a coating of biomedical implants (11). HA coatings have been prepared using different techniques including plasma spray, laser ablation, sol-gel methods, dual ion beam, and sputtering, amongst others. Plasma spray is the most common commercial technique used to coat medical implants with HA (12). Unfortunately, the coatings have some drawbacks that limit their use (13). Problems associated with this technique are the mechanical weakness of HA-substrate interface resulting in delamination and debris formation, non-uniformity of the coating requiring use of thicker films, several crystalline calcium phosphates phases present simultaneously resulting in poor temporal retention of coating characteristics, occlusion of the porous substrate surface, and resorption of the HA coating due to poor crystalline properties (14). Sputtering techniques have the advantage of producing high coating density and the ability to coat difficult geometries (15). Wolke and co-workers deposited HA using magnetron sputtering producing thin films with good uniformity and density and found that the coatings had crystallographic texture (001). In vitro tests showed that the dissolution of the coating was determined by its crystallinity (13). The effect of substrate temperature on the crystallinity of sputter deposited HA films was studied by Nelea V, et al. (16). For substrate temperatures below 300ºC the films were mostly amorphous while samples deposited at 550ºC contained mostly the crystalline HA phase. Coating by sputtering the micro-nano fibers also has the advantage of producing several morphological structures from ribbons to hollow tubes as demonstrated by Pantojas, et al. in a recent work (17).

Osteoblasts are anchorage-dependent cells and it is expected that the high surface area and porosity of electrospun fibers might aid attachment and migration of cells inside the scaffold. There have been recent efforts to combine biodegradable polymers with hydroxyapatite in scaffold constructs. Deng, et al., for example, used a mixture of PLLA and HA nanoparticles in the electrospinning solution for the formation of composite scaffolds (18). They observed a slower degradation rate as compared to pure PLLA as well as improved cell attachment and growth. Natural fibers such as collagen have also been electrospun with HA nanocrystals in solution resulting in the formation of uniform nanoparticle-filled fibers with the needle-like crystals oriented along the fibers (19).

In this work, scaffolds of PLLA/PEG (80/20) mixtures were prepared by electrospinning with the aim of producing a dense mat with excellent pore size distribution and mechanical properties to be used in biological implants. The characteristics/properties of the scaffolds are dependent on the process used for their synthesis. A clear understanding is necessary of the parameters controlling the electrospinning process, and how they affect fiber diameter, pore size distribution and microstructure. It is also expected that controlling the individual fiber nanoscale features will result in the formation of materials with superior physical and chemical properties. Fiber mats prepared at different solvent concentrations were characterized in terms of fiber diameter and surface microstructure. Variations in solvent concentration produce changes in the viscosity of the mixture and affect the resulting fiber diameter and morphology in the electrospinning process.

Present efforts in the deposition of HA on silicon substrates using the sputtering technique are also reported. The properties of the film will depend on the deposition

parameters which have to be optimized (20). The long term goal is to combine electrospinning and sputtering techniques with the aim of producing biopolymer scaffolds covered with HA nanocrystals.

Materials and Methods

Scaffolds of the 80/20 PLLA/PEG polymer mixture were fabricated by electrospinning under optimum conditions. Polymer solutions were prepared by dissolving 0.2162 g of PLLA (Mw = 152,000 g/mol) and 0.0547 g of PEG (Mw = 10,000 g/mol) onto dichloromethane/chloroform (50:50) at concentrations of 5.7%, 7%, 8%, 9% w/w. From each concentration 0.2 ml was fed into a 10 ml plastic syringe, which was controlled by a pump at a feeding rate of 0.6 ml/hr. A vertical electrospinning system was used since it improves the deposition rate and minimizes the loss of material. The distance between the syringe needle and collector was 18 cm. A high voltage of 16 kV was applied by a voltage regulated DC power supply (Gamma Research) to generate the polymer jet. The resulting fibers were collected on a previously cleaned undoped silicon wafer.

Calcium phosphate thin films were deposited by rf magnetron sputtering. The deposition systems used cryo or turbomolecular pumps to maintain background pressures below 6.6 x 10-5 Pa. A commercial hydroxyapatite target was mounted on the water cooled magnetron gun and sputtered at a power of 100 W and in a 100% argon gas atmosphere. The substrate holder was facing the target at a distance of 10 cm. The substrate holder also had the capability to heat the substrate to temperatures over 1,000°C if desired. The temperature at the substrate position was calibrated using two thermocouples, one inside the heater casing and the other at the substrate position.

