A New Composite Biomaterial Obtained by Supercritical CO_2 Assisted Process: Cellulose Acetate + Laponite®

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Supercritical phase inversion process was successfully implemented to produce loaded polymeric membranes of cellulose acetate with a synthetic clay mineral (i.e., laponite), to enhance both their mechanical and biological properties. The generated structures presented a microporous structure with a pore size ranging from 8.7 to 12.6 μm, depending on the laponite concentration: the addition of a solid in the starting solution caused a phase separation delay and a consequent pores size increasing. At the same time, the addition of laponite in form of nanodisks improved the mechanical characteristics of the material in terms of Young modulus up to 4.2 MPa. Furthermore, a relevant increase up to 80.9% on cellular adhesion (cell line BT474) was found thanks to the incorporation of laponite inside these membranes. This phenomenon can be attributed to the high surface area and the mineral properties of this clay; therefore, the inclusion of laponite in cellulose acetate membranes produced by supercritical phase inversion, drastically transformed this polymer into a suitable material for tissue engineering applications.

1. Introduction

In the last years, several studies have been proposed for the generation of composite structures characterized by several advantages. For example, different organic or inorganic compounds have been added to polymers to commute them into new materials with suitable peculiarities (Tabernero et al., 2019b). These composites can be generated by different techniques; for example, chemical crosslinking was used for composite hydrogels, electrospinning to generate fibrous composite structures and phase inversion to produce loaded membranes (Thang et al., 2016). However, these techniques are characterized by various disadvantages, such as the use of organic solvents (potential problems of toxicity), long processing times that can also take several days, and low versatility (it is hard to control the final morphology of the generated materials).

The possible application of supercritical carbon dioxide (SC-CO_2) to generate composite materials has been deeply studied (De Marco et al., 2015a). Indeed, supercritical carbon dioxide assisted techniques are characterized by specific peculiarities (Prosapio et al., 2016; Sarno et al., 2016; Baldino et al., 2017a) that improve the traditional processes, such as: low surface tension, high density, high diffusivity, low pollution and short processing times (Marra et al., 2012; Baldino et al., 2017b). For these reasons, supercritical carbon dioxide assisted techniques have been also proposed to produce porous structures; for example, starch aerogels have been obtained by supercritical carbon dioxide drying (De Marco et al., 2015b), poly-lactid-acid scaffolds by supercritical freeze extraction process (Cardea et al., 2014), alginate and gelatin aerogels by supercritical drying (Baldino et al., 2016b), cellulose acetate loaded membranes by supercritical phase inversion (Baldino et al., 2016a), polyvinylpyrrolidone fibers by supercritical assisted electrospinning (Baldino et al., 2019), etc.

In particular, among these techniques, supercritical phase inversion can strongly improve the classical phase separation process to obtain membranes. Indeed, conventional phase inversion provides the use of organic solvents and long processing times to obtain dry membranes. Moreover, non-homogenous porous structures are often produced. On the other hand, using supercritical carbon dioxide, in the role of anti-solvent for the starting polymeric solution, it is possible to quickly obtain homogeneous porous membranes and, in particular,

to control morphology and pore size changing the process parameters. Indeed, the thermodynamics and the kinetics of the supercritical phase inversion can be controlled by process pressure and temperature. Moreover, adding a solute in the starting solutions, composite structures (for example, polymer+drug, polymer+catalyst, polymer+nanoclay) can be obtained for several applications. This process has been already successfully used for generating membranes of different polymers, such as cellulose acetate (Baldino et al., 2016b), PVDF-HFP (Sarno et al., 2020) and PVA (Reverchon et al., 2008). Moreover, composite membranes for biomedical applications have been also produced, enhancing both the biological and mechanical properties of the original material. For example, levan was recently loaded into cellulose acetate membranes (Tabernero et al., 2019a), increasing both cells adhesion (from about 15% to 40%) and the final mechanical resistance of the material.

Recently, laponite has been evidenced as a material that can improve cell adhesion, cell proliferation and can promote osteogenic differentiation (Carrow et al., 2018). It is a synthetic smectite clay with low cost, already used as rheology modifier and in cosmetic applications. It is characterized by a disk-shaped nanostructure with low toxicity and, thanks to its characteristics and properties, laponite has been also loaded in different composites with the aim of improving both biological and mechanical characteristics. More information about laponite and its potential use for biomedical applications can be found elsewhere (Tomas et al., 2018). In spite of the laponite properties for developing new materials for tissue engineering, its inclusion into polymeric devices required several steps. In order to overcome this drawback, supercritical phase inversion can provide several advantages due to the possibility of producing a structure loaded with laponite in a one step processing. However, the possibility of using supercritical carbon dioxide assisted technique for processing this clay is still unexplored as well as its inclusion in composite membranes. For these reasons, in this work, the generation of composite membranes, cellulose acetate + laponite, by SC-CO_2 assisted phase inversion was proposed. The aim is to verify the feasibility of the process for the formation of these composite membranes and to study the effect of laponite inclusion on the membranes physico-chemical characteristics.

