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Development of material with strong antimicrobial activity by high pressure CO_2 impregnation of polyamide nanofibers with thymol

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ABSTRACT

This study discusses the potential of high pressure (supercritical and liquid) CO_2 impregnation to load electrospun polyamide nanofibers with thymol as an antimicrobial agent. The influence of selected pressures and temperatures on the thymol impregnation yield has been evaluated. High pressure impregnation provided superior thymol impregnation yields (up to ~60%) on nanofibers compared to conventional immersion methods (~2%). The presence of thymol in impregnated samples was confirmed by FT-IR analysis. FESEM analysis revealed that impregnation with thymol resulted in a swelling of the nanofibers. The nanofiber morphological changes strongly depended on the thymol impregnation yield. Nanofibers impregnated with thymol using high pressure CO_2 ensured excellent antimicrobial activity against Gram-negative bacterium *E. coli*, Gram-positive bacterium *S. aureus* and fungus *C. albicans*. In addition, the Higuchi release model well fitted the thymol release studies.

1. Introduction

Nanofibers produced by electrospinning have a great potential for various biomedical applications [1,2]. Due to a large surface-to-volume ratio, high porosity and good pore interconnectivity electrospun nanofibrous materials can be exploited for wound dressings, drug delivery materials, artificial blood vessels, scaffolds for tissue engineering as well as for substrates for enzymes and bioactive compounds immobilization [1]. Nanofibrous material for specific applications can be tailored by selecting the appropriate components (polymers, biopolymers, drugs etc.), components ratio and electrospinning processes parameters (voltage, humidity, distance between the spinneret and collecting plate, polymer solution viscosity, etc.) or by a physical and chemical modification after the electrospinning process [1,3–8].

Commonly, an immersion method is used for the immobilization of different compounds onto/into polymer substrates. Major drawbacks of this methodology such as the use of harmful organic solvents, uneven distribution of impregnate and low incorporation yields can be overcome by using a supercritical solvent impregnation (SSI) method [9,10]. In many cases both supercritical carbon dioxide ($scCO_2$) and liquid CO_2 may successfully replace conventional organic solvents due to the high solubility and diffusivity, low cost, environmental friendliness and ability to induce a polymer swelling and plasticizing effect. Due to the near zero surface tension, $scCO_2$ easily penetrates the substrate providing an even distribution of the active component throughout the whole volume. At the same time, CO_2 leaves the polymer due to the depressurization of the system resulting in a material without solvent traces. In addition, the drying stage which is necessary in conventional impregnation processes is avoided [11]. The effects of CO_2 in liquid and supercritical state on various polymers have been well documented [11–19].

However, the research considering its impact on electrospun nanofibers is limited at this point. So far only a few studies reported on the synthesis of nanofibers in $scCO_2$ [20,21] and the impregnation of nanofibers with metal nanoparticles, active substances or dyes in high pressure CO_2 [22–27]. Ayodeji et al. investigated the impregnation of electrospun polycaprolactone fibers with dye Rhodamine B in liquid CO_2 [23]. Their aim was to obtain a material with prolonged release of the active substance. Taking into consideration the importance of polymeric fibrous scaffolds for biomedical applications they extended the research with a particular emphasis on the release profiles of test dye Rhodamine B from different electrospun blends (polycaprolactone

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(PCL)-gelatin, PCL-PMMA, PMMA-PCL-gelatin) treated in high pressure CO_2 [24–27] in order to get better insight into release kinetics of the dye which leached out from the electrospun mats.

It has been recently reported that polyamide 6 shows good biocompatibility with various human cells and tissues [28]. Due to its similarity in chemical structure and active groups to collagen protein, PA nanofibers have been studied for the applications in bone tissue engineering [4,28]. Shrestha et al. developed polyamide/chitosan electospun nanofibrous scaffolds for tissue engineering research [6]. The antimicrobial activity of polyamide 6 electrospun nanofibrous membrane loaded with different N-halamines was also studied [29].

Keeping in mind the growing need for medical textiles with antimicrobial properties, the advantages of nanofibrous mats over conventional wound dressings (high-surface area, porosity, flexibility, etc.) and the potential of SSI as a green technology, we focused our research on the possibility of fabrication of antimicrobial polyamide 6 nanofiber mats by high pressure CO_2 impregnation with thymol. Thymol is recognized as a natural antimicrobial agent, which provides antimicrobial activity against a broad spectrum of bacteria, yeasts and fungi [10,30]. The data on SSI of nanofibers with antimicrobial agents and particularly with natural one that can be utilized for wound management are scarce. To the best of our knowledge high pressure CO_2 impregnation of polyamide 6 nanofibers has not been studied yet. The efficiency of liquid CO_2 and $scCO_2$ impregnation of polyamide 6 nanofibers with thymol was compared with conventional immersion method. In addition, the $scCO_2$ impregnation of polyamide fabric was also studied. Chemical and morphological changes of polyamide 6 nanofibers were assessed by FTIR and FESEM analyses, respectively. Antimicrobial activity of investigated samples was tested against Gram-negative bacterium *E. coli*, Gram-positive bacterium *S. aureus* and fungus *C. albicans*.

