Confocal Raman imaging as a useful tool to understand the internal microstructure of multicomponent aerogels

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Abstract

This work shows the characterization of (nano)cellulosic aerogels prepared from *Posidonia oceanica* waste biomass by means of Confocal Raman Microscopy (CRM). For this aim, aerogels were prepared by simple freeze-drying of aqueous dispersions of 4 (nano)cellulosic fractions with different purification degrees, tested at 2 different concentrations (0.5% and 2%). These were then coated with PLA in order to improve their hydrophobicity and subjected to oil sorption-desorption experiments. Both univariate and multivariate analyses, including an approach based on comparing the spectra with those of reference materials and another one based on automatic detection of components, were compared in terms of the quality and the accuracy of the information provided. Univariate analysis only provided accurate information in the simplest systems (native (nano)cellulosic aerogels), while multivariate analyses facilitated the detection of the different components even for the most complex structures. Automatic identification of components was selected as the optimal methodology, although it also underestimated the abundance of the components with the least intense Raman spectra (cellulosic clusters) in the presence of PLA and oil. Comparison with the reference materials resulted in unrealistic images for the most complex systems. Micron-sized regions of concentrated cellulose were detected using CRM, being more abundant in the denser aerogels. Results also confirmed that PLA was preferentially located close to the surface, while oil could penetrate deeper along the matrix. Overall, the results showed the potential of Raman imaging as a novel approach for the characterization of complex biopolymeric aerogels.

Keywords:

Raman microscopy, hyperspectral imaging, cellulose, multivariate analysis, renewable.

1. Introduction

Aerogels are extremely light and porous materials with high surface area and low density, which are useful for a wide range of applications due to their high absorption and adsorption capacities¹⁻³. Although aerogels have been traditionally made from inorganic materials^{4,5}, their application as adsorbent/absorbent structures (pads) in food packaging requires the search of alternative and more sustainable materials with properties that resemble those from the commercially used synthetic polymeric pads. In this sense, cellulose is being explored as one of the most promising biopolymers for the development of high performance aerogels⁶⁻⁹. Cellulose can be extracted from several resources such as cotton, spruce or wood¹⁰⁻¹². However, these resources usually compete with their agricultural purpose and so become unsustainable. As a result, alternative sources such as aquatic biomass and, in particular, waste biomass, have been recently proposed as sustainable sources for the extraction of cellulosic fractions¹³⁻¹⁵.

Posidonia oceanica (*P. oceanica*) is a marine seagrass endemic to the Mediterranean Sea whose native form can be beneficial for other marine organisms and seashores. However, during its lifecycle, *P. oceanica* leaves detach off their stems and, after being dragged by marine currents, accumulate on the coasts, generating a residue which is detrimental for the quality of the beaches, tourism and local economy¹⁶. In an effort to valorise this residue, its utilization as a renewable source of cellulose and nanocellulose has been recently proposed¹⁷. The common processes for the extraction and purification of cellulose from plant-derived biomass generally involve aggressive Soxhlet, alkali and acid treatments¹⁸. Interestingly, less purified cellulosic and nanocellulosic fractions have been obtained by simpler extraction protocols, in which both the Soxhlet and alkali treatments were omitted. These fractions were reported to contain lipidic and hemicellulosic components¹⁷, whose presence was beneficial for the preparation of

aerogels, significantly affecting their microstructure. These (nano)cellulosic aerogels exhibited lower density and higher surface area than the pure (nano)cellulose aerogels, being capable of adsorbing large amounts of oil; however, they presented very poor performance in contact with water. In this context, a simple PLA dipping strategy was developed in order to increase their hydrophobicity and mechanical performance, yielding PLA-coated aerogels with remarkable oil-sorption capacity (more than 900% w/w) making them suitable for food-packaging applications as adsorbent pads or for oil spill cleaning applications ¹⁹.

Unarguably, the microstructure of these materials has a critical impact on their functional and mechanical properties. Although a preliminary analysis of the microstructure of the (nano)cellulosic aerogels and the effect of PLA coating was carried out in our previous work ¹⁹, the study was only performed on the surface of the materials. Confocal microscopy techniques allow visualisation of different thin optical sections of samples and, thus, can provide three-dimensional information without physically sectioning the samples or disturbing their internal structure ²⁰. As opposed to the more commonly used Confocal Scanning Laser Microscopy (CSLM) technique, which often requires the use of fluorescent dyes that can potentially alter the microstructure of the specimens ²¹, Confocal Raman Microscopy (CRM) is based on the principles of Raman spectroscopy, where each pixel of the resulting images comprises the chemical information obtained from the Raman spectra collected from each spot in the sample. Therefore, it can simultaneously provide visual and compositional information of the mapped sample area. The usefulness of CRM to study the microstructure of different systems such as plant cell membranes ²² or even specific food components present in bread or cheese has been already demonstrated ^{23,24}. However, to the best of our knowledge, the study of aerogels by CRM represents an innovative approach.

When mapping the distribution of different components in a material through CRM, there are different data processing approaches to transform the information of the Raman spectra into an image, and the selected method can have a critical impact on the quality and accuracy of the information obtained. These methods can be divided in univariate and multivariate. On one hand, univariate methods consist of analysing the sample by taking into account a small concrete region of the spectrum and is the methodology most commonly followed and reported in the literature ^{22,25,26}. On the other hand, multivariate analyses are more complex approaches which allow taking into account the whole spectra of the analysed area and, thus, consider all the chemical information available ^{23,24}.

This work explores the potential of CRM to study the microstructure of PLA-coated (nano)cellulosic aerogels produced from *P. oceanica* waste biomass by optimized protocols. The aerogels were studied before and after the PLA dipping process, as well as the internal distribution of oil within the PLA-coated aerogels after oil immersion. Different data processing approaches (univariate and multivariate) were applied and compared in order to establish the optimal methodology for the analysis of this type of materials through CRM. CLSM was used as a complementary technique to compare the results obtained from CRM.

2. Materials and methods

2.1. Raw material

Biomass waste material consisting of *Posidonia oceanica* leaves was collected in the sea shore of Calpe (Alicante, Spain) in January 2018, washed with water to remove sand and salts, and stored under refrigeration conditions (4° C) until further use.

2.2. Preparation of cellulosic fractions and nanocrystals

A purification procedure described in previous works ^{14,18,27}, consisting of a Soxhlet extraction, followed by acid bleaching and an alkaline treatment, was carried out to sequentially remove cell wall components from *P. oceanica* waste biomass and obtain pure cellulose (F3). Additionally, less purified holocellulosic fractions were produced by omitting the alkaline treatment (F2) or both the Soxhlet and alkaline treatments (F2A). Subsequently, these three fractions were subjected to an acid hydrolysis ¹⁷, yielding nanocellulosic fractions (NANO F3, NANO F2 and NANO F2A).

2.3. Production of the aerogels

Cellulosic and nanocellulosic aerogels were produced from aqueous dispersions of the (nano)cellulosic materials at concentrations of 0.5% and 2% (w/v). The dispersions were freeze-dried to remove water and obtain the (nano)cellulosic primary scaffolds. These aerogels, which were selected due to their high sorption capacity and good processability ¹⁹, were coded according to the (nano)cellulosic fraction used (F3, NANO F3, NANO F2A and NANO F2). The aerogels were then coated with PLA using a proprietary technology ^{19,28} to form the final hybrid structures (F3 + PLA, NANO F3 + PLA, NANO F2A + PLA and NANO F2 + PLA). All the aerogels were stored in equilibrated relative humidity cabinets at 0% RH and 25°C for at least three days prior to their characterization.

2.4. Oil immersion

Small pieces (~1cm²) of the PLA-coated (nano)cellulosic aerogels were immersed in sunflower oil for at least 1 minute. Oil excess was carefully withdrawn until the material stopped dripping. Oil-immersed aerogels were coded as F3 + OIL, NANO F3 + OIL, NANO F2A + OIL and NANO F2 + OIL. These aerogels were then stored at ambient

conditions and placed on top of filter paper to accelerate oil release. After 7 days, equilibrium was considered to be reached for the oil desorption process, according to previous studies ¹⁹, and the equilibrated aerogels were also characterized and named as F3 + OIL (f), NANO F3 + OIL (f), NANO F2A + OIL (f) and NANO F2 + OIL (f).

2.5. Confocal laser scanning microscopy (CLSM)

Sunflower oil was stained by adding 0.1% (v/v) of Nile Red in ethanol (1:10 Nile Red:Oil) and vortexed vigorously until a homogeneous solution was achieved. Aerogels were then immersed in the stained oil for at least 10 minutes in order to maximize oil sorption. Immersed aerogels were then visualized using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) with 5x dry objective (numerical aperture of 0.12) and the pinhole set at 1 Airy Unit. Nile red was excited at 488 nm using an Argon laser, and the emission filter was set at 560-600 nm. A second filter set at 488 nm was also used to observe the light reflected by the cellulosic materials. Leica LAS AV software (v 2.7.3.9723) was used to acquire digital images of 1024 x 1024 pixels in size at day 1 and 7 after immersion. Samples were placed in darkness conditions between imaging to avoid fluorescence loss.

2.6. Confocal Raman microscopy (CRM)

2.6.1. Equipment and software

An Alpha300 R confocal Raman microscope (WITec, Germany) equipped with a 532 nm laser and an ultra-fast Raman imaging CCD camera was used for all the CRM analysis. The Raman shift was calibrated using silicon. Project Five software v5.0 (WITec, Germany) was used for image processing and analysis.

2.6.2. Acquisition of single Raman spectra of reference materials

Single Raman spectra of the reference materials were initially obtained. Pieces of the (nano)cellulosic aerogels (0.5% and 2%), raw PLA pellets and an oil droplet were placed on microscopy slides. Additionally, the original (nano)cellulosic fractions were oven-dried at 60 °C for at least 2 h in order to obtain denser materials. This was expected to result in more intense Raman spectra, given that the aerogels were mainly composed by air ($> 98\% \text{ v/v}^{19}$). These samples were named “dry (100%)” and also mounted on microscopy slides. A 50x microscope objective (0.55 numerical aperture) was then used to collect the Raman spectra of the samples at 5 different points, with 20 accumulations of 1 s of integration time each and laser power of 30 mW. The raw spectra were processed using the cosmic ray removal correction function of the software (filter size 3, dynamic factor 8) and a shape function for the background subtraction (shape size 250, noise factor 1). Finally, the 5 spectra of each sample were averaged and added to a library to be used as reference spectra.

2.6.3. Confocal Raman imaging of the aerogels

2D micrographs (depth scans, i.e. cross section images) of the four different (nano)cellulosic aerogels and their respective PLA-coated aerogels were obtained using a 10x objective (0.25 numerical aperture). Areas of $200 \mu\text{m} \times 200 \mu\text{m}$ (X, Z) were scanned at 150 points per line and 150 lines per image, and spectra were collected with a laser power of 25 mW and an integration time of 0.25 s, which resulted in a measurement time of approx. 40 minutes. Three different cross sections were scanned for each aerogel. Hybrid PLA-coated aerogels were also imaged in triplicate after oil immersion and oil release (after 7 days).

Image z-stacks were also obtained for the aerogel which previously showed the most optimum performance (NANO F2A 2%)¹⁹, in order to study the three-dimensional distribution of components in the original aerogel. In addition, images were acquired for the PLA-coated aerogel before (NANO F2A 2% + PLA) and after oil immersion (NANO F2A 2% + OIL). In this case, volumes of 200 μm x 50 μm x 200 μm (X, Y, Z) were scanned at 100 points per line, 25 lines per image and 100 layers per stack with an integration time of 0.35 s per point, resulting in a measurement time of approx. 8 hours. The laser power was fixed at 30 mW while 10x was the selected objective.

2.6.4 Bright field imaging

Optical 2D micrographs of the aerogels were also obtained using the bright field mode of the confocal Raman microscope, with the 50x objective on the aerogels' raw surface, after PLA coating and after oil immersion and release. Surface areas of 1000 μm $\times$ 1000 μm (X, Y) were imaged at 1000 points per line x 1000 lines per image.

2.7. Image processing and analysis

After cosmic ray removal and background subtraction, performed as described in Section 2.6.2, the spectra from the depth scans were processed following three different procedures to map the spatial distribution of the different components (i.e. cellulose, PLA and oil), the first two based on those previously explored by Huen, Weikusat, Bayer-Giraldi, Weikusat, Ringer and Lösche²³. These three methods were then compared to evaluate differences in the information provided.

2.7.1. Integration of single Raman bands (univariate analysis)

In the first method, single Raman bands characteristic for each component were integrated. Monochromatic images were generated, where the intensity of the pixels was proportional to the area of those individual bands in each measurement point. The specific spectral ranges selected for each component are marked in blue in Fig. 1, and are identified by their central value in the text and figures. Every filter used had a width of 60 cm^{-1} except the one centred at 2955 cm^{-1} whose width was 30 cm^{-1} in order to avoid overlapping with nearby bands. Additionally, a combined image was created overlapping the resulting images of single filters derived from each component at every aerogel.

2.7.2. Multivariate analysis based on reference materials

The second method considered the full Raman spectra rather than single bands using the True Component Analysis tool of the software. This function is based on the basis analysis algorithm, and describes each measured spectrum of a hyperspectral dataset by a linear combination of reference spectra, as depicted in equation 1, where $\vec{S}_i$ is spectrum i from the hyperspectral dataset, $\vec{H}_i$ is the mixing values for spectrum i , $\vec{E}_i$ is the error spectrum and $\hat{B}$ is the matrix of basis (reference) spectra. The mixing values are fitted by the method of least squares minimizing the expression shown in equation 2.

$$\vec{S}_i = \hat{B}\vec{H}_i + \vec{E}_i \quad \text{Eq. 1}$$

$$(\vec{S}_i - \hat{B}\vec{H}_i)^2 = \text{Minimum} \quad \text{Eq. 2}$$

In practice, the spectra of the reference materials are the inputs, and the outputs are one image for each component spectra showing the intensity distribution for this spectrum. For this analysis, every single spectrum expected to be present in each sample was added

as inputs (i.e. all cellulosic spectra in raw aerogels, cellulosic spectra + PLA in PLA-coated aerogels and cellulosic spectra + PLA + oil in immersed PLA-coated aerogels both before and after oil release). ImageJ-win64 software was utilised for calculating the relative abundance (%) of each component in every image by analysing their coloured histogram. Briefly, the distribution and area (in pixels) of each colour was obtained and compared to the total area of the image.

2.7.3. Multivariate analysis based on automatic component identification

A third method based on the automatic component identification was carried out. This method also used the True Component Analysis function of the software but without providing any reference spectra. Instead, the software was asked to select a first spectrum from the whole dataset (the most intense) as a base spectrum and calculate the residual image. If the residual image is structured, subsequent cycles of adding components automatically and calculating the corresponding residual images are performed until it was considered that the residual image showed only noise. Figure S1 (Supporting Information) shows an example of a final residual image. The components automatically detected were then demixed, compared with a library built with the reference spectra, and assigned to the one with the highest similarity index ($\geq 90\%$) using the True Match software. Again, ImageJ software was used for calculating the relative abundance (%) of each component in every image by analysing their coloured histogram. In both the second and third methodologies, each sample point was assumed to proceed from a linear combination of the single components of the systems analysed.

2.7.4. 3D image analysis

The individual 100 layers of the z-stack were processed using the third method (Section 2.7.3) and exported as a stack. The different components were later combined using ImageJ-win64 software.

2.8. Statistics

Analysis of variance (ANOVA) followed by a Tukey-b test were used when comparing more than two data sets, after confirming the homogeneity of variances by the Levene test using IBM SPSS Statistics software v.26. All data have been represented as the average $\pm$ standard deviation. Significant differences ($p \leq 0.05$) are denoted by showing the data provided in tables with different letters.

3. Results and discussion

3.1. Raman spectra of single components

Spectra of every single component were obtained and are shown in Fig. 1, and the most representative peaks are referenced in Table 1. Due to the high similarity between the different (nano)cellulosic fractions, as shown in Fig. S2 (Supporting Information), pure cellulose (F3) was selected as the most representative one in Fig. 1. As expected, there was a substantial difference between (nano)cellulose spectra depending on the concentration used to prepare the samples (0.5% or 2% in the case of the aerogels, and 100% assumed for the dry fraction). While only two peaks at 480 cm^{-1} and 2430 cm^{-1} could be observed at the lowest concentration (0.5%), the characteristic peaks of cellulose at 1100 cm^{-1} and 2900 cm^{-1} (Table 1) became more intense in the aerogels prepared from 2% (nano)cellulose dispersions, and were even more evident in the dried cellulose (Figure

1). The spectra of the 0.5% aerogels, thus, seemed to arise from light scattering phenomena as a result of the crystalline nature of the cellulosic materials, rather than corresponding to the Raman spectrum of cellulose itself. Given that air comprised >99% of the volume of these structures¹⁹, these aerogels can be considered very diluted systems with very weak Raman signals. This suggested that the selected instrumental conditions were not able to identify the components present in the lightest hydrogels, so these spectra will be referred as “cellulose background” throughout the manuscript. On the other hand, in the case of the (nano)cellulose dried in the oven, additional peaks in the 100-500 cm⁻¹ and 1400 cm⁻¹ regions were present in accordance with those extensively reported for cellulose in the literature (Table 1)²⁹. However, there were no major differences found between the spectra of the different (nano)cellulosic fractions. As observed in Fig. S2 (Supporting Information), NANO F2A showed slightly broader peaks than the rest in the 1200-1500 cm⁻¹ region (dry spectrum), while the 2970-2840 cm⁻¹ region became sharper in the spectrum of its 2% aerogels, suggesting more abundance of the C—H stretching vibrations instead of C—C—H bending, CH₂ wagging and C—O—H bending in comparison with the other (nano)cellulosic fractions. This can be attributed to the presence of a minor fraction of lipids in this material¹⁷. It is worth highlighting that the relative intensities of the Raman spectral bands from the (nano)cellulosic aerogels were more sensitive to the density of the material than to the differences in composition, despite the presence of a significant fraction of hemicelluloses (in both NANO F2A and NANO F2) and lipids (NANO F2A) that were resistant to the sulphuric acid hydrolysis¹⁷.