2. Materials and methods

Cellulose acetate (50000 Da of average molecular weight) and acetone were bought by Sigma-Aldrich. Laponite® RD (laponite) was provided by the Department of Inorganic Chemistry of the University of Salamanca (purchased from Kremer Pigmente, Spain). CO_2 (purity 99.9%) was bought by Morlando Group S.R.L. (Sant'Antimo, NA, Italy).

2.1 Membrane production by supercritical phase inversion

Cellulose acetate solutions were prepared in acetone at a concentration of 20% w/w. Then, laponite, at different concentrations, was added in suspension since it is not soluble in the solvent used. In particular, two different suspensions were prepared at 20 and 50% w/w laponite with respect to cellulose acetate.

The supercritical phase inversion process was performed in a home-made apparatus previously described (Reverchon et al., 2007); the operative conditions (35°C and 200 bar) were selected considering previous works (Tabernero et al., 2019b) in which these parameters were optimized for the formation of homogeneous cellulose acetate loaded membranes. A constant flow rate of carbon dioxide of 1.5 kg/h was used and the experiment lasted 5 h. At the end of the experiment, the vessel was slowly depressurized up to atmospheric pressure, and the polymeric composite structures, cellulose acetate + laponite, were collected.

2.2 Laponite characterizations

Laponite samples were analyzed by thermogravimetric analysis with a SDT Q600, USA (TA Instruments), under N_2 atmosphere at a flow-rate of 20 mL/min. The apparatus worked in a range of temperature from 20ºC to 900ºC (10ºC/min).

A Perkin-Elmer Spectra One spectrometer was used for recording the infrared spectra, using KBr as pellet.

A Siemens D-500 diffractometer was used for determining the powder XRD diagrams. This equipment operated at 40 kV and 30 mA with a filtered radiation Cu Kα from 2º to 65º. The selected scan speed was $2º \cdot min^{-1}$.

2.3 Membrane characterizations

Membrane samples were morphologically characterized using a scanning electron microscope (SEM mod. LEO420, Assing, Italy).

Tensile tests were performed using an INSTROM 4301 (Instron Int. Ltd., High Wycombe, UK) on membrane samples of 2 cm x 1.5 cm (length x thickness). A 100 N load cell was used with a gauge length and grip separation of 3 mm.

Cell adhesion tests were performed following a surface seeding protocol. The cell line BT474 was used for this purpose. Cells were cultured in DMEM medium (Dulbecco´s modified eagle medium) at 37ºC and using an incubator with 5% v/v of CO_2. After cells cultivation, the composite membranes were put into the microwell plate and 100 µL (with 8000 cells) were added, to perform 3 h of incubation process. Then, 1 mL of supplementary medium was added to completely submerge the membranes: cells were incubated for 24 h at 37ºC. The membranes were rinsed with Phosphate Buffer Saline (PBS) and retired from the microwell plate. Then, MTT was added (110 µL) and incubated for 1 h. The medium and the MTT were subsequently retired and 0.5 mL of Dimethyl sulfoxide (DMSO) were added to dissolve the formed formazan salts. After measuring the absorbance at 550 nm of the controls and the wells without the membranes, the process finished; the value of adhered cells on the membrane was determined by the absorbance difference between these values.

3. Results and discussion

In the first part of the work, the attention was focused on the characterizations of laponite. TGA curves are reported in Figure 1.
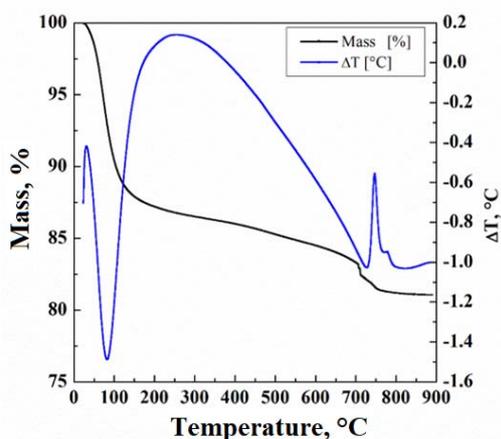

Figure 1: TGA analysis of laponite.

