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A New Composite Biomaterial Obtained by Supercritical CO_2 Assisted Process: PVDF-HFP + Levan

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A supercritical CO_2 (SC-CO_2) assisted phase inversion process was tested to load a fructose exopolysaccharide with amphiphilic properties (i.e., levan) in membranes of polyvinylidenefluoride-co-hexafluoropropylene (PVDF-HFP). The aim was to investigate the effect of levan on the morphological and biological properties of PVDF-HFP membranes. Different loadings of levan from 0 to 50% w/w were tested: PVDF-HFP + levan membranes characterized by a foam-like structure were generated, with an increase of overall porosity from 75 to 90%. The effect of levan addition on mechanical and biological characteristics of PVDF-HFP membranes was also studied: a decrease of the mechanical resistance from 12 to 1 MPa and an increase of cells (colon cancer cell line HTC-116) adhesion from 8% to 35% were measured. In conclusion, SC-CO_2 assisted process was able to generate composite structures potentially useful for biological applications, characterized by a homogeneous distribution of levan and by improved biological properties.

1. Introduction

In recent years, several processes have been improved by the use of supercritical carbon dioxide (SC-CO_2) (Marra et al., 2012; Sarno et al., 2016). Indeed, thanks to SC-CO_2 peculiarities, such as low surface tension, low pollution, high solvent power, high diffusivity, etc., the disadvantages of traditional processes have been overcome (Prosapio et al., 2016). In particular, SC-CO_2 based processes have been used in micronization (De Marco et al., 2015b), drying (Cardea et al., 2014), electrospinning (Baldino et al., 2019), membranes formation (Baldino et al., 2016a), extraction (Baldino et al., 2017a; Baldino et al., 2017b), etc. Specifically, the potential of using SC-CO_2 as a drying agent has been demonstrated in several works (De Marco et al., 2015a). This technique is suitable to produce pure or composite aerogels, with a nanofibrous structure, from hydrogels and/or interpenetrated polymer networks. Chitosan, alginate or a mixture of alginate-gelatin are some examples of aerogels that were obtained using a SC-CO_2 assisted drying process (Baldino et al., 2016b).
On the other hand, supercritical assisted phase inversion can be used to produce porous structures, called membranes. This technique involves the use of a mould, in which the polymeric solution is poured. After that, the solution is put in contact with SC-CO_2 to induce a phase separation phenomenon to produce a porous membrane, as occurs with conventional phase inversion techniques (Reverchon et al., 2008).
Cellulose acetate, cellulose acetate loaded with graphene oxide and polyvinylidenefluoride-co-hexafluoropropylene (PVDF-HFP) are some examples of solids processed using this technique, obtaining membranes with proper properties concerning pores size, pore interconnectivity and mechanical properties (Naddeo et al., 2018). Changing the experimental parameters, such as pressure, temperature or polymer concentration it is possible to control the membranes morphology and pore size and interconnectivity. Generally, according to these articles, a pressure of about 200 bar and a temperature around 35ºC are appropriate to successfully obtain regular porous membranes with interconnected pores (Cardea et al., 2009).
In spite of the broad range of materials that can be processed by supercritical phase inversion, this technique has been barely used with exopolysaccharides (Tabernero et al., 2017). These highly hydrophilic and organic

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compounds are relevant for biomedical applications due to their special characteristics, such as biocompatibility, biodegradability and even rheological and antitumoral properties. Among these polymers, levan is the only fructose exopolysaccharide with amphiphilic properties. This polymer is obtained because an enzyme, called levansucrose, can break the glucose-fructose bond, under certain experimental conditions, to polymerize after that the fructose residues (Sezer et al., 2011).
Levan has shown a great potential for different applications for its characteristics: adhesive properties, prebiotic properties, anti-inflammatory properties; moreover, several articles highlighted its potential as a carrier for drug delivery systems (Sezer et al., 2017). On the other hand, until now, this fructose polymer has not been included in composite membranes to study its effect on morphological properties; at the same time, the possibility of using SC-CO_2 to process this material, or any polymer with self-assembly, is still unexplored.
Based on the previous facts, in this work, the use of supercritical phase inversion for producing composite PVDF-HFP membranes loaded with levan is proposed. Levan will be produced by means of a cell-free system and will be subsequently included into PVDF-HFP solutions as nanoparticles. The obtained membranes will be characterized morphologically by scanning electron microscopy (SEM), mechanically by tensile tests and biologically by cell adhesion.

2. Materials and methods

2.1 Levan production

Levan was produced without cultivating the typical microorganisms for this purpose (*Bacillus subtilis* or *Zymomonas mobilis*) and without using a medium culture with a huge number of compounds. In this case, a straightforward enzymatic reaction was performed, with Fructosyltransferase 68S from Bacillus subtilis (NATE-1384, purchased from Creative enzymes, NY, USA). Reactors of 100 mL at 37ºC were used with an enzyme concentration of 0.2 mg L-1 and an initial concentration of sucrose of 90 g L-1. Experimental conditions were used previously (Szwengiel et al., 2016). After 3 days of reaction (the maximum conversion was obtained), the polymer was isolated from the solution following a methodology based on the use of ethanol (3 volumes per each volume of reaction media). The obtained mixture was stored for 24 h at -20ºC, and then a centrifugation process (10 minutes at 10,000 rpm) was performed to separate the supernatant. Finally, the pellet was dried by lyophilization (Telstar, -55ºC, 0.05 bar).

2.2 Levan characterizations

Since levan can formed a colloidal dispersion in dimethylsulfoxide (DMSO), a first characterization was performed to study levan particle size distribution in DMSO. TEM microscopy (Zeiss EM902 at 80 kV) was used for this purpose. Levan samples were prepared by loading a droplet of the dispersion on a grid with a subsequent drying process at air.
Particle size and Z-potential of the polymer was determined by DLS (Zetasizer nano, Malvern Instruments).

2.3 Membranes production

Supercritical phase inversion apparatus and methodology were already described elsewhere (Reverchon et al., 2007). Basically, solutions of PVDF-HFP (Sigma-Aldrich with an average molecular weight of 400000 Da) at 20% w/w in DMSO were prepared; then, different amounts of levan were added generating suspensions of levan in PVDF-HFP/DMSO solutions that were kept under agitation at 30°C for 24 h to obtain a proper homogenization. Different loadings of levan were processed: PVDF-HFP solution in DMSO without levan (0% levan), PVDF-HFP in DMSO + 20% w/w levan with respect of PVDF-HFP (PVL20) and PVDF-HFP in DMSO + 50% w/w levan with respect of PVDF-HFP (PVL50).
These suspensions were subsequently placed in a cap that was put inside a home-made high pressure vessel, filled with CO_2 (purity 99.9%, bought by Morlando Group S.R.L., Sant'Antimo, NA, Italy) up to 200 bar by a high-pressure pump (Milton Roy – France). After that, the vessel was heated up to a temperature of 35°C. These conditions were kept for 5 h and, then, the system was slowly depressurized. Finally, the vessel was opened and the caps with the processed materials were recovered (Reverchon et al., 2008).

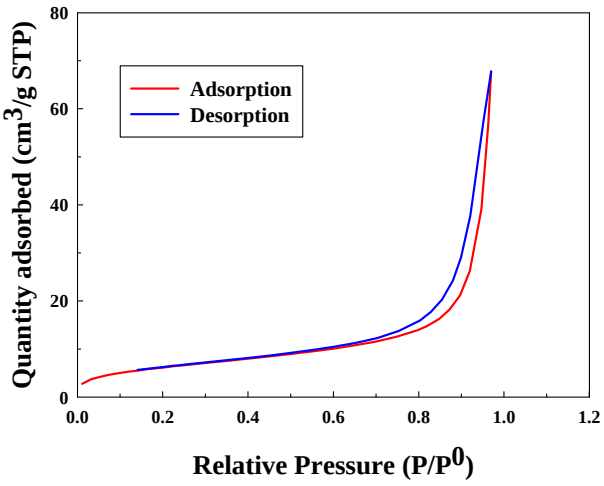
2.4 Membranes characterization

A scanning electron microscope (SEM, mod. LEO420, Assing, Italy) was used to analyze the morphologies of the loaded PVDF-HFP membranes.
Tensile tests were performed using an INSTROM 4301 (Instron Int. Ltd., High Wycombe, UK). A pulling force was applied on sample of 2 cm of length and 1.5 cm of thickness, with a 100 N load cell at 1.5 mm/min with a gauge length and grip separation of 3 mm.
Cell adhesion tests were performed by surface seeding protocol. The colon cancer cell line HTC-116 was used for this purpose. Cells were cultured in DMEM medium (Dulbecco´s modified eagle medium) at 37ºC and

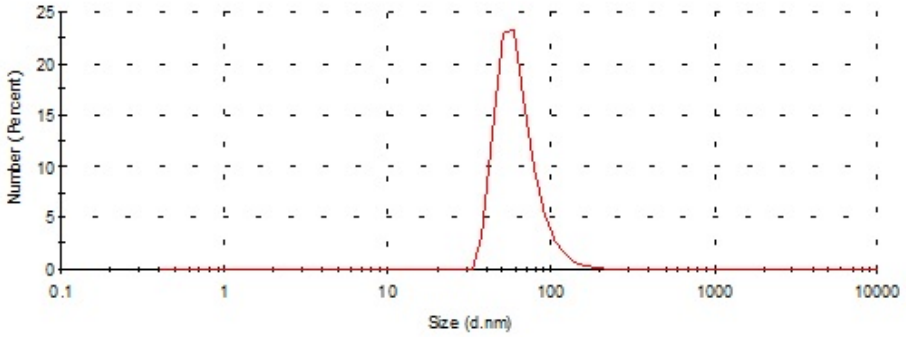
using an incubator with 5% v/v of CO_2. After cells cultivation, membranes were put into the microwell plate and 100 µL (with 8000 cells) were added, to perform 3 h of incubation process. Then, 1 mL of supplementary medium was added to completely submerge the membranes: cells were incubated for 24 h at 37ºC. The membranes were rinsed with Phosphate Buffer Saline (PBS) and retired from the microwell plate. Then, MTT was added (110 microliters) and incubated for 1 h. The medium and the MTT were subsequently retired and 0.5 mL of Dimethylsulfoxide (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 were evaluated by the absorbance difference between these values.

3. Results and discussion

In the first part of the experimentations, the attention was focused on the results concerning the levan production. A yield of about 20% (mg polyfructose/mg initial sucrose) was obtained; this value is relevant and confirms the successful of levan production process (Tabernero et al., 2017). According to the performed characterizations, levan had a BET surface area of 23 m^2/g and a pore size of about 15 nm.
Moreover, nitrogen adsorption results indicated that levan showed a type II isotherm (figure 1a) that is characteristic of a non-porous material with unrestricted monolayer-multilayer adsorption. On the other hand, according to DLS measurements, in figure 1b is reported the particle size distribution of levan in a colloidal suspension of DMSO: a particle size of about 100 nm and a Z-potential of about –3 mV were found.



a



b

Figure 1: a) Levan adsorption isotherm; b) Particle size distribution of levan in DMSO.

Subsequently, TEM and SEM analyses were performed on levan samples: figure 2a shows a TEM image of levan nanoparticles in DMSO; whereas a SEM image of the uprocessed levan is illustrated in figure 2b. According to figure 2a, levan nanoparticles were smaller than 100 nm. This difference can be explained referring to the drying process that must be performed before the use of TEM; i.e., air drying caused a decrease of levan particles size.

On the other hand, SEM image of unprocessed levan showed a broad range of particles size (from 2 to 10 microns); although it is not showed in the figure, some particles of more than 100 microns were also obtained.

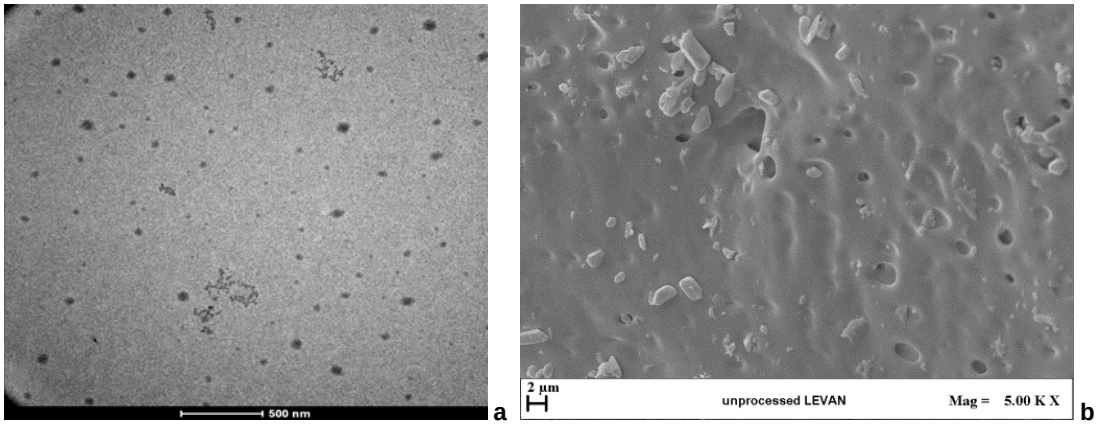


Figure 2: a) TEM image of levan nanoparticles in DMSO and b) SEM image of unprocessed levan.

In the second part of this work, morphological analyses were performed to verify the effect of levan nanoparticles addition to PVDF-HFP membranes. As it possible to observe by SEM images reported in figure 3, a drastic change was evidenced when levan concentration increased in the polymeric membrane. In particular, passing from pure PVDF-HFP membrane (figure 3a) to PVDF-HFP membrane containing 50% w/w levan (PVL50, in figure 3b), structural modifications occurred. Indeed, a porous structure with an average pore size of about 20 microns was obtained when PVDF-HFP was processed alone (figure 3a); whereas a foam-like structure with sensibly larger pores was obtained in the case of PVL50 composite membranes (figure 3b). However, SEM images did not allow to evidence the presence of levan in the composite structure; this phenomenon is due to the size of the levan particles in DMSO (i.e., 100 nm), that are probably embedded in the polymeric matrix. Similar results were also obtained for PVL20 samples.

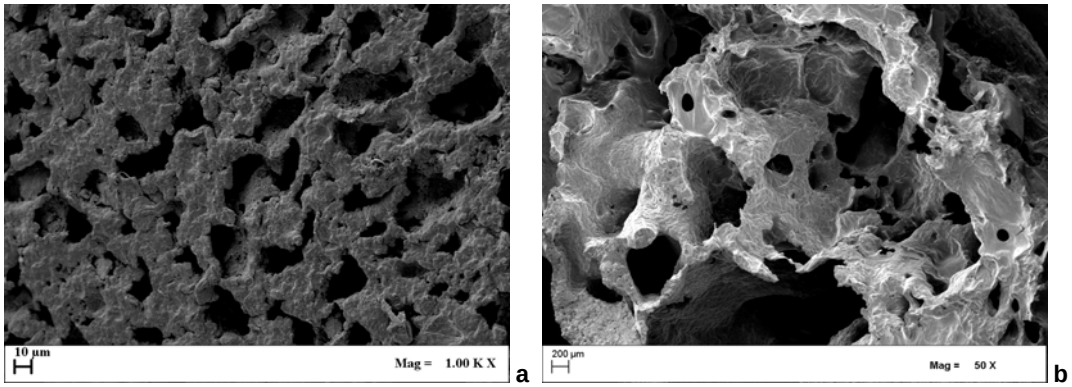


Figure 3: SEM images of a) PVDF-HFP and b) PVL50.

This is an unexpected result since it is very difficult to obtain a foaming-like structure processing PVDF-HFP, due to its characteristic temperatures. From the literature (Baldino et al., 2016a), it is known that the presence of a suspended solute in the starting polymeric solution can cause a delay of the phase inversion mechanism that can lead to the formation of larger pores like the ones observed in figure 3b. Moreover, the high affinity between levan and the solvent used (i.e., DMSO) can further slow down the separation process, facilitating the formation of larger pores similar to the ones typical of foam-like structures.

Tensile tests were performed on pure PVDF-HFP membranes and on PVDF-HFP + levan composite membranes; the results are shown in figure 4. The inclusion of levan in the structure reduced the resistance of the material. This phenomenon can be attributed to the change of the membranes internal structure, already observed in figure 3. The larger open pores created a less compact membrane and, as a consequence, a decrease of its mechanical performances. As can be observed in figure 4, a membrane of PVDF-HFP without levan needs a stress of around 2 MPa for producing an elongation (lambda) of 0.5 units. However, the same

stress provided an elongation of about 1.0 units for PVL20 composite membrane and greater than 2.0 units for PVL50 composite membrane.

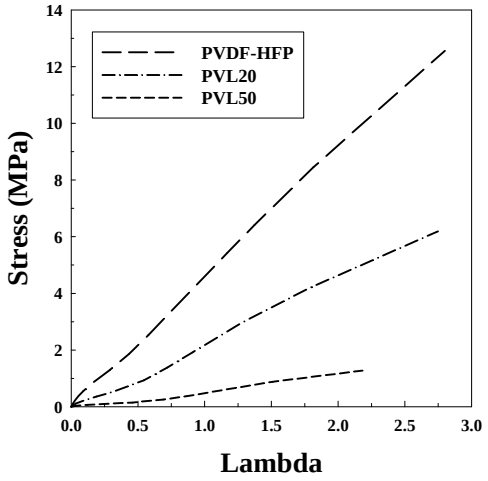


Figure 4: Tensile test for the PVDF-HFP-levan systems: PVDF-HFP, PVL20, PVL50.

Finally, cell adhesion experiments were performed on these membranes. These experiments were performed to obtain information about the possibility of using levan as a material in composites for increasing cell adhesion, for biomedical applications. Table 1 shows that pure PVDF-HFP membranes were characterized by a cell adhesion value of 8%, confirming the low affinity between the pure polymer and cells; whereas increasing the levan percentages from 0 to 50%, a large increase of cell adhesion was obtained. In particular, on PVL20 composite membranes a value of 30.9% and on PVL50 composite membranes a value larger than 35% were measured. This is an interesting result, because it confirmed as the SC-CO_2 assisted phase inversion is a process capable to generate porous composite structures suitable for tissue engineering applications, whereas the increase of cell adhesion was produced thanks to levan properties and its chemical composition.

Table 1: Cell adhesion results.

Membrane	Cells adhesion, %
PVL	8.0
PVL20	30.9
PVL50	35.1

4. Conclusions

Composite membranes of PVDF-HFP loaded with a fructose polysaccharide (levan) were successfully generated by a SC-CO_2 assisted phase inversion process. Levan formed a colloid when it was put in contact with DMSO (nanoparticles of about 100 nm), with a low value of Z potential, but without providing aggregation phenomenon. The levan colloidal suspension was included in membranes of PVDF-HFP modifying the internal structure and creating larger pores. This morphology caused a mechanical resistance reduction of the membranes; on the other hand, the addition of levan in PVDF-HFP membranes drastically improved the biological behavior of these composite materials, reaching cells adhesion values over 35%.

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