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Effect of halloysite nanotube structure on physical, chemical, structural and biological properties of elastic polycaprolactone/gelatin nanofibers for wound healing applications

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ABSTRACT

Nanofibrous elastic material based on the blend of hydrophobic poly(ε-caprolactone) (PCL) and hydrophilic gelatin (Gel) reinforced with halloysite nanotubes (HNTs) was prepared by electrospinning process by respecting principles of "green chemistry" required for tissue engineering and drug delivery carriers. Three different kinds of HNTs with similar aspect ratio, but different length and inner diameter were examined to explain the effect of HNT concentration and geometry on a structure, morphology, chemical composition, mechanical properties and biocompatibility of nanostructured materials. Reinforcing effect of each type of HNTs has been confirmed up to 6 wt%. However, the highest improvement of mechanical properties was exhibited by addition just 0.5 wt% of HNTs. All HNT modified nanofibers have been confirmed as non-cytotoxic based on the interaction with mouse fibroblasts NIH-3T3 cells and therefore suitable for biomedical applications, e.g. as wound healing coverings with controlled drug delivery.

1. Introduction

Controlled drug delivery system represents multidisciplinary scientific approach to contributing to human health care [1]. Drugs can be loaded into or on the surface of drug carriers. Among the promising drug carriers, nanofibers due to their unique structure can be included nanofibers [2]. Biodegradable electrospun nanofibers can be prepared from natural (e.g. collagen, gelatin, chitosan, elastin, hyaluronan or cellulose derivate) or synthetic polymers (e.g. polycaprolactone, polylactide acid, poly(lactic-*co*-glycolic acid), polyethylene glycol or polyvinyl alcohol) or their blends [3]. Polycaprolactone (PCL) is a synthetic, biodegradable, bioresorbable, semi-crystalline, non-cytotoxic and hydrophobic polymer belonging to polyesters. The PCL is a highly desirable candidate for medical applications by reason of its slow degradation, biocompatibility and FDA approval [4]. Due to good mechanical properties and because of its compatibility with a wide range of drugs, PCL is largely investigated for controlled drug delivery system [5]. However, hydrophobic behavior of PCL carriers often results in a low cell adhesion and proliferation and suffers by slow biodegradation rate [6]. Contrary, highly hydrophilic gelatin (Gel) is natural protein exhibiting many outstanding properties, such as its biological origin, biocompatibility and biodegradability [7]. In a literature, there are many studies about the preparation of nanofibers from PCL/Gel polymer mixture [8–15]. However, in these works fluorinated solvents, which are generally classified as toxic and irritant were used. Especially, often mentioned 2,2,2-trifluoroethanol solvent has been found toxic for blood system [16]. There are only few papers engaging in electrospinning of PCL/Gel from alternative solvents, e.g. Gautam et al. [17] published solvent system containing a chloroform/methanol mixture for PCL and acetic acid for gelatin. Respecting principles of "green chemistry" as requirements of tissue engineering, it is necessary to use non-toxic solvents.

Halloysite nanotubes (HNTs) are a two-layered aluminosilicate material with empirical chemical formula $Al_2Si_2O_5(OH)\cdot nH_2O$ [18]. For polymer nanocomposites, the most important is tubular halloysite with typical dimensions: external diameter of 50–80 nm, cylindrical pore between 10 and 15 nm (called lumen), length of about 1 μm and aspect ratio (ratio of HNT length to HNT diameter) in the range of 1–50 [19]. Due to the high aspect ratio HNTs are excellent reinforcement materials [20,21].

HNTs have been found as environmental friendly, biocompatible for a range of microbial, cell culture and animal models, non-toxic and

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inexpensive clay nanomaterial [22–24]. HNTs are safe if added to cell cultures, microworms or infusoria at the concentration of up to 0.5 mg·mL^{-1}, which can be removed from an organism with macrophages [25–28]. Due to their elongated shape and different chemical properties on the surface and in the cylindrical lumen, natural HNTs can be loaded with a number of chemically and biologically active substances and/or drugs [29–42]. Up to date, numerous polymer nanofibers including HNTs for various applications, from conventional reinforcements to drug delivery nanocarriers, were prepared [43–50]. To summarize, there is no versatile type of natural HNTs allowing applications as both reinforcement and nanocarrier. Thus, the selection of HNT structure and geometry primarily depends on specific application. Whilst HNTs with the highest aspect ratio and uniform tubular shape are the most appropriate for improving mechanical properties, HNT types with the highest inner diameter and absence of any impurities are most suitable as nanocarriers for medical applications.

In our recent work, it has been found that using only 0.5 wt% HNTs has achieved exceptional improvement of mechanical properties. Moreover, we revealed that HNTs significantly influenced the morphology of nanofibers, their mechanic properties and changes in thermal stability [51,52]. In the present study, we thoroughly investigated the effect of three different types of HNTs on the structure, morphology, chemical composition, mechanical properties and hydrolytic stability of electrospun PCL/Gel nanofibers. In vitro proliferation test and live/dead assay using fluorescence microscopy on NIH-3T3 fibroblasts were also performed to know the compatibility of nanofibers with mammalian cells. The selection of HNT type as well as organic solvent for electrospinning process was guided by potential application of PCL/Gel/HNTs nanofibers as a drug delivery system. Based on these criteria three types of HNTs (New Zealand – HNT NZ, Slovakia – HNT SK and Sigma-Aldrich – HNT SA) with similar aspect ratio (in the range of 3.9–5.3) but different length (in the range of 200–1200 nm) and inner diameter (in the range of 20–100 nm) have been used for nanofiber preparation.

2. Experimental procedure

2.1. Materials

Gelatin (Gel - Type B, BioReagent, powder from bovine skin) and polycaprolactone (PCL - M_n = 80 kDa) were purchased from Sigma Aldrich. Acetic acid (99% p.a.) was obtained from Penta s.r.o, Czech Republic. The tubular halloysite from three different deposits has been used. Halloysite Premium (HNT NZ) was received from Imerys Tableware Asia Ltd., New Zealand; Halloysite from Biela Hora in Slovakia (HNT SK) was grounded into powder using a laboratory ball mill. Next the powdered halloysite was purified and sieved. Third halloysite (HNT SA) was sourced from Sigma Aldrich.

2.2. Fabrication of electrospun mats

PCL/Gel solution was prepared by adding calculated amount of PCL (6 wt%) and Gel (6 wt%), weight ratio 1:1, into the 25 mL of acetic acid. PCL/Gel/HNTs mixtures were obtained by the addition of different amount of HNTs (0.5, 1.0, 3.0, 6.0 and 9.0 wt%) to the PCL/Gel solution. Then, prepared mixtures were stirred overnight. Because of strong van der Waals force among HNTs resulting in bundles, it was crucial to homogenously disperse HNTs in liquid polymer matrix to acquire satisfactory dispersion in final nanofibers. Therefore, immediately before the electrospinning process, mixtures were homogenized in ultrasound bath for 15 min. Electrospun mats were made on the laboratory machine Nanospider™ NS LAB 500S (Elmarco, Czech Republic) at room temperature and relative humidity of 60% using rotary spinning electrode with the speed of 6 rpm. The distance between spinning and collecting electrode was kept at 150 mm. Nanospider™ technology is based on needle-free, high voltage (up to 80 kV) and free liquid surface electrospinning process producing uniform non-woven nanofiber mats [53].

2.3. Characterization

Nanofiber morphology was investigated employing scanning electron microscope (SEM) Tescan MIRA3 (Tescan, Czech Republic). If not specified otherwise, all observations were made in the secondary electron emission mode at 10 kV acceleration voltage. For better resolution, nanofibers were coated with a 10 nm gold layer. The surface elemental analysis was determined by EDX Spectroscopy (Oxford Instruments) and evaluated by Aztec 2.1a software. Fiber diameter was carried out using ImageJ software. One analysis consisted of 50 measured values in random position in a SEM image.

Aspect ratio (ξ) of HNTs was calculated using Eq. (1). Where l is HNT length and d is HNT diameter.

$$\text{Aspect ratio } \xi = l/d \tag{1}$$

Attenuated total reflectance Fourier-transformed infrared spectroscopy (ATR-FTIR) was applied for the chemical composition characterization of obtained mats. ATR-FTIR spectra were measured employing Bruker Vertex80V (Bruker Optics) spectrometer using diamond ATR-crystal. Each spectrum consists of 128 scans in the range between 4000 and 650 cm^{-1}. Obtained spectra were analyzed by OPUS software (version 6.5) using “rubber-band” baseline correction and normalization at diamond absorbance (2300–1850 cm^{-1}).

Swelling ratio of 1 × 1 cm nanofiber specimens was gravimetrically evaluated in ultrapure water at laboratory temperature. In regular time interval (1, 3, 5, 10, 15, 20, 30, 45, 60, 150 and 180 min) swollen pieces were removed, the excess surface water was quickly swiped out by blotting paper and reweighed. The water uptake (WU) of nanofibers was defined according to the Eq. (2), where w_s is the weight of swollen sample at the given time and w_d is the weight of dry sample. The resulting value is an average of 5 measurements.

$$\text{WU}[\%] = [(w_s - w_d)/w_d] * 100 \tag{2}$$

Mechanical properties of prepared nanofibers were evaluated by Zwick/Roell-Z010 test instrument (Zwick/Roell, Germany) with a 10 N load cell at crosshead speed of 5 mm·min^{-1} at ambient temperature (20 °C). All samples were cut into rectangles with dimensions of 30 mm × 5 mm. At least six samples were tested for each type of nanofibers and data were averaged to get the standard deviation. The thickness of samples was measured with a micrometer (Prominent s.r.o., Czech Republic).

NIH-3 T3 mouse fibroblasts (ECACC, UK) were maintained in Dulbecco's Modified Essential Medium (Sigma Aldrich) supplemented with 10% fetal bovine serum, 2 mmol·L^{-1} L-glutamine and penicillin/streptomycin (100 U·mL^{-1} and 100 U·mL^{-1}, respectively) at 37 °C under 5% CO_2 atmosphere. Cells were harvested by trypsinization in 0.25% trypsin-EDTA (0.25% (w/v) trypsin)/(0.02% EDTA in PBS, pH = 7.4). Before the cell proliferation testing, selected nanofibers were fitted and placed on the bottom of 48 well plate, sterilized under the UV for 10 min and kept for one hour in cell culture medium. The medium was then removed and fibroblasts were seeded on nanofiber samples at cell density of 5000/well. Cell proliferation was quantified at 1, 3, 7 and 14 days using the MTT proliferation assay (Sigma Aldrich) according to the manufacturer's instructions. Briefly, cells were incubated at the appropriate time and then gently washed twice with pre-heated PBS. The mixture of 150 μL of culture medium and 50 μL of MTT (5 mg·mL^{-1} in PBS, pH 7.4) was added into each well containing samples. After 4 h, medium with MTT was removed followed by the addition of 10% sodium dodecyl sulfate to solubilize the crystals of produced formazan by cells. 100 μL of solutions from each well were transferred into a new 96-well plate and the absorbance was measured at 570 nm with the microplate spectrophotometer (Beckman Coulter Paradigm). In addition, live/dead staining was performed. The samples