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Development of PLGA-PEDOT Mixed Polymeric Scaffolds and their Impregnation with Natural Extracts using Supercritical CO_2

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Conjugated materials are increasingly becoming a vital component in the production of therapeutic scaffolds through the creation of foams. This is due to the unique properties they possess that are not commonly found in other traditional polymers used in the industry. This preliminary study investigates the formation of conductive and porous scaffolds of PLGA/PEDOT:PSS and their impregnation with an ethanolic extract of mango leaves using supercritical CO_2. The effect of various process parameters, such as pressure, temperature, contact time, and polymer ratio, on the formation of the scaffolds and their properties were studied. The results were analyzed using scanning electron microscopy, DPPH assay, and the Folin-Ciocalteu method for polyphenols. The produced scaffolds showed various pore sizes (10 to 100 µm), and high antioxidant capacities and impregnation of the phenolic compounds were achieved. The results suggest that the scaffolds and the proposed technique have potential for various biomedical applications due to their porous structure and antioxidant capacities.

1. Introduction

The current research interest on composite 3D structures has increased considerably, especially due to the support for cell attachment that they provide to in vitro culture cells (Drury and Mooney, 2003). The flexibility and porosity of these structures are their most important properties for their use in tissue engineering, and polymers or mixtures of these are commonly used materials, owing to their mechanical properties (Chan and Leong, 2008). To mimic the extracellular matrix, materials must have an internal structure with interconnected pores or large voids, called scaffolds (Lawrence and Madihally, 2008).

Recent research has shed light on the advantages of utilizing biodegradable and conductive copolymers to facilitate cell proliferation and tissue repair (Wu et al., 2016). The presence of conductivity in organisms plays a crucial role in maintaining biological processes such as nerve signal transmission and tissue healing. Consequently, the potential of using electrical stimulation to enhance these processes is gaining recognition (Korupalli et al., 2021). Conductive polymers, such as polyaniline, polythiophenes, polypyrrole or poly(3,4-ethylenedioxythiophene) (PEDOT), have a positive effect on cell proliferation and adhesion with or without electrical stimulation and demonstrate a high degree of biocompatibility (Yu et al., 2022). PEDOT:PSS, a polymer studied in this work, has been incorporated into scaffolds with different techniques, such as electrochemical polymerization surrounding a pre-synthesized scaffold, vapor-phase polymerization for neural stem cell proliferation and differentiation or freeze-drying embedding PEDOT:PSS in porous materials, achieving improved mechanical properties through the addition of different aggregates (Richardson-Burns et al., 2007).

Conductive polymers lack important characteristics for tissue engineering such as strength, flexibility, solubility, or durability. To overcome these limitations, conductive polymers are often combined with non-conductive polymeric compounds, which provide mechanical properties required for tissue repair. Poly(lactic-co-glycolic acid) (PLGA), polylactide (PLA), polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), polyvinyl acetate (PVAc)

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or polycaprolactone (PCL) are the most commonly used compounds (Marsudi et al., 2021). Among these, PLGA is the most widely used for biomedical objectives, thanks to its mechanical properties, adjustable biodegradability ratio, and facility of processing in different techniques (Ishaug et al., 1997; Shen et al., 2008). PLGA has excellent processability, which facilitates the production of scaffolds with various pore sizes, and adequate mechanical properties, biocompatibility, and degradability to promote the regeneration of tissues. However, its degradation can lead to a local acidic microenvironment (Pan and Ding, 2012).
Additionally, studies in the literature suggest that bioactive capabilities can be added to porous scaffolds by impregnating them with compounds from natural compounds (Satpayeva et al., 2022). For example, a natural extract from mango leaves with pharmacological properties considered an excellent agent for the treatment of degenerative diseases, such as Alzheimer's or cancer was used in the present work (Fernández-Ponce et al., 2015). Supercritical carbon dioxide ($scCO_2$) foaming is employed for the formation of functional porous scaffolds. CO_2 has a high dissolution in polymers and is a non-toxic, inexpensive, and reusable compound. Therefore, this technique is considered a green process (Jacobs et al., 2008). In the supercritical state, CO_2 has properties of density, diffusivity, and low viscosity that allow it to permeate the polymeric matrix, causing plasticization of the matrix by decreasing its glass transition temperature. After a controlled contact time, depressurization of the system leads to supersaturation, with the subsequent cell nucleation phenomenon during phase separation, resulting in the formation of the porous scaffold structure (Kosowska et al., 2022; Valor et al., 2022). To carry out foaming and impregnation in a single step, the polymer mixture and the extract are introduced concurrently and maintained in contact with supercritical carbon dioxide ($scCO_2$) to facilitate its permeation into the polymer and induce a plasticizing effect that facilitates the impregnation process. The objective of this preliminary research is to developed conjugated systems with PEDOT and PLGA with the incorporation of mango leaves extracts, to use in tissue engineering. The authors evaluated the influence of temperature, pressure or contact time on the morphology, total impregnated compound and its antioxidant capacities.

2. Materials and methods

2.1 Materials

PLGA (lactide:glycolide 75:25) (poly(lactic-co-glycolic acid)) with Mw 76,000-115,000 and PEDOT:PSS (Poly(2,3-dihydrothieno-1,4-dioxin)-poly(styrenesulfonate)) 3.0-4.0% in H_2O were purchased from Sigma-Aldrich (Spain). CO_2 (99.8% purity) was supplied by Linde (Spain). DPPH (2,2-diphenyl-1-picrylhydrazyl), ethanol (99.5%), Folin–Ciocâlteu reagent (FCR) and Na_2CO_3 were supplied by Sigma–Aldrich (Madrid, Spain).

2.2 Production of ethanolic extract from mango leaves

The mango leaf extract was obtained through maceration extraction using a sample of 40 g of dried and crushed mango leaves. Ethanol (99.5%) was used as a solvent for 3 hours at a mild temperature of 45°C with agitation to avoid degradation of the leaf compounds. Ethanol was chosen for its high solubility in the supercritical phase, making it easy to remove from the final product. The resulting ethanolic suspension was filtered using commercial filter paper and then stored in darkness at 4 °C prior to assay. A rotary evaporator was employed at 45°C to achieve the desired total concentration.

2.3 Supercritical foaming-impregnation process

The experiments involving polymer foaming and impregnation were conducted using a SSI pilot plant (Thar technologies), as illustrated in Figure 1. The equipment is equipped with a high-pressure pump and a thermal bath, enabling CO_2 injection in its liquid state. A temperature-controlled electric heat exchanger, located just before the CO_2 inlet to the 257 mL foaming vessel, helps regulate the temperature. The required pressure of the entire system is controlled through an automatic backpressure regulator valve. Finally, the system includes a micrometric valve, which enables adjustment of the vessel depressurization rate and CO_2 venting through a cyclonic separator.
The PLGA and PEDOT:PSS polymer mixture was processed using a mechanical press to produce tablets, which were placed on an aluminum support. 3 mL of the ethanolic extract of mango leaves were introduced into the vessel without being in direct contact with the polymer mixture. Then, supercritical CO_2 was injected into the vessel under the desired conditions for the foaming/impregnation process. The mixture was maintained under supercritical conditions for a determined contact time, causing the polymer to plasticize. The system was then depressurized at a controlled rate using the micrometric valve, resulting in the foaming and impregnation of the compounds present in the extract through the formation of a porous structure inside the tablet.

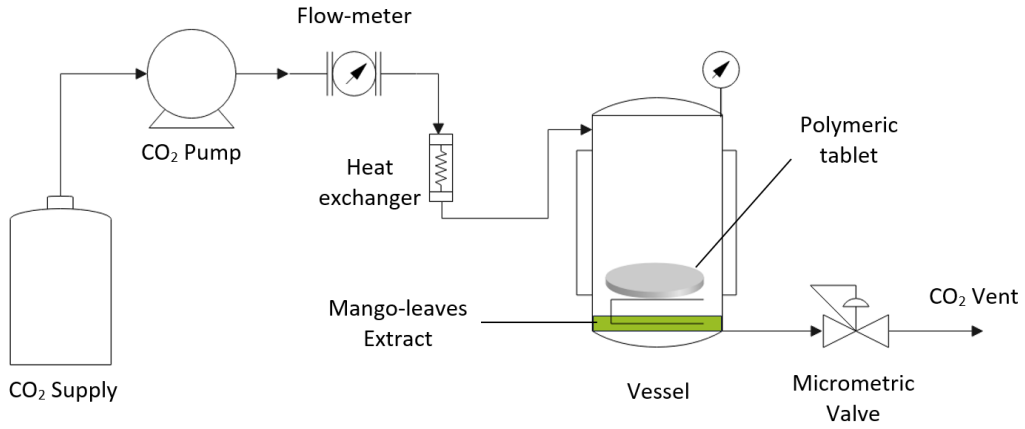


Figure 1: Schematic diagram of SSI pilot plant.

A total of seven experiments were conducted to investigate the impact of various parameters such as polymer ratio, temperature, pressure, and contact time on the scaffold formation process and their properties. The conditions used in each experiment are shown in Table 1. The tablet was always made using 25 mg of PLGA, while the amount of PEDOT was varied in some experiments. The depressurization rate was kept constant at 0.5 MPa/min, while the concentration of the extract used was always 20 mg extract/mL ethanol.

Table 1: Conditions used for the foaming/impregnation process.

Run	PEDOT (μL)	Pressure (MPa)	Temperature (K)	Time (h)
1	100	12	318	0.5
2	50	12	318	0.5
3	50	20	318	0.5
4	50	20	333	0.5
5	50	20	318	2
6	50	20	318	6
7	50	20	318	24

2.4 Scanning electron microscopy

The morphology and pore size of the foamed scaffolds were analyzed using a Scanning Electron Microscope (SEM). A Nova NanoSEM 450TM (Elecmi, Zaragoza, Spain) with an accelerating voltage of 15 kV was used for the analysis. To enhance conductivity and improve image quality in SEM, a 10 nm film of gold was coated on a cross-section of each sample before analysis. The SEM images were processed using Scion image software.

2.5 Antioxidant activity of extract and scaffolds

The antioxidant activity was calculated in order to evaluate the success of the runs carried out, for both mango leaves extract and the impregnated scaffolds. The methodology employed was derived from the one suggested by Brand-Williams and Scherer (Brand-Williams et al., 1995; Scherer and Godoy, 2009), but with necessary adaptations. The method was adapted to determine the antioxidant capacity of the impregnated scaffolds. A specific quantity of processed scaffolds was submerged in 4 mL of a solution containing 6×10^{-5} mol/L DPPH and left for 2 hours. The absorbance of the DPPH at the beginning and end of the process was measured at 515 nm. The results were expressed as % IO per 100 mg of impregnated scaffold, where % IO represent the percentage of DPPH inhibition, Eq (1). The analyses were carried out in triplicate.

$$\% \text{ Inhibition Oxidation} = \frac{(A_0 - A_i)}{A_0} * 100 \quad (1)$$

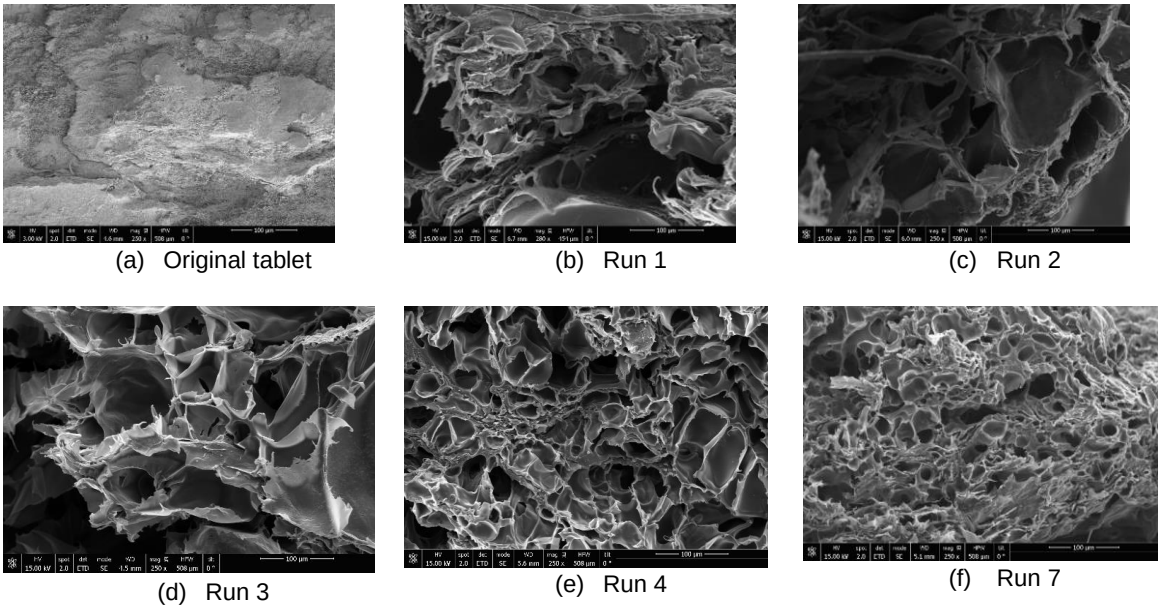
2.6 Total polyphenol content impregnated

The analysis of total phenolic content impregnated in the scaffolds was conducted using a modified version of the Folin-Ciocalteau method, as described by Singleton and Rossi (Singleton et al., 1999), for microplates. The

total release of polyphenols presents in scaffolds was achieved via 5-hour stirring in ethanol, and a 12.5 µL aliquot from this release was mixed with 12.5 µL of Folin-Ciocalteu's reagent and 200 µL distilled water, then shaken for 5 minutes. Subsequently, 25 µL of a sodium carbonate solution (20% w/v) was added, and the mixture was stirred for another 5 minutes. After 60 minutes of incubation at room temperature in the absence of light, absorbance was measured at 725 nm by means of a Synergy HTX multi-mode reader (Aligent, Biotek, VT, USA). A calibration curve was established using gallic acid (20–300 µg/mL). The analyses were performed in triplicate to ensure reproducibility.

3. Results and discussion

Several successful experiments were carried out in which key variables in both the supercritical foaming process and the impregnation of bioactive compounds present in the extract were modified, including temperature, pressure, contact time, and the ratio of conjugated polymers used. Figure 2 shows scanning electron microscopy images of the experiments where impregnated scaffolds were obtained. Generally, the foaming/impregnation process was able to modify the morphology of the initial material, resulting in porous structures, moderately ordered, with several pore size distributions depending on the conditions used. The morphology of the original tablet Figure 2(a) can be observed, in which it appears that PEDOT:PSS forms a film throughout the system, in which no pores are apparently observed at the magnification used. The pore sizes obtained ranged from 12 µm in run 7 to larger pores of up to 80-100 µm, as observed in run 2. The increased use of PEDOT seems to lead to a smaller pore opening when comparing the results of test 1 with those of test 2. The ability to modify sample porosity with slight changes in operational parameters makes supercritical foaming an attractive alternative for scaffold formation in tissue engineering. In addition, all samples showed a conductivity in the order of 10^{-5} S/cm in the 2-point conductivity method, so that an acceptable spreading of the PEDOT:PSS over the whole surface was obtained.



(a) Original tablet (b) Run 1 (c) Run 2

(d) Run 3 (e) Run 4 (f) Run 7

Figure 2: SEM images of (a) original PLGA/PEDOT:PSS tablet and formed scaffolds (1,2,3,4 and 7).

The obtained results with respect to the antioxidant capacity exhibited by the scaffolds when subjected to the DPPH oxidation test as well as the amount of polyphenolic compounds impregnated are shown in Table 2. It can be observed that the increase in pressure has a positive effect when comparing runs 2 and 3 (12 and 20 MPa, respectively) under isothermal conditions. In cases where the impregnation vessel contains an excessive amount of active compound, the saturation of the CO_2-phase with the compound can be maintained. As the pressure (or density) of CO_2 increases, the solubility of the active compound in the supercritical CO_2-phase also increases, resulting in a higher concentration of the active compound in the CO_2-phase. This generates a greater concentration gradient of the active compound between the CO_2-phase and the polymeric matrix, which promotes the active compound impregnation in the material. Additionally, the swelling phenomenon of the polymer is enhanced with increased CO_2 pressure, which facilitates the diffusion of the active compound inside

the polymer. This positive effect of pressure on the active compound loading has been reported in several studies (De Souza et al., 2014).

Table 2: Percentage of inhibition in the DPPH test (%IO) and total content of impregnated polyphenols (TPC).

Run	% Inhibition Oxidation	TPC (mgGAE/g sample)
1	41.76	209.30
2	35.42	176.05
3	48.11	218.48
4	12.56	60.23
5	49.77	209.58
6	58.56	234.56
7	39.21	196.16

Regarding the effect of temperature, the temperature was increased up to 60ºC under isobaric conditions to test its effect. A lower antioxidant activity and impregnation of compounds were clearly obtained. Increasing the temperature under isobaric conditions may potentially hinder the impregnation of the active compound into the polymer matrix. This is because as the temperature increases, the solvent power of CO_2 decreases, resulting in a decrease in the amount of active compound dissolved in the supercritical CO_2 phase. Studies have reported a decrease in active compound incorporation in various polymeric materials when the temperature or pressure was increased beyond certain limits. For example, an increase in temperature from 40 to 60 °C at 15 MPa caused decreased incorporation of flax oil in β-glucan aerogels (Comin et al., 2012).
Foaming/impregnation experiments were also carried out at different times while keeping pressure and temperature constant. Both the impregnation of phenolic compounds and the % inhibition in the oxidation of DPPH in the samples increase with contact time, reaching maximum performance at 6 hours (Run 6). The incorporation of active compounds into polymers using $scCO_2$-assisted impregnation involves both thermodynamic and kinetic processes. The attainment of equilibrium is dependent on thermodynamic factors such as the concentration gradient of active compounds and the physical-chemical properties of the polymer and fluid phase, including their chemical affinity. On the other hand, the time needed to reach equilibrium is controlled by the kinetics of the impregnation process. It may also be due to degradation of the bioactive compounds over time as indicated in several papers in the literature (Almeida et al., 2013; Pantić et al., 2016).

4. Conclusions

In this preliminary work the formation of PLGA/PEDOT:PSS using the supercritical foaming technique and its impregnation with a high antioxidant extract of mango leaves was achieved. The influence of pressure, polymeric ratio, temperature and contact time on the final activity of samples was studied, always under supercritical conditions. Scaffolds with a porous structure were obtained by modifying their initial morphology, reaching pores ranging from 10 to 100 µm. Successful impregnation of bioactive compounds into the scaffolds with proven antioxidant activity was obtained. The positive effect of pressure and time was observed, while increases in temperature led to lower compound loadings. Therefore, supercritical CO_2 foaming and the properties of the obtained scaffolds have high potential for use in tissue engineering.

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