A JEOL JSM-6360 scanning electron microscope (SEM) was used to analyze the as-spun and coated fibers. Micrographs were taken at random areas at low, medium and high magnifications and at different locations to verify that they represent the morphology of each sample. The diameters of the fibers were measured from the micrographs using commercial image analysis software. An energy dispersive spectrometer (EDS) attached to the SEM was used to obtain information about the sample composition. A voltage over 7 kV was used to minimize the substrate interference as described elsewhere (21). X-ray diffraction (XRD) was performed on a Bruker Hi-star System with a General Area Detector. The sample angle was set at $\omega=20°$ while the detector angle was set at $2\theta=45°$. The two dimensional diffraction frames were integrated to obtain the one-dimensional diffractogram.

Results

Electrospinning

Figure 1 represents a typical electrospun mat prepared by electrospinning. Fibers are deposited in a non-woven dense mat with voids between fibers producing the porosity of the scaffold. Fiber mats can be deposited over large areas with a thickness that can be controlled by the amount of deposited material. Fibers are long, continuous and without the formation of beads which are typical of solutions with very low polymer concentration. The diameters of the fibers obtained at different polymer concentrations are given in Table 1. Average fiber diameters range from 650 to 830 nm with a slight increase as a function of polymer concentration.

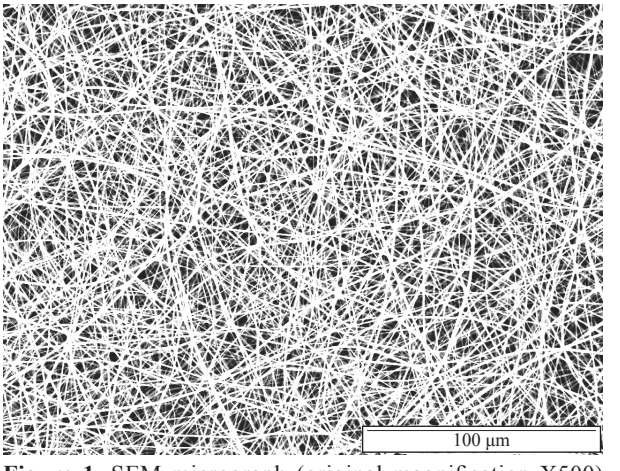

Figure 1. SEM micrograph (original magnification X500) of a typical electrospun scaffold composed of an 80/20 mixture of PLLA/PEG showing the randomly oriented fibers composition.

Table 1.

Blend Solution Concentration (%w/w)	Fiber Diameter (nm)
5.7	680 ± 200
7.0	650 ± 300
8.0	740 ± 360
9.0	830 ± 220

Figure 2 shows a SEM micrograph of the fiber mats produced at different polymer concentrations in the electrospinning solution. For low polymer concentration (5.7% w/w) fibers were flat with a curved and bent structure (Figure 2A). Relatively large voids with a range of sizes are observed between fibers. Also, individual fibers appear to fuse at their bonding points with other fibers indicating

that the fibers were still wet when they reached the collector, thus producing fused junctions and acquiring the flat shape of the substrate. This fused junction morphology can be beneficial to the mechanical integrity of the scaffold as it can provide reinforcement to individual fibers. For slightly higher polymer concentration, 7% w/w, fibers are straighter but the flat shape and fused junction morphology are maintained (Figure 2B). Mats produced at 8% w/w, Figure 2C, also show straight fibers but individual fibers overlap each other instead of fusing at their junction. In addition, some degree of fiber alignment is present.

Finally, for high polymer concentration (9%), figure 2D, cylindrical shaped fibers with a porous surface are obtained. At this polymer concentration the fibers solidify before reaching the substrate and maintain their cylindrical shape. Fibers produced by electrospinning usually have a smooth surface but under certain deposition conditions surface features may appear. The formation of pores in this system has been related to the rapid phase separation during electrospinning where the solvent rich regions apparently transformed into surface pores after evaporation (22). The average pore size along the fiber axis is 5.44 ±0.82 μm while perpendicular to the fiber axis is 2.59±0.82 μm.