2. Materials and methods

2.1. Materials

Polyamide 6 nanofibers (PA NFs, 38.5 g m^{-2}) were used as a substrate for thymol impregnation. Uniform PA 6 nanofibrous veils were produced by electrospinning on an in-house designed multinozzle system according to previous studies [31–33]. A linear lateral motion of the electrospinning nozzles perpendicular to the production direction guarantees the uniformity of the nanofibrous veils. A 1:1 solvent mixture of 98–100 v% formic acid (FA) and 98 v% acetic acid (AA) was used to spin a 16 wt% PA 6 solution. Both solvents and polymer were obtained from Sigma-Aldrich and used as received. The average diameter of the PA 6 nanofibres was 206 ± 29 nm.

Desized and bleached polyamide 6 fabric (PA, 150 g m^{-2}) was also studied. PA fabric was initially cleaned in a bath containing 0.5% nonionic washing agent Felosan RG-N (Bezema). Liquor-to-fabric ratio was 50:1. After 15 min of washing at 50 °C, the fabric was rinsed once with warm water (50 °C) for 3 min and three times (3 min) with cold water. The fabric was then dried at room temperature.

Thymol (purity > 99%) was supplied by Sigma-Aldrich Chemie GmbH (Germany). Commercial CO_2 (purity 99%) used as a fluid for impregnation experiments was purchased from Messer-Tehnogas, Serbia. Ethanol 96% (Sani-Chem, Serbia) was used as a solvent for thymol in conventional immersion experiments.

2.2. Impregnation using high pressure CO_2

Impregnation of PA NFs and PA fabric with thymol was performed by static method in a high-pressure view chamber (Eurotechnica GmbH, Germany) described elsewhere [30]. The experimental setup for a high pressure impregnation process is shown in Fig.1. PA NFs or PA fabrics were cut into pieces (1.5 cm × 3.0 cm). Thymol was placed in a glass recipient on the bottom of the chamber. The initial mass of thymol in the vessel was 1.00 g. The sample (PA NFs or PA fabric) was placed in the porous basket above thymol. In order to prevent thymol splashing during decompression, thymol and substrate were separated by a Teflon fabric. Experiments were performed with $scCO_2$ at a temperature of 35 °C and pressures of 10 and 20 MPa as well as with liquid CO_2 at 25 °C and 7 MPa. The temperature of 35 °C was chosen in order to keep solubility of thymol in $scCO_2$ as high as possible [30] at selected pressures of 10 and 20 MPa. At pressures higher than 20 MPa the partition coefficient of thymol (ratio of thymol concentration in the polymer and $scCO_2$ phase) may decrease due to its high affinity towards $scCO_2$ [34]. Namely, pressure increase leads to an increase of thymol solubility in $scCO_2$ [30] which may cause decrease of impregnation yields at higher pressures [34]. Operating time was varied in the range from 0.5 h to 4 h. Decompression rate applied at the end of each experiment was 1.4 MPa min^{-1}. All experiments were performed in triplicate. The mass of impregnated thymol (m_{th}) was determined gravimetrically by measuring the samples before and after impregnation. Thymol impregnation yield (I) of PA NFs and PA fabric was calculated according to Eq. (1):

$$I = \frac{m_{th}}{m_0 + m_{th}} \cdot 100(\%) \quad (1)$$

where m_0 is the initial mass of PA NFs or PA fabric.

The influence of impregnation on dimensional changes of PA NFs samples was determined by measuring their length and width before and after treatment. The measurements were performed in triplicate using electronic digital caliper (Womax, Germany) with accuracy ± 0.02 mm. Dimensional change (ΔS) was calculated according to Eq. (2):

$$\Delta S = \frac{S_I - S_0}{S_0} \cdot 100\% \quad (2)$$

where S_I is the surface of thymol impregnated sample and S_0 is the surface of the sample before impregnation.

2.3. Conventional immersion method

The samples of PA NFs and PA fabric (1.5 cm × 3.0 cm) were immersed in 20 mL of ethanol/water mixture (14:6) containing previously dissolved 0.181 g of thymol. After 30 min long immersion the samples were squeezed between rolls and dried at room temperature. The impregnation yield was calculated according to Eq. (1).

2.4. Morphological and chemical characterization of PA NFs

The surface morphology of PA NFs before and after $scCO_2$ treatment and after thymol impregnation was analyzed by Field Emission Scanning Electron Microscopy (FESEM, Mira3 Tescan). The samples were sputter coated with a thin layer of Au prior to analysis.

Fourier transform infrared (FTIR) spectra of the control PA NFs and PA NFs impregnated with thymol were recorded in the ATR mode using a Nicolet 6700 FTIR Spectrometer (Thermo Scientific) at 2 cm^{-1} resolution, in the wavenumber range from 500 to 4000 cm^{-1}.

2.5. DSC analysis of PA NFs

Melting profiles of the PA NFs before and after the SSI with thymol were determined by differential scanning calorimetry (DSC) analysis using a TA Instrument differential scanning calorimeter thermal analyzer (DSC Q10). The instrument was calibrated using indium for temperature and heat flow calibration. The samples (3–4 mg) placed in sealed aluminum pans were heated from the room temperature up to 300 °C at a heating rate of 10 °C min^{-1}, using a nitrogen purge gas at 50 mL min^{-1}. The first heating cycle was analyzed for melting temperature (T_m), degree of crystallinity (χ_m) and melting enthalpy (H_m) which can be defined as an area under the melting curve. The degree of crystallinity of the samples was determined in accordance with Eq. (3) [35]: