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Use of polypyrrole-polystyrene membranes for extracting DNA from plant tissues

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ABSTRACT

We describe the preparation of a membrane composed of polypyrrole-polystyrene (PPy-PS) and its application in DNA extraction. We adopted the electrospinning technique to prepare polystyrene (PS) membranes, which we used as substrates for incorporating polypyrrole chains through an *in situ* chemical procedure. As a model system, we initially investigated the use of PPy-PS membranes for the extraction of salmon sperm DNA from aqueous solutions. These studies have shown that the PPy-PS membrane has a maximum adsorption capacity of 236.0 mg of DNA per gram of PPy after 30 min of exposure to a DNA solution (100 mg/L). We incorporated the PPy-PS membranes into centrifugation columns, which we used to carry out experiments for extracting and purification of DNA from curly lettuce leaves. The protocol was initially optimized by first examining the most appropriate concentration of the three components of the lysis buffer (Tris/HCl, NaCl, and EDTA-Na). We then investigated the most adequate volumes of the concentrated surfactant solution (SDS 20%) and that used in the protein and polysaccharide precipitation step (5 M potassium acetate, pH 6.3), factors that directly influence the quality and quantity of the fraction of DNA obtained. For curly lettuce leaves, both in their mature and young stages, the yield and purity of the DNA purified using the PPy-PS membrane were comparable to those obtained using a commercial kit. In both cases, the collected DNA samples presented excellent integrity and quality. These results are suggestive that these composite membranes are competitive with the commercial kits available for the extraction and purification of DNA from plants.

1. Introduction

Several molecular biology studies (such as cloning, identification of genotypes, and others in the genetic area) involve the extraction and purification of DNA as an initial stage [1]. This process is especially challenging when dealing with plant tissues due to the presence of a rigid cell wall and a large fraction of contaminants and secondary metabolites (polysaccharides and polyphenols, among other chemical compounds). These characteristics of plant tissues make the DNA extraction of vegetables more difficult and laborious than in the case of samples of animal origin. The isolation of good quality DNA samples relies on the adjustment of the extraction and purification procedures according to the degree of contaminants, as the presence of secondary metabolites can interfere with subsequent applications, such as amplification in a polymerase chain reaction (PCR) [2–5].

Currently, one can classify the different methodologies for isolating DNA from plants into three categories: standard CTAB method (cetyltrimethylammonium bromide), rapid DNA extraction methods, and commercial DNA isolation kits [6]. The CTAB method, first proposed by Murray and Thompson in 1980 [7], has since been adapted to suit different applications [8–12]. Although it can be considered a standard method for producing high-quality DNA extracted from a wide variety of plants, the corresponding protocols are time-consuming, requiring many steps of centrifugation and incubation and the use of solvents such as phenol-chloroform, which are toxic and can contaminate the purified DNA [13]. Given the need to improve the extraction time and avoid the use of reagents harmful to human beings and the environment, the development of new protocols as an alternative has become essential.

Rapid extraction methods, which are generally based on the use of alkaline solutions (NaOH), Tris-HCl, and centrifugation processes for DNA purification, involve simple and fast procedures, at the cost in terms of efficiency, since the purity and yield of the material obtained are generally low [14,15].

More recently, commercial kits have become available for the efficient and simple extraction and purification of DNA from plants, result-

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ing in a high-quality fraction of collected material. In general terms, commercial kits are based on solid-phase extraction methods, in which the DNA strand binds to a solid matrix that has an affinity for nucleic acid molecules under chaotropic conditions [16,17]. This process avoids the use of toxic organic solvents, allowing purification essentially free of contaminants. Most kits use rotating columns, where the solid material is attached to the inside of the column to facilitate DNA ligation, washing, and elution. The active material is silica in the form of a solid matrix, usually a glass fiber filter or a membrane [13]. At present, commercial kits for the extraction of DNA from plants have also incorporated functionalized magnetic materials, for simplifying the purification step by the use of an external magnet. Nevertheless, commercial kits remain quite expensive for regular use in research laboratories, stimulating the search for new solid matrix materials that could be used in more economical extraction protocols [18–20]. There continues to exist the need for improved methods of DNA extraction and purification, which could combine simplicity in the execution to a good level of efficiency.

In this work, we propose the use of a functionalized membrane as an alternative solid matrix material for the extraction and purification of DNA from plant samples. For the preparation of the membranes, we adopted the electrospinning method, a versatile and low-cost technique that allows the use of different materials to obtain micro and nanofibers organized in films that have high porosity and high surface area [21]. These characteristics make membranes an interesting adsorbent material, which although used in different areas [22–24], has not been widely applied in the capture and purification of DNA [25,26].

Currently, conductive polymers (PCs) have received a lot of attention, as they have unique characteristics, such as the possibility of changing their properties according to the conditions of the medium, a low density, and resistance to corrosion [27]. For these and other reasons, PCs have found the most diverse applications, such as energy storage devices [28], sensors, and biosensors [29,30]. Among the conducting polymers, polypyrrole (PPy) has been widely used in the field of biotechnology [31,32], since that, in its protonated form, PPy can form relatively stable hybrid structures with dissolved anions and polyanions, eventually allowing the capture of macromolecules, such as DNA chains [33]. This polymer is usually obtained in the form of a black powder that tends to agglomerate, a characteristic that limits its processability for practical applications [34]. For this reason, in the present study, we followed the strategy of deposition of PPy nanostructures on polystyrene membranes prepared by the electrospinning technique and we investigated its use in the capture of salmon sperm DNA in an aqueous medium and later expanded its application in extraction and purification of DNA from plant samples, using a spin column configuration. To the best of our knowledge, the use of these composite membranes for the practical and simple extraction of DNA has not been previously discussed in the literature.

2. Material and methods

2.1. Materials

We purchased polystyrene (PS) (MW=250 kDa) from Acros Organic (USA) and pyrrole and salmon sperm DNA from Sigma-Aldrich (USA). Dimethylformamide (DMF), polyvinylpyrrolidone (PVP, K-30), acetic acid (CH_3COOH), and Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) were obtained from Dinâmica (Brazil). Hydrochloric acid (HCl), sodium hydroxide (NaOH), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-Na), and sodium chloride (NaCl) were acquired from the Brazilian company Química Moderna. Sodium phosphate monobasic (NaH_2PO_4) and bibasic sodium phosphate (Na_2HPO_4) were acquired from Nuclear (Brazil). Potassium acetate (CH_3COOK) was obtained by AppliChem (Germany) and absolute ethanol (EMSURE – Merck, Germany). Glycine, sodium dodecyl sulfate (SDS), tris (hydroxymethyl) aminomethane (Tris), Tris-Acetate-EDTA (TAE) 40X, Agarose, blue/orange loading dye 6X, diamond nucleic acid dye, and 100bp DNA Ladder were purchased from Promega (USA). SYBR Green dye, PureLink™ RNase A (20 mg/mL), the PureLink™ Genomic Plant DNA Purification Kit, ITS (Internal transcription spacer) primers: forward 5′-TCC GTA GGT GAA CCT GCG G -3′ and reverse 5′- TCC TCC GCT TAT TGA TAT GC -3′ [35] was obtained by ThermoFisher Scientific (USA). The lettuce leaves were purchased at a local supermarket in Recife. We used all reagents as received, with exception of the pyrrole monomer, which was distilled under reduced pressure before use. High purity water (Millipore, USA) was used in all experiments.

2.2. Preparation of the PPy-PS membranes composites

The PS membranes were obtained by the electrospinning technique method. For this, we initially prepared a 30% w/v polymeric solution in DMF, which was subjected to a magnetic stirring for 12 hours, at a temperature of 85 °C. After that, we added a 2 mL volume of the solution to a 3 mL syringe (Beckton Dickinson, USA), to which a metallic needle was subsequently attached. The experimental apparatus was mounted in a horizontal configuration, using a NE-4000 syringe pump (New Era, USA). The operational parameters for the production of the membranes were adjusted to a distance of 20 cm between the needle and the collector (covered with aluminum foil), a flow of 0.5 mL/h, and a voltage of 17 kV. The membrane formed after 4 h of preparation was collected and allowed to dry for 12 h at room temperature. We then subjected the membranes (cut as 18 cm x 10 cm rectangles) to a thermo-mechanical treatment aiming the improvement of their mechanical properties. After being kept under a ~2 kg weight with a cross-section of 20 cm x 12 cm in an oven at 80 °C for 30 h, the membranes become more compact and robust.

The PS membranes were then modified through an *in situ* polymerization of pyrrole in aqueous media. For this, we initially cut them into 0.75 cm x 3.00 cm strips, which were weighed and subjected to a 6 min air plasma treatment using a PDC-002 plasma cleaner (Harrick, USA), operating under a power of 29.6 W, with a flow rate that varied in the (141.58-258.17) L/h range and a 300 mTorr vacuum, to confer a hydrophilic character to their surface. Then, four PS strips were initially kept for 15 minutes under stirring in a beaker containing 30 mL of a 0.49 mmol of an aqueous suspension of the pyrrole monomer. We then added 20 mL of a 0.05 M (aqueous solution of iron (III) chloride hexahydrate and allowed the polymerization process to proceed for 2 h under constant stirring at 5°C. The resulting PPy-PS composite membrane strips were repeatedly washed with deionized water and then dried at room temperature. Finally, we weighed them to estimate the amount of PPy effectively deposited. A schematic view of the steps followed in preparing the membrane and coating with the polypyrrole can be seen in Figs. SI-1 and SI-2, respectively.

2.3. Characterization methods

We used a MIRA 3 scanning electron microscope (TESCAN, Czech Republic) to assess the morphology of the prepared membranes, with the average diameter of the fibers being estimated with the aid of the Image J software. Using a 1 μm-accurate MDC-25J digital micrometer (Mitutoyo, Japan), we measured the thickness at the middle and extremes of each one of three identical strips, adopting then the average value. A Series 2400 voltage source (Keithley, USA) was used for the two-point electrical measurements. The UV-vis absorption spectrum (200-1200 nm) and chemical composition of the composite membrane were analyzed using a UV-2600 spectrophotometer (Shimadzu, Japan) and IRTracer-100 equipment (Shimadzu, Japan) operating at a resolution of 4 cm^{-1} in the 4000-400 cm^{-1} range (64 scans). To analyze the wetting properties of the membranes we used a CAM 100 goniometer (KSV, Finland), after depositing 5 μL of deionized water in 5 distinct regions of their surface, at room temperature. The DNA yield and purity were determined using a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific,

USA), following the characteristic absorption of 260 nm and the purity ratios $A_{260/280}$ and $A_{260/230}$, respectively.

2.4. DNA adsorption experiments in a model system

As an initial test on a model system, we investigated the use of the hybrid PPy-PS membranes to capture salmon sperm DNA strands dissolved in aqueous solutions. For this, we prepared a stock solution by dissolving 20 mg of salmon sperm DNA in 100 mL of deionized water and adjusting (200 mL) the final concentration to 100 mg/L with the addition of a glycine/HCl buffer solution (pH 2.8) [36]. We then immersed individual 0.75 cm x 3.00 cm strips of the PPy-PS composite membrane (each one containing 0.3 mg of PPy) in separate tubes containing a 2 mL aliquot of the stock solution. The tubes containing the DNA solution and the membrane were agitated at room temperature with the aid of an orbital shaker operating at 300 rpm. All experiments were performed in triplicate. To better establish the ability of the PPy-PS membrane in capturing the DNA chains, we performed a set of benchmark experiments where a plasma-treated pristine PS membrane was exposed to a 100 mg/L DNA solution for 1 h. The equations used for estimating the percentage and capacity (q_e) of DNA adsorbed on the PPy-PS membrane can be found in Section 2 of the Supplementary Information.

We examined how the variation in the interaction time would affect the performance of the PPy-PS as an adsorbing agent, by measuring the 260 nm absorption of aliquots (2 μL) taken at different moments along 120 min. Once the time required for equilibrium was determined, we examined the effect of the initial DNA concentration (5-100 mg/L). The experimental data were adjusted to the isothermal models of Langmuir, Freundlich, and Temkin, in addition to the pseudo-first, pseudo-second-order kinetics model, Morris-Weber, and Boyd [37–40].

2.5. DNA elution

We examined how easily the fraction of nucleic acid that had been captured by the membrane could be eluted. For this, we initially saturated the membranes with salmon sperm DNA (50 mg/L) and washed them with 1 mL of deionized water. Different elution solutions were evaluated – Tris/SDS (50 mM Tris/8.7 mM SDS, pH 8.0), Tris/EDTA-Na (TE, 10 mM Tris/1 mM EDTA-Na, pH 8.5), and Phosphate/Phosphate (87 mM Na_2HPO_4/13 mM NaH_2PO_4, pH 7.6), following a procedure in which 2 mL of each solution was added separately to a tube containing the membrane/DNA. Subsequently, the tube was placed on an orbital shaker operating at 300 rpm for 30 min and the supernatant was collected for UV-vis analysis.

2.6. Fluorescence microscopy

To monitor the DNA adsorption-elution processes, we used an Axio Imager 2 microscope (Zeiss, Germany) equipped with 450-490 nm excitation and 515-565 nm emission to obtain images of the PPy-PS membrane at different moments of the corresponding steps. For this, 2 μL of the SYBR Green dye was added to 2 mL of a glycine/salmon sperm DNA solution (50 mg/L) pH 2.8. Subsequently, we allowed the adsorption process to occur for 30 min and then washed the membrane. Finally, the DNA was eluted using a Tris/SDS (pH 8.0) solution. As a control experiment, we also allowed a membrane to interact for 30 min with 2 mL of the glycine/SYBR Green solution without the presence of DNA.

2.7. Process of extraction and purification of DNA from vegetable tissues

As a test system for the use of PPy-PS membranes in the extraction of DNA from plant samples, we used lettuce leaves of the curly type in their mature stage.

2.7.1. System preparation

We performed the purification of the DNA extracted from lettuce leaves using a system based on centrifugation columns, similar to those found in commercial DNA extraction kits. These columns are composed of one support to fix the adsorbent material (where the DNA will bind) and a microtube to retain the liquid (Fig. SI-3).

2.7.2. Optimization of the DNA extraction and purification process from plant tissues

The extraction and purification process consisted of initially breaking the rigid cell walls with the aid of an extraction buffer, followed by cell lysis. The next step was the precipitation of contaminants, such as proteins and polysaccharides, followed by DNA binding to the adsorbent material fixed to the column support. After washing the system, the DNA was finally eluted. The extraction buffer used in the process consists of a mixture of five components: SDS, Tris/HCl, NaCl, EDTA-Na, and PVP. The relative concentration of each of these components is a crucial parameter to determine the yield and purity of the final DNA strands obtained. Thus, to establish the best conditions, we implemented a factorial design (2^3) [41] in which two levels (– and +) of concentration were assigned to three fundamental components of the extraction buffer (Tris/HCl (pH 8.0), NaCl, and EDTA-Na), totaling eight experiments (I-VIII, as shown in Table SI-1). In all experiments, fixed volumes of 15 μL of anionic surfactant SDS at 20% and a concentration of 4% of PVP were used. All possible variations for the lysis buffer (I-VIII) were tested in triplicate according to the steps shown in Fig. 1. The detailed DNA extraction and purification protocol (adapted from Ref. [17]) and the specific solutions (Table SI-2) used in this process can be found in Section 3.2 of the Supplementary Information.

After testing all variations of the lysis buffer (I-VIII), the concentration and purity of the DNA fraction obtained in each experiment were analyzed by a UV-vis spectrophotometer. The protocol chosen was the one in which we obtained the highest concentration of DNA and the one in which the purity was close to ideal. As reported in the literature [42], for a DNA sample to be considered pure the ratio $A_{260/280}$ [$A_{260/230}$] must be close to 1.8 [in the range of 2.0-2.2].

After defining the best parameters for the lysis solution, we evaluated how the variation of the volume used of the 20% SDS surfactant and the 5 M potassium acetate (pH 6.3) solution (in the 15-62.5 μL and 50-200 μL range, respectively) would affect the amount and purity of the DNA fraction extracted. With this optimized protocol (i.e., the Lysis solution of Experiment VIII, and 1.5% SDS (25 μL) and 0.8 M potassium acetate pH 6.3 (50 μL) solutions), we were able to initiate the experiments, by first comparing the yield and purity of DNA extracted from young and mature leaves of curly lettuce, always referring to the baseline results of when pristine PS membranes were used. The purification protocol so established was then adopted to extract DNA from leaves of the purple, iceberg, and butterhead varieties of lettuce.

Finally, we compared the yield and purity of the DNA fraction extracted from young and mature leaves of curly lettuce leaves by use of our PPy-PS membranes to those obtained by following the recommended protocols of a commercial extraction kit (PureLink™ Genomic Plant DNA Purification, ThermoFisher Scientific, USA). These experiments were also performed in triplicate, and the quality of the collected DNA fraction was assessed by performing the amplification of a specific DNA sequence using a polymerase chain reaction (PCR).

The purified DNA samples were submitted to PCR amplification, using the ITS primers listed in Section 2.1. To prepare the master mix, we added 1.5 μL of purified DNA, 10 μL Fast SYBR Green Master Mix, 1.5 μL (10 pmol) of the forward primer, and 1.5 μL (10 pmol) of the reverse, plus 5.5 μL of RNase-Free Water, totaling a volume of 20 μL. As a negative, we used a sample consisting of 1.5 μL of RNase-Free Water. After adding the samples to the wells, they were subjected to the following cycling conditions: (i) 50°C for 2 min; (ii) 95°C for 3 min; (iii) and 40 sequential amplification cycles consisting of steps of 95°C for 3 s, 60°C for 30 s, and a final 4°C cooling step using a StepOnePlus ther-

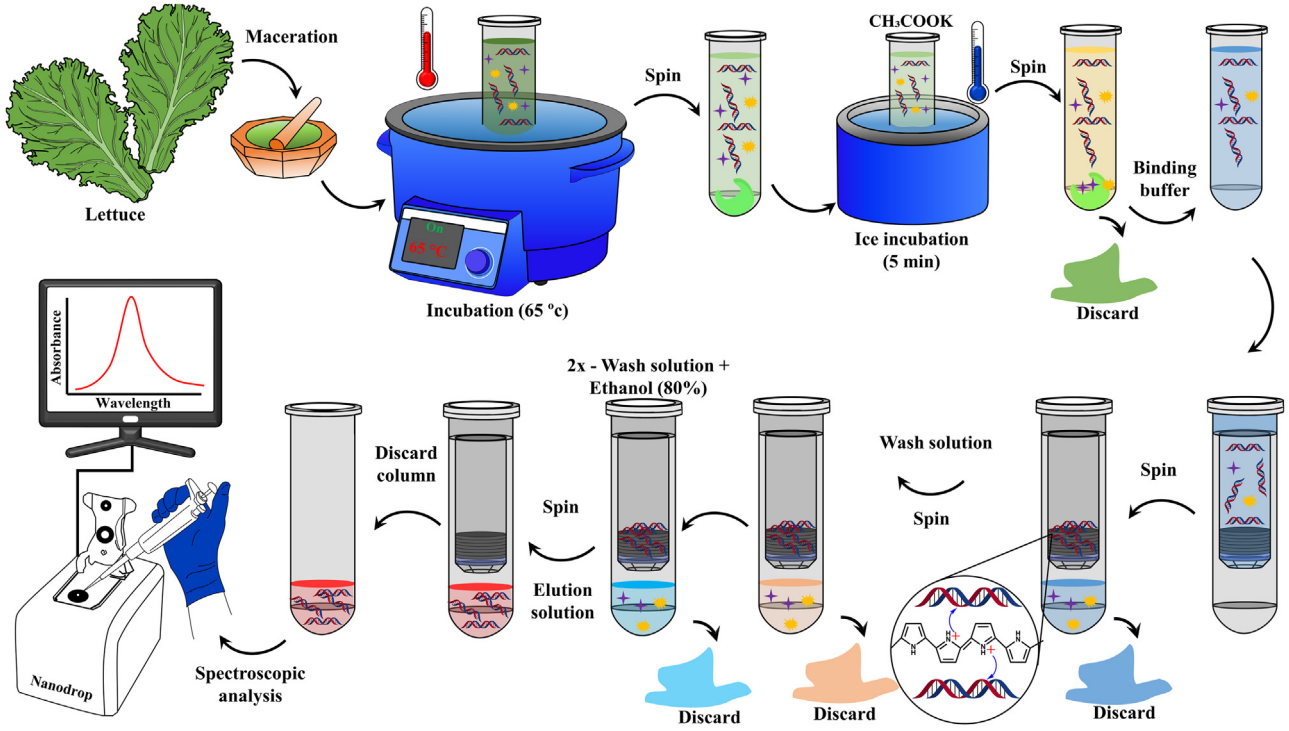

Fig. 1. Steps followed in the process of extraction and purification of DNA from lettuce leaves.

mocycler (Applied Biosystems, USA). The amplified samples were then purified using the QIAquick PCR kit (Qiagen, USA), and later analyzed by use of the electrophoresis technique. To implement these latter experiments, we used a 1% agarose gel and the diamond nucleic acid marker in a K33-15H horizontal vat (Kasvi, Brazil), followed by the addition of TAE buffer (0.5 X). Then, the DNA samples (8 μL) were mixed with the Blue/Orange 6X carrier (2 μL) and carefully placed into the wells of the equipment. Finally, we applied a voltage of 60 V for 15 min, and then 100 V for approximately 90 min. After the run, we took the gel to an MB-16 (Maestrogen, USA) UV (302 nm) transilluminator to capture the images of the DNA bands formed. Both PCR amplification and electrophoresis experiments were performed in duplicate.

3. Results

3.1. Characterization of polypyrrole/polystyrene membranes

3.1.1. Morphology, hydrophilicity degree, and electrical properties

In Fig. 2A, we show a collection of strips of the pristine PS and PPy-PS membranes, with the latter exhibiting the characteristic black color due to the incorporated polypyrrole chains (I), a 20,000 x SEM image that indicates that the PS mats are relatively free from defects and exhibit homogeneous fibers with an average diameter of (2.40 ± 0.35) μm (II), a 20000 x SEM micrograph of the PPy-PS membrane that reveals that the polymer is deposited as a thin layer on the surface of the PS fibers (III), and the surface of a PPy-PS fiber at higher magnification (50,000 x) (IV).

The thermally treated PS mats exhibited a high hydrophobicity, with an average contact angle of $\theta_c = (128.9 \pm 3)^\circ$. However, the plasma treatment favors the incorporation of PPy chains, and the resulting hybrid membrane becomes hydrophilic, exhibiting a contact angle close to 0°.

As a further confirmation of the successful incorporation of the PPy chains, and to better characterize the physical properties, we investigate the electrical conductivity of the PPy-PS membranes. As shown in the Supplementary Information, these films, with an average thickness of (0.119 ± 0.003) mm, exhibit an Ohmic behavior, with an electrical resistivity estimated to be of the order of (15.67 ± 3.22) Ω.m (Fig. SI-4).

3.1.2. Spectroscopic characterization

We collected the FTIR spectra of the PS and PPy-PS membranes to investigate their chemical composition (Fig. 2B). In the spectrum of the PS membrane (curve I), the peaks at 3060 cm^{-1} and 3026 cm^{-1} are associated with the C-H vibration of the aromatic ring [43], and those at 2855 cm^{-1} and 2922 cm^{-1} are related to the C-H stretching vibration of the CH_2 groups [44,45], while the peak at 1602 cm^{-1} is attributed to the aromatic C = C stretch. The peak at 1494 cm^{-1} corresponds to a flexion vibration of the C-H and the one at 698 cm^{-1} can be attributed to the mono-substituted aromatic rings [46]. In the spectrum corresponding to the PPy-PS membrane (curve II), one can identify the characteristic peaks of both PS and PPy. The peaks of 1553 cm^{-1} and 1492 cm^{-1} can be attributed to the stretching vibrations in the polypyrrole rings. The stretching vibration C-N can be seen at 1181 cm^{-1}. Finally, the bands at 1045 cm^{-1} and 1316 cm^{-1} are attributed to the deformation of the C-H bond [47,48].

In Fig. 2C, we present the UV-vis spectrum of the PS membrane (curve I), where one can see the polystyrene characteristic peaks at 212 nm and 258 nm, due to the presence of benzene rings. In the case of the PPy-PS membranes (curve II), one can observe the peaks characteristic of the protonated form of polypyrrole. The peak at 462 nm is attributed to the π-π^* transition of the benzenoid rings, while the peak at 880 nm corresponds to the presence of bipolarons characteristic of the conductive state of polypyrrole.

3.2. DNA adsorption experiments in a model system

3.2.1. Initial test and effect of interaction time and initial DNA concentration

As shown in Fig. SI-5A, the PS membrane used as a control was able to capture only 5.4% of the salmon sperm DNA, a value much lower than that obtained by use of the PPy-PS composite membrane (35.4%), As shown in Fig. SI-5B, the adsorption on the PPy-PS membrane proceeds at a faster rate in the first minutes of interaction, when most of