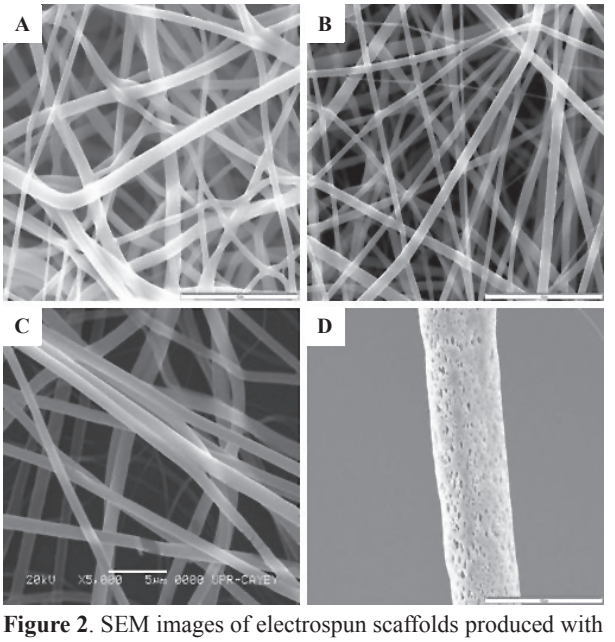

Figure 2. SEM images of electrospun scaffolds produced with different polymer concentration; A: 5.7% (x5000, 10μm bar), B: 7% (x5000, 10μm bar), C: 8% (x2000, 10μm bar), D: 9% (x500, 100μm bar). The produced morphologies included flat curved fibers with fused bonding points (A), flat straight fibers with fused bonding points (B), flat straight fibers without fused bonding points (C) and cylindrically shaped fibers without fused bonding points at intersections (D).

Sputtering

Thin films composed of CaP were deposited by sputtering on top of silicon in order to analyze its composition, crystallinity and morphology. Previous results already reported in literature showed that it is possible to deposit HA films by sputtering without the necessity of an external heat treatment to induce the growth of the apatite crystals (21). The apatite phase was obtained by in-situ heating during the deposition of the film at 600°C but the crystallinity was poor as observed by XRD. A magnetron gun with high strength permanent magnets was added to the system to increase the plasma ion density and, consequently, the deposition rate. Several samples were deposited at a pressure of 1.33 Pa, for two hours, and at a substrate temperature ranging from room temperature to 600°C. The diffractogram for samples prepared with substrate temperatures below 400°C did not show sharp peaks indicating that the films were amorphous. Figure 3 shows the diffractogram for samples deposited at substrate temperatures of 400, 500 and 600°C. For the sample deposited at 400°C the high intensity peaks of the HA crystalline phase are present and the peak intensities indicate partial (002) texture, typical of hexagonal structures. The small number of counts and peak broadening suggests nanocrystalline sizes that are typically formed in low mobility processes such as sputtering. Deposition at higher temperatures and direct observation of crystals by Transmission Electron Microscopy are needed to confirm these results.

Figure 4 shows the EDS spectrum typical of CaP samples prepared by sputtering. The silicon peak is produced by the substrate. The ratio of calcium to phosphorous is calculated from the peak intensities and plotted as a function of substrate temperature as shown in Figure 5. Excess calcium is observed when compared to the Ca/P ratio of 1.67 for the stoichiometric crystalline HA. This excess could be the result of different sputtering rates for Ca and P at the target and, consequently, can be modified by changing plasma process parameters such as deposition pressure and sputtering power. It could also depend on the history of target processing that alters the target stoichiometry if sputtering rates are not identical for the different components.

Discussion

Electrospinning is a technique which produces extremely fine fibers and is easy to implement in a laboratory. This technique can be used to fabricate scaffolds with fiber diameters ranging from several microns down to several hundred nanometers. Pores in the structure are formed by the space between differently oriented fibers lying on top

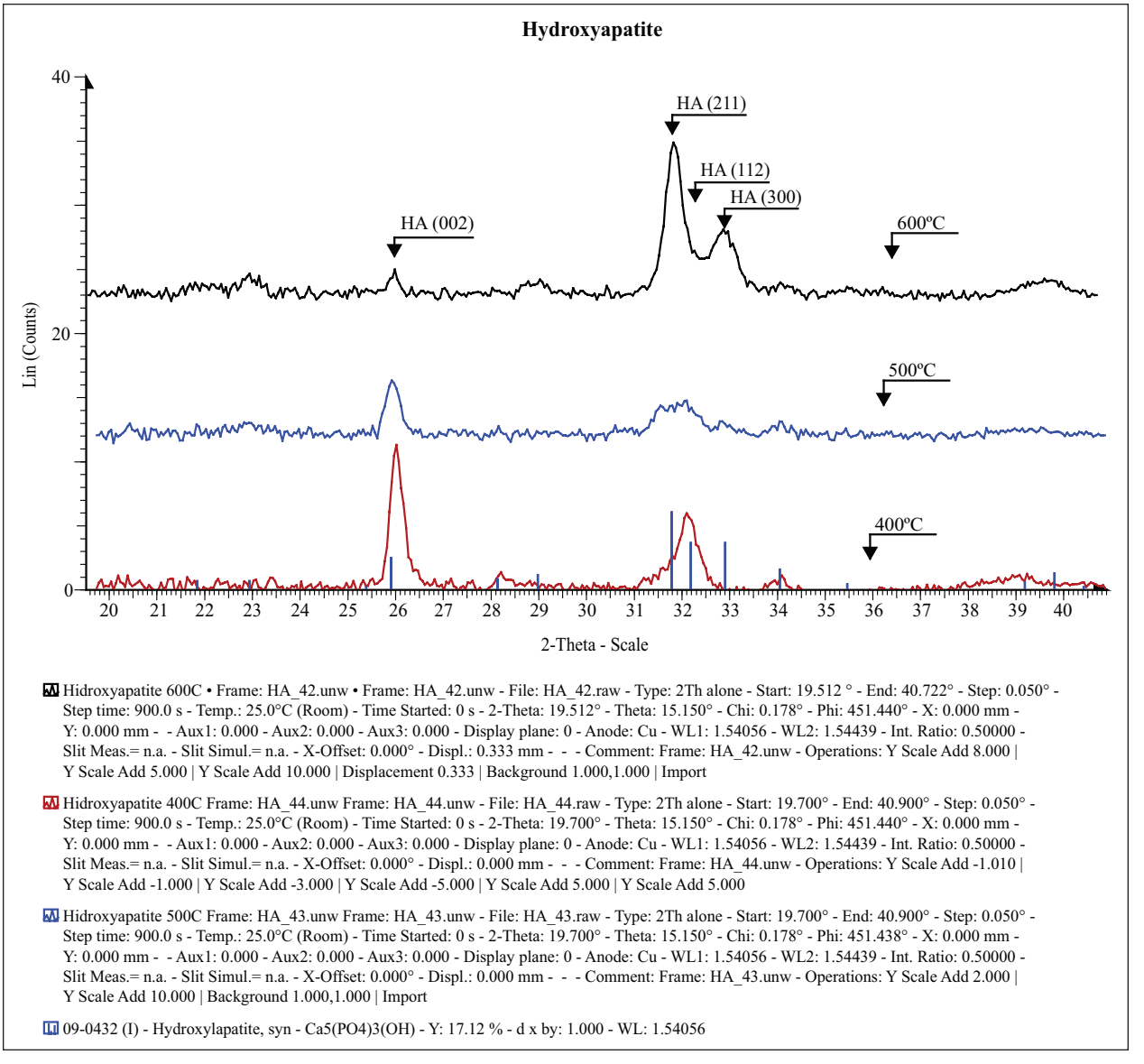

Figure 3. XRD of samples deposited at substrate temperatures of 400°C, 500°C and 600°C. The markers on top of the 2-Theta-Scale correspond to the peak positions and relative intensities from HA 09-0432 standard card.

of each other. For cell ingrowth the range of required pores sizes will depend on the type of cell but, for the majority, the pore diameter is in the range of 25 to100 μm. Pore sizes for the scaffold prepared at 7%, for example, has pore sizes ranging from 12 μm to 800 nm. An advantage of using scaffolds with cm long fibers for cell growth is that, even if the pores are too small for cell migration, the fiber structure allows the cells to push the surrounding fibers aside to expand the holes since the small fibers offer little resistance to cell movement.

The viscosity of the solution, as controlled by changing the polymer concentration, has been found to be one of the biggest determiners of fiber size and morphology. Only a small variation in fiber diameter is observed in our samples suggesting that, to better control size another deposition parameter, such as the feeding rate or the type of solvent has to be explored. In general, fibers produced by electrospinning solutions with low polymer concentration or low viscosity are observed to be flat with fused junction between fibers. This fused junction morphology can improve the mechanical stability and structural integrity of the scaffolds. This is an important factor in its design since it is expected to provide biomechanical support during the process of tissue regeneration and structure degradation.

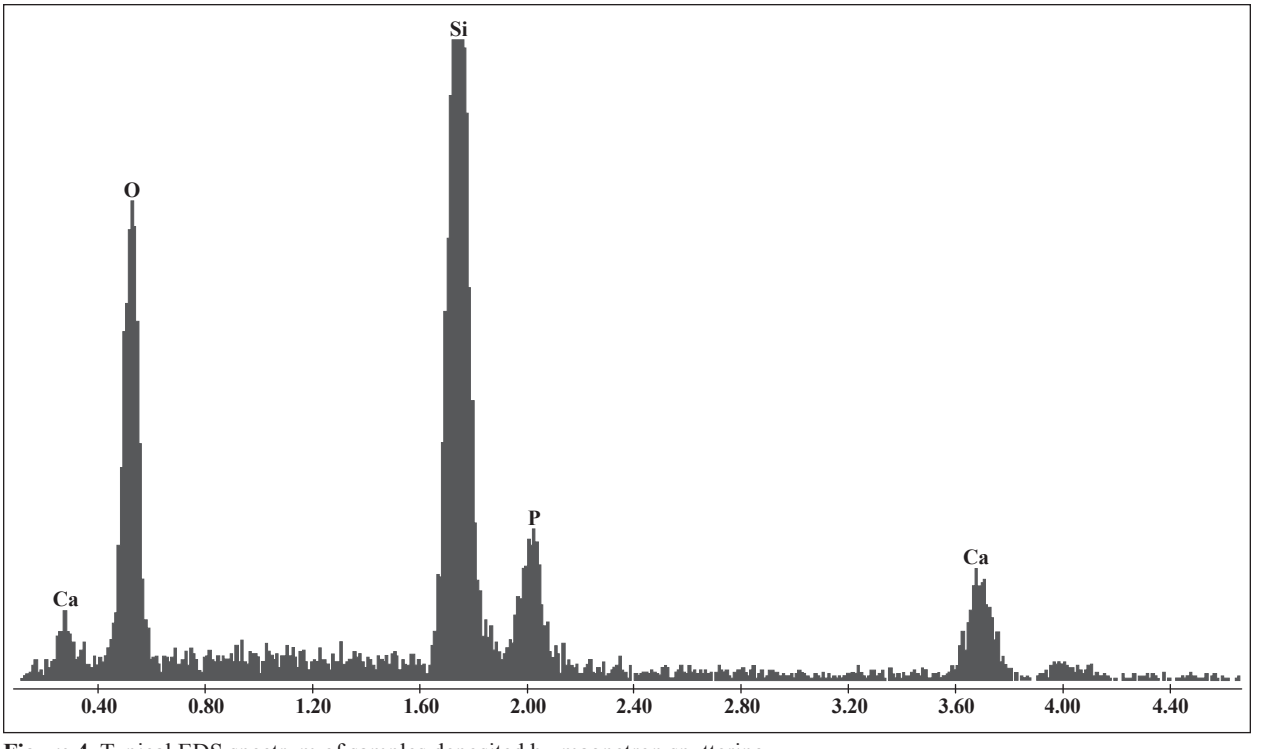

Figure 4. Typical EDS spectrum of samples deposited by magnetron sputtering.