In figure 1 it is possible to observe a first mass loss that can be associated to water removal at about 90°C (endothermic process); whereas the second mass loss at about 720°C can be due to solid dehydroxylation (also endothermic). Finally, the last decomposition step at 750°C (exothermic) can be due to a change of phase from laponite to enstatite and silica.

Subsequently, the laponite was subjected to infrared spectroscopy in order to obtain a typical infrared spectrum where, on the abscissa, there is the wave number of the incident photon, and, on the ordinate, the transmittance. The vibrations during this technique can be of two types: stretching of the chemical bond and deformation of the bonding angle.
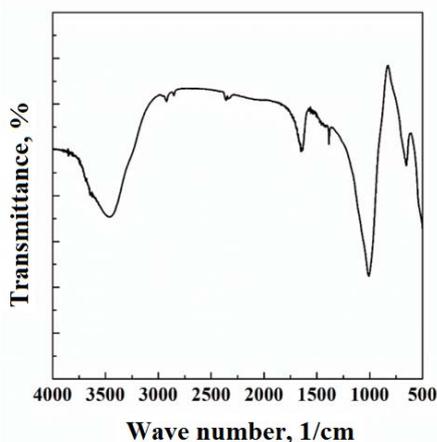

Figure 2: Infrared spectrum of laponite.

The data obtained from the FT-IR absorption spectrum, shown in figure 2, reveal that laponite had a stretching zone at 3690 cm^{-1} (MgO-H), 655 cm^{-1} (Mg-O) and 1010 cm^{-1} (Si-O), together with the bands corresponding to the modalities of elongation and bending of water molecules. Furthermore, at bands close to 1000 and 1100 cm^{-1}, changes were observed in the Si-O stretching region.

The starting silicate was also analyzed by X-ray diffractometry in order to define the crystalline phases, based on the presence or absence of the characteristic peaks. The diffraction spectrum of the tested sample is shown in figure 3, at a scanning speed equal to 2°/min.
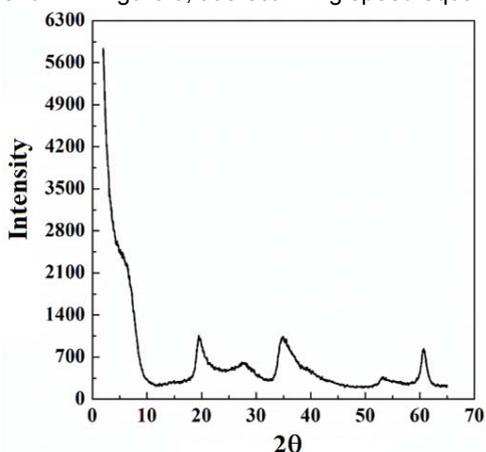

Figure 3: X-ray diffractometry of laponite.

The diagram for powdered laponite doesn't show an high order in its basal reflex, but only a flexion at 2θ = 6°, confirming the semi-crystalline structure of the silicate. Furthermore, the intralayer reflexes, independent of the stacking of silicate layers, are clearly highlighted (Lazzara et al., 2009).

After the characterization of laponite, cellulose acetate-laponite composite membranes were analyzed. In figure 4, SEM images of cellulose acetate membranes (figure 4a) and of cellulose acetate + laponite composite membranes (figure 4b and 4c) are reported. Observing these SEM images, two important considerations can be evidenced: 1) cellular structures were generated for all membranes; this result confirms that the supercritical carbon dioxide phase inversion allowed to generate uniform and regularly porous composite membranes. Moreover, the kind of morphology suggests that a binodal decomposition occurred during the phase separation, with the nucleation and growth of the polymer-lean phase into the polymer rich-phase with the subsequent solidification of the polymer-rich phase; 2) after laponite addition, the membranes morphology was not modified; the addition of laponite did not affect the phase inversion process and the mechanism of membranes formation. This result was not obvious, since the addition of laponite nanodisk could modify the membrane formation mechanism, influencing its final morphology. Moreover, since the presence of laponite is not evidenced by SEM images, probably, the loaded nanodisks were embedded inside the polymeric matrix.
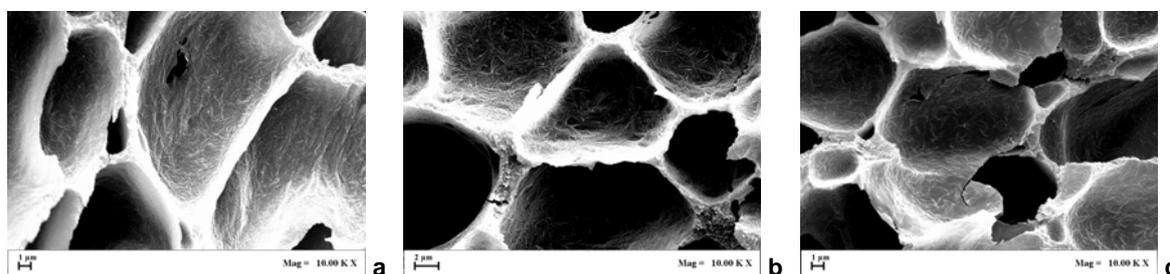

Figure 4: SEM images of cellulose acetate membrane section (4a) and cellulose acetate-laponite membrane section (4b, 20% w/w laponite; 4c, 50% w/w laponite).

On the other hand, the addition of laponite influenced the pores size of the cellulose acetate membranes. Indeed, the membranes without laponite (figure 4a) showed an average pore size of about 8.7 µm; whereas increasing the amount of loaded laponite at 20% w/w (figure 4b) and 50% w/w (figure 4c), an increase of average pore size to 11.8 and 12.6 µm was also observed. This result is summarized in figure 5, where the

membranes pore size distributions are reported. This average pores size increase can be attributed to the presence of the laponite in the starting suspensions; indeed, the presence of laponite nanodisks delayed the demixing process during the membrane formation process, causing a larger growth of the polymer-lean phase inside the polymer-rich phase; i.e., the demixing process was slower and the pores had more time to grow inside the membranes. A similar phenomenon has been already reported in the literature on a similar system (Baldino et al., 2016b).
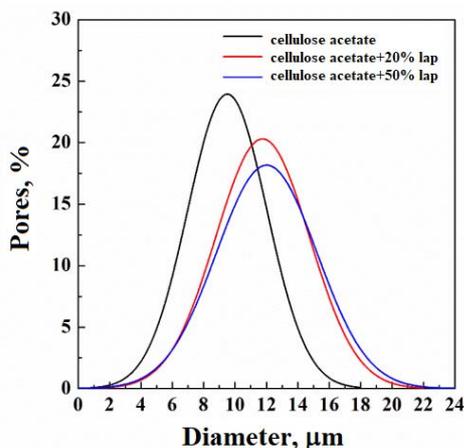

Figure 5: Pore size distribution of cellulose acetate and cellulose acetate+laponite membranes.

Once verified the effect of laponite addition on the membranes morphology, the mechanical properties were studied. In particular, Young modulus was calculated by the Instron apparatus, as reported in 2.3 paragraph. The results obtained are reported in Table 1.

Table 1: Mechanical results

Membrane	Young modulus, MPa
cellulose acetate	0.6
cellulose acetate +20% laponite	3.6
cellulose acetate +50% laponite	4.2

As it is possible to observe from Table 1, the addition of laponite in the cellulose acetate membranes produced an increase of the Young modulus. This result is relevant and can be correlated to the characteristics of the laponite nanodisks that allow to increase the elasticity performances of the membranes, making them potentially useful for biological applications.

Subsequently, the biological aspects of the composite membranes were studied. Cells adhesion on membranes was calculated and the results are shown in table 2. Also in this case, the results obtained are promising; indeed, starting from low values of cells adhesion for pure cellulose acetate (about 16%), it was possible to increase this value up to 81% adding laponite in the membranes. In particular, when cellulose acetate + 20% w/w laponite membranes were tested, a cell adhesion value of about 54% was measured; whereas a cell adhesion value of about 81% was found the cellulose acetate + 50% w/w laponite membranes. This surprising result can be due to the peculiarities of the laponite, in particular to laponite high surface area and to its mineral properties that facilitate cell adhesion confirming the positive biological behavior of this inorganic material.

Table 2: Cell adhesion results

Membrane	Cells adhesion, %
cellulose acetate	16.2
cellulose acetate +20% laponite	54.1
cellulose acetate +50% laponite	80.9

4. Conclusions

SC-CO$_2$ phase inversion process was successfully tested to produce laponite loaded cellulose acetate porous structures. The obtained membranes presented a homogeneous cellular structure and the inclusion of laponite positively influenced the morphology of the membranes, increasing the mechanical resistance of the material in terms of Young modulus. The large increase in cells adhesion puts in evidence the huge possibilities of supercritical fluids process to generate composites for biomedical applications in a step processing.

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