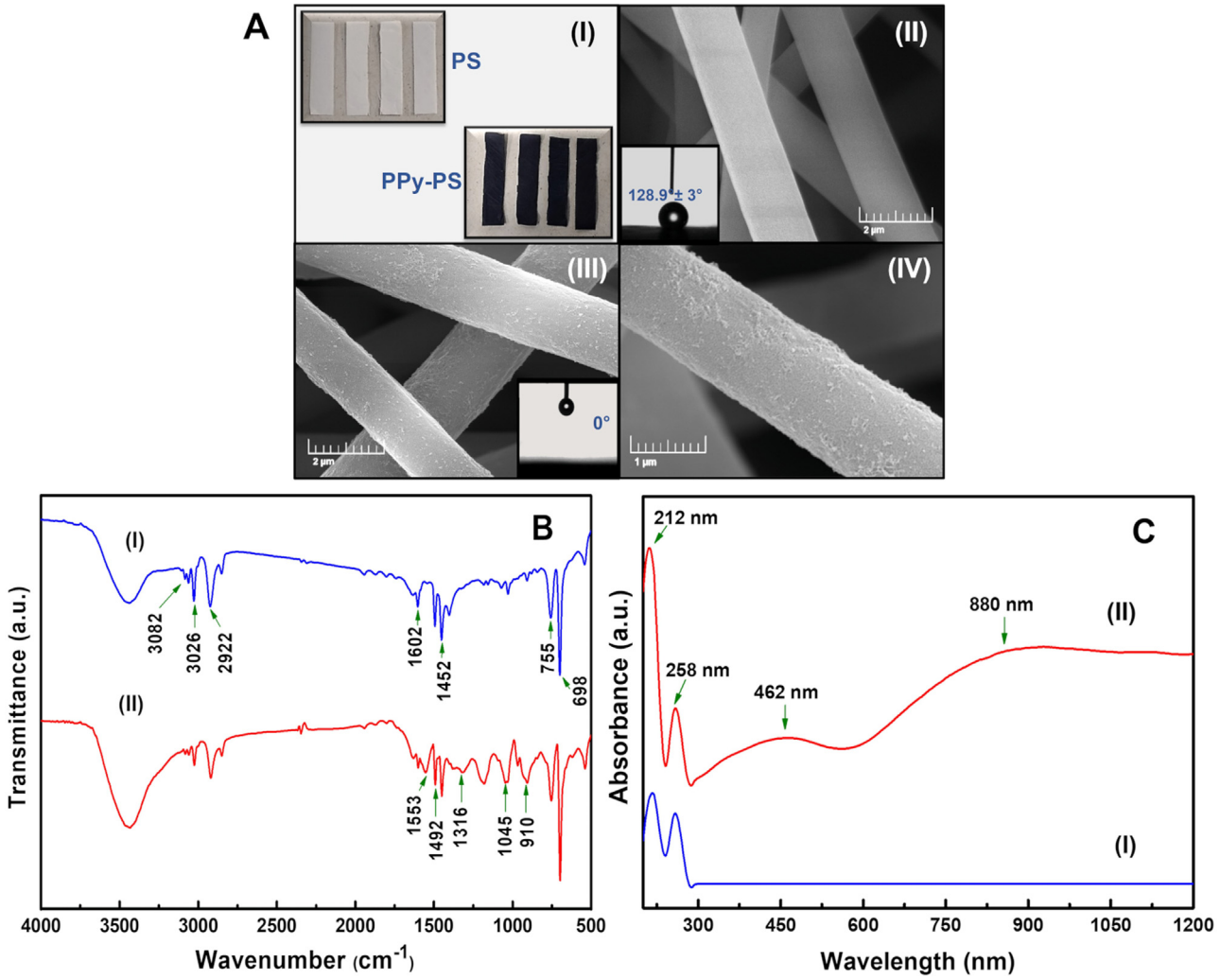

Fig. 2. (A) Collection of PS and PPy-PS membrane strips (I). SEM micrograph and contact angle (inset) of PS (II) and PPy-PS (III) membranes. (IV) Higher magnification (50000 x) of an individual PPy-PS fiber. (B) FTIR and (C) UV-vis spectra for PS (I) and PPy-PS (II) membranes.

the active sites of the membrane are still available. For an initial DNA concentration of 100 mg/L, an equilibrium adsorption capacity q_e of the order of 236.0 mg/g (i.e., mg of DNA per gram of PPy) was reached in 30 min.

In Fig. SI-5C, we show how the variation of the initial DNA concentration adsorption affects the capacity at equilibrium (q_e) and the degree of adsorption. When the amount of initial DNA is increased, a decrease in the percentage of adsorption and an increase in q_e are observed. For example, for an initial concentration of 10 mg/L [65 mg/L], 100% [53.2%] of the amount of dissolved DNA was captured by the membrane, with an adsorption degree of 179.0 mg/g [228.0 mg/g]. The corresponding values change to adsorption of 35.5%, and a q_e of 236.0 mg/g, for an initial DNA concentration of 100 mg/L.

3.2.2. Adsorption isotherms

For a better understanding of the adsorption of the DNA molecules on the PPy-PS membrane, we adjusted the corresponding experimental data to the Langmuir, Freundlich, and Temkin isotherm models – see Fig. SI-6. From the data presented in Table SI-3, one can observe that our data is best fitted by the Langmuir model, for which we obtained a correlation coefficient of 0.996 and lower AIC (Akaike Information Criterion) and RMSE (Root Mean Squared Error) values. In fact, by using the Langmuir model, we estimated the maximum adsorption capacity as 237.5 mg of DNA per gram of PPy, a value close to the experimental result (236.0 mg/g). In Fig. 3A, we show the fitting of the variation of the limiting adsorption capacity q_e as a function of the initial DNA concentration according to the Langmuir model.

3.2.3. Adsorption kinetics

To investigate the prevailing mechanisms in the kinetics of the adsorption process, we adjusted our experimental data to the pseudo-first-order, pseudo-second-order, Morris-Weber, and Boyd kinetic models - see Fig. SI-7. In Table SI-4, we present the corresponding optimized parameters. From the corresponding coefficients of determination (R^2) and AIC and RMSE values, it can be seen that the adsorption kinetics is best described by the pseudo-second-order model. This is confirmed by the fact that the corresponding estimated value of the maximum adsorption capacity (238.0 mg/g) is quite close to the experimental value (236.0 mg/g). In Fig. 3B, we present the adjustment of the limit adsorption capacity variation q_e as a function of the time allotted for the interaction accordingly to the pseudo-second-order model, with the linear fit for this model being shown in the inset.

3.3. Elution experiments

We examined how efficient would different alkaline aqueous solutions be in deprotonating the PPy chains, thus, promoting the elution of the adsorbed DNA in the membrane. Although the pH values of the alkaline solutions were close, there was a significant difference in the DNA

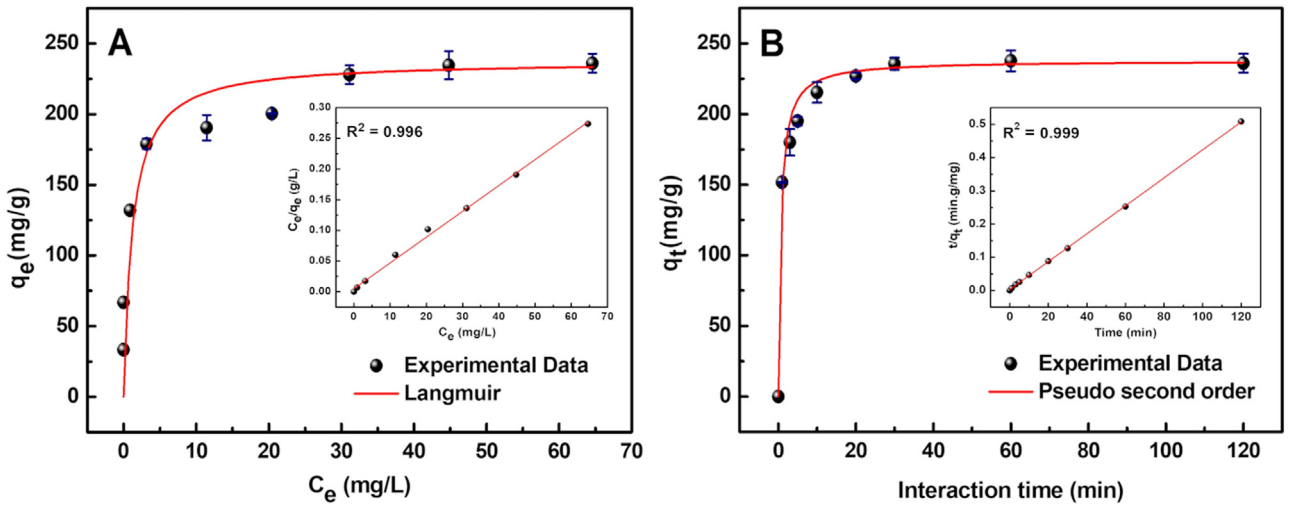

Fig. 3. Fitting of the experimental data of the DNA adsorption by the PPy-PS membrane according to (A) the Langmuir adsorption isotherm model; (B) the Pseudo-second-order kinetic model. (In the inserts, the corresponding linear fittings.)

elution percentages. As the results that are shown in Fig. SI-8 reveal, the highest elution percentage (~73.6%) was obtained when a Tris/SDS (pH 8.0) solution was used. Elutions of the order of 50% and 37.5% would result from the use of Tris/EDTA-Na (pH 8.5) and Phosphate/Phosphate (pH 7.6) solutions, respectively. In all cases, the presence of ions in the solutions favors the exchange with the phosphate groups of the DNA, especially the long-chain sulfate anions of the SDS surfactant.

3.4. Fluorescence microscopy

We used a fluorescence microscope to capture images of the membrane, allowing us to follow the DNA adsorption and elution process (Fig. SI-9). While the PPy-PS membrane appears dark (Fig. SI-9A), small green dots with low intensity can be visualized after interaction with the SYBR Green dye (Fig. SI-9B). On the other hand, after exposure to the aqueous solution containing dissolved salmon sperm DNA strands, we can observe a large number of brighter green dots (Fig. SI-9C). As expected, there is an absence of these bright spots after the elution step (Fig. SI-9D). The corresponding 3D images of the measured fluorescence are shown as insets in Figs. SI-9B, SI-9C, and SI-9D.

3.5. Comparison with other DNA adsorbents

To better assess the performance of the hybrid PPy-PS membranes as competitive materials for purifying salmon sperm DNA, we compared their operational parameters, such as adsorption capacity, elution rate, and the times required for the adsorption and elution processes to reach the equilibrium, with those of different adsorbents (Table SI-5). Based on these results, we can affirm that after 30 min of interaction the proposed membrane has an excellent adsorption capacity (236.0 mg/g) when compared to other materials. In particular, the MSP ZrBF [γ-Fe_2O_3/PEDOT MNC] adsorbent has a capacity similar to that found by the composite membrane, with an estimated value of 238.6 [248.7] mg/g; however, in both cases, the time for the adsorption process to reach equilibrium was longer, around 480 [360] min. In the elution process, one could manage the PPy-PS membrane to release 73.6% of the adsorbed DNA in 30 min, parameters that are competitive relative to the performance of other materials. For instance, a higher efficiency (89.0% [89.5%]) was observed for MSP ZrBF [MSP Fe_3O_4/SiO_2], but in a longer time (240.0 [60.0] min). The good adsorption and elution capacity of the composite PPy suggest that this is a promising adsorbent material to be used in the extraction of DNA strands from plant tissues.

3.6. DNA extraction from vegetables

3.6.1. Protocol optimization

The results obtained by performing the 2^3-factorial design adopted to optimize the extraction and purification of DNA from curly lettuce leaves by using the PPy-PS membranes can be seen in Fig. 4A. We found that experiment VIII, where the highest concentrations of the control factors (Tris/HCl, NaCl, and EDTA-Na) of the cell lysis solution were used, corresponds to the best condition when an average DNA concentration of 70.9 mg/L was obtained with an appropriate degree of purity (with an $A_{260/230}$ ratio in the 2.0-2.2 range). However, we observe that the average $A_{260/280}$ ratio of 1.6, while still acceptable, indicates a slight presence of proteins or other contaminants. Statistical analysis was performed using the Statistica 10 software (Statsoft, USA) to obtain the Pareto graph (Fig. SI-10). The graph showed which of the three factors analyzed (concentration of Tris/HCl, NaCl, and EDTA-Na) or their interactions should be statistically significant according to the variation between the two chosen levels. The red line at 0.05 indicates the statistical significance limit (Fig. SI-10).

In the optimization of the protocol for isolating DNA from lettuce leaves, we adopted conditions of experiment VIII (+++) and varied the volume of the surfactant (SDS 20%) in the lysis buffer. As the results that are shown in Fig. 4B indicate, we observed a slight increase in the DNA concentration (to approximately (76.6 ± 7.5) mg/L), with no noticeable change in the $A_{260/280}$ and $A_{260/230}$ ratios when 25 μL of SDS was used (corresponding to a final concentration of ~1.5%). We also evaluated the influence of varying volumes of the potassium acetate solution. By changing the volume of the solution, we controlled the final concentration of the potassium acetate solution (50 μL/0.8 M, 130 μL/1.6 M, and 200 μL/2.0 M). In Fig. 4B, it is possible to observe that, when using a smaller volume (50 μL), there is a considerable increase in the DNA concentration (average of (147.4 ± 9.6) mg/L) and an improvement in the $A_{260/280}$ ratio (average of 1.7 ± 0.01).

When the optimized purification protocol was used for extracting DNA from mature lettuce leaves using pristine PS membranes, only a small fraction of DNA was captured (~ 35.4 mg/L), with a purity of 1.5 and 2.0 for $A_{260/280}$ and $A_{260\ /230}$, respectively (see Fig. SI-11).

3.6.2. Effect of leaf maturity and lettuce type on the DNA yield

To obtain DNA fractions from plant tissues in larger quantities and with good quality, whenever possible is preferable to use young leaves as the source material. To establish these effects on our PPy-PS membrane-based protocol, we compared the amount of purified DNA concentration

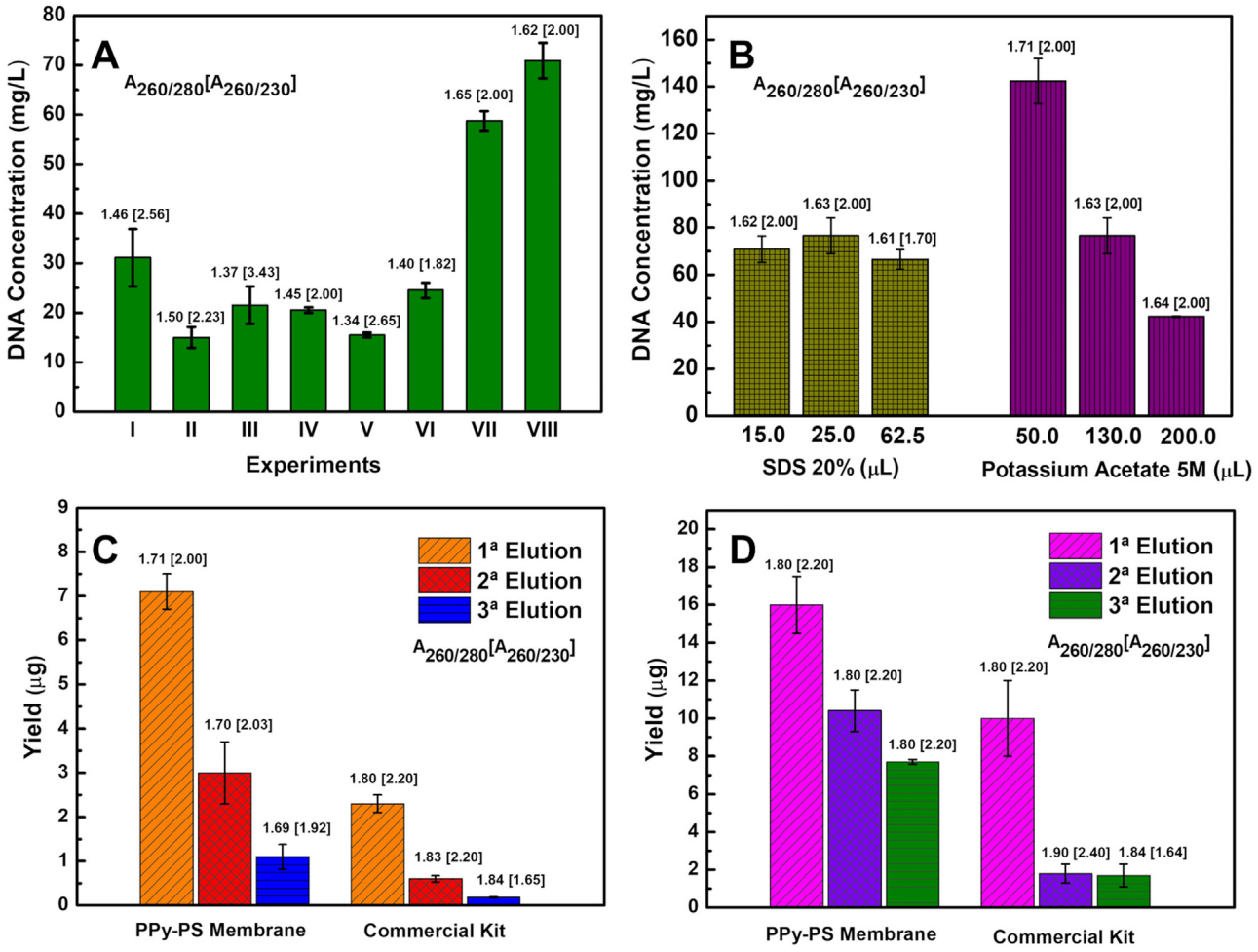

Fig. 4. (A) Concentration of extracted DNA for the different factorial assays and effect of the volume variation of the relevant parameters in different assays during the optimization of the extraction protocol (B). Comparison of DNA yield for the DNA extraction of mature (C) and young (D) lettuce leaves using the PPy-PS membrane and the PureLink™ Genomic Plant DNA Purification kit, after three successive elutions.

when mature and young lettuce leaves were used. As expected, we obtained a larger DNA concentration of DNA ((328.5 ± 1.6) mg/L), with a higher degree of purity ($A_{260/280}$ [$A_{260/230}$] = 1.8 [2.0]), when using young lettuce leaves. When performing similar experiments with the pristine PS membranes, the adsorption capacity was only 83.1 mg/L and the corresponding purity was $A_{260/280}$ [$A_{260/230}$] = 1.6 [2.1] (Fig. SI-11).

Differences in the texture of the leaves of different types of lettuce can affect the yield and quality of the extracted DNA fraction. While for iceberg lettuce we estimated an average concentration of 74 mg/L and ratios of 1.7 [2.0], for purple and butterhead lettuce we obtained values of 289.0 mg/L (1.8 [2.0]) and 233.8 mg/L (1.8 [2.0]), respectively.

3.6.3. Yield comparison with a commercial kit

To evaluate the efficiency of PPy-PS membranes in extracting nucleic acids from plant tissues, we compared the yields of their use in the purification of DNA from young and mature leaves of curly lettuce with those obtained by following the extraction protocol of a dedicated commercial kit (Fig. 4). In both cases, we subjected the extracted DNA fraction to three consecutive elutions.

As the results that are shown in Fig. 4C for mature lettuce leaves indicate, a larger amount of DNA was extracted by using PPy-PS membranes (a total of 11.2 μg, corresponding to partial fractions of 7.1 μg, 3.0 μg, and 1.1 μg after each consecutive elution) than using the commercial kit (a total of 3.2 μg after partial extractions of 2.3 μg, 0.7 μg, and 0.2 μg). When the source material corresponded to young lettuce leaves (Fig. 4D), we again achieved a larger amount of DNA for each elution using PPy-PS membranes (a total of 34.1 μg, corresponding to partial fractions of 16.0 μg, 10.4 μg, and 7.7 μg after each consecutive elution) than using the commercial kit (a total of 13.5 μg after partial extractions of 10.0 μg, 1.8 μg, and 1.7 μg).

In Fig. 4C and D, we also show the corresponding $A_{260/280}$ and $A_{260/230}$ ratios. The average values found (~1.7-1.9 and ~ 1.6-2.4) reveal the excellent DNA quality for both materials. As can be seen in the graph, the lowest value (1.6) of the $A_{260/230}$ ratio was obtained in the 3rd and last elution, when we used the commercial kit for young and mature lettuce leaves.

Overall, these results suggest that PPy-PS membrane-based protocols appear as a promising alternative for the extraction and purification of DNA fractions from plant tissues.

3.6.4. DNA integrity and quality

In both cases, i.e., when using the PPy-PS membrane and the commercial kit, we subjected the collected DNA fraction using to electrophoresis experiments, for the evaluation of its corresponding integrity. In all cases, the results reveal the presence of well-defined DNA bands with similar intensities, confirming that the purification steps do not induce the denaturation of the collected DNA in the different types of lettuce leaves examined (Fig. 5A). We point out that identical bands were obtained when the commercial kit was used to extract DNA from young curly lettuce leaves.

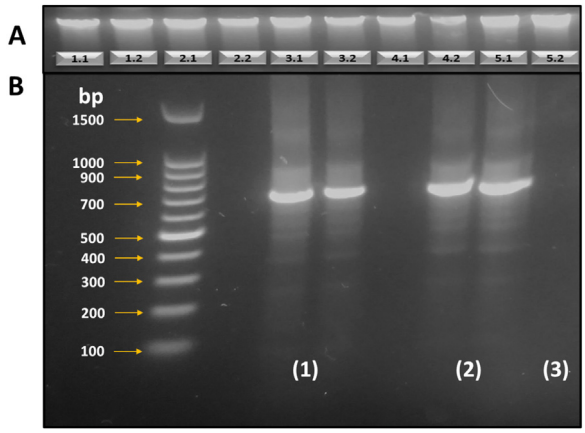

Fig. 5. (A) Results of electrophoresis runs (in duplicate) for DNA extracted from young lettuce leaves using the PPy-PS membrane for curly (1.1 and 1.2), purple (2.1 and 2.2), iceberg (3.1 and 3.2), and butterhead (4.1 and 4.2) lettuce, and of curly lettuce, using the commercial kit (5.1 and 5.2). (B) PCR amplification results for curly lettuce DNA samples, using PPy-PS membrane (1), the PureLink™ Genomic Plant DNA Purification kit (2), and for the negative sample (3).

As an additional test of the quality of the curly lettuce DNA samples obtained, we submitted them to PCR amplification experiments, a procedure known to be extremely susceptible to the presence of contaminants. Afterward, we subjected the amplified samples to electrophoresis runs in agarose gel. In all cases (Fig. 5B), one can identify intense bands with a size of approximately 700 base pairs (bp), as should be expected for the set of primers used in the ITS region. Once again, the results obtained after using a PPy-PS membrane (1) were identical to those resulting from the use of a commercial kit (2). As expected, in the negative control (3) no extraneous bands were present. The successful amplification of the collected samples demonstrates the good purity and integrity of the purified DNA fraction, which is therefore of adequate quality for further use in subsequent molecular biology procedures.

4. Discussion

The diversity of plant species is a severe obstacle to the definition of a generic protocol for the extraction of large quantities of good-quality DNA from vegetable tissues. In recent years, the search for methods of extraction and purification of plant DNA that are simple, low-cost, and avoid the use of toxic solvents such as chloroform has grown considerably [13,17]. In addition to being a time-consuming procedure, the extraction method that uses toxic solvents not only can generate contamination in the resulting DNA sample but also exposes the operator to risks of long-term health problems.

In this work, we show the use of the PPy-PS composite membrane as an active material for capturing DNA molecules extracted from plant tissue dissolved in aqueous media. The results of the morphological and spectroscopic characterization revealed the success of polypyrrole incorporation on the surface of the fibers that compose the PS membrane [43–50]. From this, it was possible to explore the properties of polypyrrole in its protonated state to electrostatically interact with the phosphate groups of DNA molecules [51]. The initial experiments of DNA adsorption of commercial salmon sperm in batch mode showed that the presence of PPy chains on the surface of the PS membrane fibers favored a marked increase in the adsorption percentage. The PPy-PS membrane exhibited a maximal adsorptive capacity of 236.0 mg/g after the interaction reached equilibrium in just 30 min. After that, by varying the initial concentration of DNA we verified that there was a decrease in the degree of adsorption with the increase in the amount of DNA initially in the solution. While a progressive reduction in the number of available active sites on the surface can explain the decrease in the adsorption degree, the driving force of the mass transfer from the solution to the adsorbent process becomes more intense with a higher initial DNA concentration. The greater number of nucleic acid molecules available to interact with the active sites on the membrane results in a higher q_e [52].

When we fitted the data to the Langmuir isotherm model, we found that the calculated adsorption capacity was close to the experimental value. This result indicated that the double strands of DNA adsorb homogeneously on the surface of the membrane, forming a monolayer [52,53]. Furthermore, examining the corresponding adsorption kinetics, it is easy to see that the pseudo-second-order model was the one that best fit our experimental data, a result that supports the hypothesis that DNA strands are adsorbed by the PPy-PS membrane according to chemical processes [54]. In the present case, the PPy chains are doped (in this work, with Cl^- ions), and the amine groups of PPy are highly protonated [55], as confirmed by UV-vis spectroscopic analysis through the 880 nm band corresponding to polarons and bipolarons [50]. In the presence of DNA, the Cl^- counterions associated with the PPy chains are replaced by the phosphate groups of the DNA, in an ion exchange process. After studying the adsorption process, we investigated the possibility of eluting the captured DNA fraction, a step that is extremely important in a DNA purification protocol. The efficiency of this process depends on parameters such as pH and ionic strength [56]. The higher elution percentages obtained in the experiments using the Tris/SDS elution solution can be associated with the longer chains of the surfactant that favor the exchange with the phosphate groups of DNA. An interesting result of the DNA adsorption and elution process was shown in the fluorescence microscopy images after using the SYBR Green dye, which exhibits strong fluorescence after interaction with double-stranded nucleic acids [57]. From this, we could confirm the occurrence of the steps of capture and release of DNA from the membrane surface. The success of the batch adsorption and elution experiments using commercial salmon sperm DNA gave us the motivation to investigate the use of these composite membranes for DNA purification from real samples.

In the literature, several authors have discussed the difficulties in purifying DNA from plant samples, especially those with a high degree of contaminants, such as polysaccharides, proteins, polyphenols, and others [58]. In this work, we adapted the PPy-PS membrane in a column centrifuge system since this method can be easily operated with the aid of a centrifuge. We adopted this setup to optimize a protocol for extracting and purifying DNA from vegetable samples, using mature curly lettuce leaves as a template. The results of the factorial design showed that at high concentrations of the three components initially studied, we have a greater amount of purified DNA. Specifically, the statistical analysis indicated that the DNA concentration is increased mainly when using the lysis solution with the highest concentration of NaCl and EDTA-Na and interactions (NaCl and EDTA-Na, Tris/HCl and EDTA-Na), as indicated by the positive value of the standardized effect (NaCl and EDTA-Na = 9.80; NaCl = 8.95; EDTA-Na = 8.52, and the interaction of Tris and EDTA-Na = 3.97. It is suggested that, for example, NaCl at high concentrations can remove proteins from DNA, which in turn, protected by EDTA-Na, remain free in the solution. With the expectation of further optimizing the protocol, and obtaining purer DNA samples with higher yields, we examined the final concentration of SDS (to obtain greater efficiency in breaking the cell membrane [59]) and of potassium acetate, responsible for the precipitation of proteins and polysaccharides. The results revealed that there was no significant change in the DNA concentration when we changed the concentration of SDS. On the other hand, when using a low final concentration of potassium acetate (0.8 M) a significant increase in DNA concentration was evident, with a slight improvement in the $A_{260/280}$ ratio (related to the presence of proteins in the DNA samples). We suggest that when using a lower final concentration of this reagent, there is greater precipitation of the complex formed by the surfactant SDS-protein and polysaccharides [60], leaving the DNA free of these contaminants in the solution. On the other hand, at higher final concentrations (1.6 M and 2.0 M), the excess ions present in the solution can interfere with the precipitation process, or even allow

the interaction of DNA with the complex formed, leading to a decrease in the final yield of purified DNA. As expected, the PS membrane used as support contributed minimally to the adsorption of the DNA chains, since lower values of concentration and purity ($A_{260/280}$) were obtained when pristine PS were used in the purification process.

When the optimized protocol was tested for young lettuce leaves, a greater amount of DNA with better quality was obtained. This result is in agreement with the pertinent literature, which suggests that whenever possible it is preferable to use young leaves as the source material. Mature leaves generally contain a greater degree of species such as polyphenols and polysaccharides [61], which act as contaminants in DNA extraction protocols, characteristics that can result in a lower yield and DNA quality. We also evaluated the protocol for different types of young lettuce leaves, and we realized that the use of plant tissues that have a thicker texture, such as iceberg-type lettuce, represented an obstacle to the initial step of manual homogenization in the extraction protocol leading to a lower yield than those obtained by purple and butterhead [62] young lettuce leaves, which have a softer texture. In this case, liquid nitrogen should be used to aid in the homogenization time [63].

We compared the yield and quality of purified DNA from curly lettuce leaves in their mature and young state when we applied the PPy-PS membrane protocol and a commercially available kit. The results showed that when using the PPy-PS membrane in the DNA purification process, for both cases (mature and young lettuce leaves) a higher DNA yield was obtained relative to that resulting from the use of a commercial kit. In general, the evaluated protocols produced DNA samples with the optimal purity required for the later use in molecular biology protocols, as was shown in PCR amplification, in which expected amplicon sizes were obtained [35,64]. The results achieved showed the importance of the protocol optimization study presented in this work. By varying the concentrations of the reagents, it was possible to identify the optimal concentrations that result in a good yield and the purity more suitable for subsequent DNA applications.

5. Conclusion

In this work, we report a simple method for the preparation of hybrid PPy-PS membranes manufactured after exposing the electrospun PS films to an *in situ* chemical polymerization of the pyrrole monomer. The PPy-PS membranes exhibited an excellent affinity for DNA dissolved in aqueous media, reaching an adsorption [elution] capacity of 236.0 mg/g [73.6%], values that are comparable to those obtained by the use of other adsorbents discussed in the literature. Preliminary studies of the DNA extraction protocol from mature [young] lettuce leaves showed that the PPy-PS membrane has an excellent yield of 7.1 [16.0] μg after a first elution, a favorable result relative to the yield obtained with the commercial kit (2.3 [10.0] μg). Furthermore, the quality and purity of the purified DNA were confirmed through the process of PCR and electrophoresis. Thus, the simple and fast protocol for extracting DNA from lettuce leaves suggests that PPy-PS membranes represent a promising alternative for extracting DNA from plant tissues.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.bbiosy.2022.100060.

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