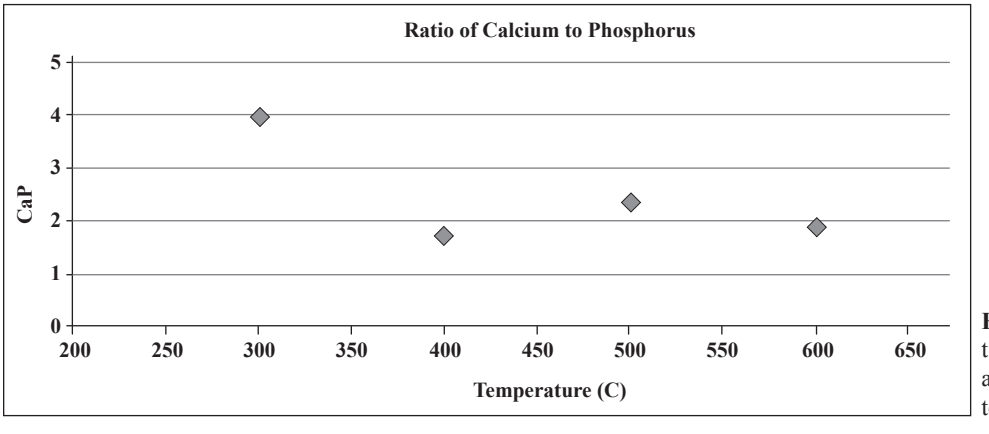

Figure 5. Plot of calcium to phosphorous ratio as a function of substrate temperature.

Fibers from solutions with high polymer concentration or high viscosity have cylindrical shapes and overlapped with each other instead of fusing at their junctions. Surface features can also be controlled by the selection of polymer concentration as porous fibers are formed when phase separation occurs during the electrospinning process. For tissue engineering it may advantageous if the surface is porous instead of smooth. Surface pores, for example, can function as anchoring points for cells, modifying the wetting properties, increasing surface area, or influencing the kinetics of biodegradation of the scaffold. It is possible to generate pores during the electrospinning process by choosing particular solvents or solvent mixtures, by varying the humidity, or by using polymer mixtures (23).

Electrospinning also allows for the alignment of the scaffold's fibers through a number of possible modifications to the system including the use of a rotating drum as collector and the application of designed voltages to avoid the fiber whipping effect. Fiber alignment is desirable in applications where cell growth guidance is desired as in the case of neural cells. As cells move across

a surface, physical and chemical cues can be used to cause the cells to preferentially adhere in a given direction. Physical cues, e.g. microchannels, are a standard technique for aligning cells in the field of tissue engineering. Fabrication of these surface microchannels by techniques such as electron beam lithography are time consuming, requires expensive equipment and the surface coverage is small. Electrospinning may provide a vast improvement over the current microscale alignment methods by moving tissue engineering into the nanoscale domain.

Meanwhile, deposition of calcium phosphate by sputtering appears to be a promising process for coating biomedical implants. The preliminary results demonstrated in this article point to the formation of a HA coating on top of a suitable implant material, e.g. titanium, by in-situ heating. The methodology for coating the biopolymer scaffolds requires sputtering the calcium phosphate without substrate heating to prevent the thermal destruction of the scaffold. After deposition, the coated scaffold can be heated in air to promote the growth of HA nanocrystals.

Resumen

Matrices extracelulares compuestas de una mezcla de dos polímeros biodegradables, ácido poli láctico (PLLA) y polietileno-glicol (PEG), fueron preparados por "electrospinning". Matrices tridimensionales de fibras entrelazadas para aplicaciones en biomedicina fueron formadas. Una mezcla (80/20) de PLLA/PEG fue disuelta a concentraciones de 5.7%, 7%, 8% y 9 % en solución para electrospinning. La estructura y morfología de las matrices fue investigada con microscopia electrónica de barrido. Matrices compuestas de fibras con diámetros desde 600 nm hasta 800 nm fueron obtenidas al variar la viscosidad de la solución. Para bajas concentración de polímero, las fibras son planas con las intersecciones entre fibras fusionadas. Para altas concentraciones de polímero, las fibras son cilíndricas con las fibras sobrepuestas. Para las matrices formadas a una concentración de 9% las fibras individuales contienen poros nanométricos en su superficie. En el caso de aplicaciones en la formación de huesos, películas finas de hidroxiapatita fueron preparadas por "magnetron sputtering" de radiofrecuencias sobre substratos de Silicio calentados a temperaturas entre 300-600 °C. Estos resultados sugieren que es posible fabricar matrices de biopolímeros mediante la combinación de las técnicas de "electrospinning" y "sputtering".

Abbreviations and acronyms

PLLA = poly(L-lactic) acid
PEG = polyethylene glycol
ECM = extracellular matrix
PGA = polyglycolides
HA = hydroxyapatite
CaP = calcium phosphate
SEM = scanning electron microscope
EDS = energy dispersive spectrometer
XRD = X-ray diffraction

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