INSERT FIGURE 1 ABOUT HERE

3.2. 2D Raman micrographs (depth scans) of the (nano)cellulosic aerogels

3.2.1. Impact of the analysis method on the extent of structural information obtained

In order to evaluate the suitability of the different methodologies described in sections 2.7.1, 2.7.2. and 2.7.3 to carry out a compositional analysis using Raman imaging, the same aerogel was analysed using the three different methodologies.

For the univariate analysis, different spectral ranges were selected depending on the complexity of the samples ((nano)cellulosic aerogel, (nano)cellulosic aerogel + PLA and (nano)cellulosic aerogel + PLA + oil). The band at 2430 cm^{-1} was selected to identify the cellulose background, while both 2900 cm^{-1} and 1100 cm^{-1} bands were selected for identifying denser cellulosic regions (tagged 2% and 100% respectively, to emphasize the significant differences in density). Although the 2900 cm^{-1} vibrational band was clearly much more intense than that at 1100 cm^{-1} , the latter was preferentially selected as it did not overlap with the oil spectra (cf. Figure 1). In any case, both bands were finally tested in order to show the possible differences and evaluate how band selection can affect the final analysis.

With respect to the oil and PLA spectra, PLA presented similar intensity than dry cellulose while the oil spectrum was much more intense. The Raman spectra of fats are generally intense due to the abundance of C-C single and double bonds in these compounds, derived from the presence of long non-polar acyl chains in their structures³⁰. Again, in order to avoid overlapping, the vibrational band positioned at 1660 cm^{-1} was selected in the case of the oil. For PLA, its most intense band at 2955 cm^{-1} was selected.

Figure 2A shows a representative depth scan of one of the aerogels prepared from 2% NANO F2A dispersions, analysed by the three different methods. With the univariate analysis the image obtained using the band at 2430 cm^{-1} did not provide any structural

information, as cellulose background was present throughout the aerogel (Fig. 1). However, the images obtained using the characteristic bands of cellulose (i.e. 1100 and 2900 cm^{-1}) showed different regions where the polysaccharide was clearly concentrated (especially with the 1100 cm^{-1} filter) (Fig. 1). Similarly, the combined images obtained from the multivariate analyses showed a major contribution of the cellulose background (green), while the spectra identified as cellulose (2% in white and 100% in yellow) were jointly located forming clusters closer to the aerogels' surface. For the simplest systems, such as these uncoated aerogels, there was no visual difference between the images obtained using both multivariate analysis methodologies. Indeed, the combined image obtained from the overlapping of the single filters showed a high similarity with respect to the multivariate analyses, suggesting this methodology as a valid alternative for the (nano)cellulosic aerogels. Overall, cellulose distribution was easily observed in every coloured combined image with minor differences that did not impede determining its approximate distribution according to its concentration, although the quality and precision of the final image seemed to be somewhat improved using the multivariate analysis.

Figure 2B shows a representative depth scan of a PLA-coated NANO F2A aerogel prepared from a 2% dispersion of the nanocellulosic fraction, again analysed by the three different methods. With regards to the univariate analysis and after incorporating the PLA, neither the spectral range centred at 2430 cm^{-1} nor the one at 1100 cm^{-1} (cellulose background and cellulose) were affected by the addition of the new component. However, the image obtained using the 2900 cm^{-1} filter was affected by the presence of PLA, whose spectrum presented its most intense peak at 2955 cm^{-1} (Fig. 1). Consequently, the information about the presence of denser cellulose clusters close to the aerogel surface was lost in this image (see circled areas in Fig. 2B), which is the inverse of the one

obtained using the 2955 cm^{-1} peak. In this context, the univariate analysis was more sensitive to the overlapping of bands in the spectra of different components. Thus, the 1100 cm^{-1} filter was selected as the most appropriate one in terms of observing cellulose in PLA-coated aerogels. However, the combined image of the filters did not show an optimum contrast for determining the distribution of the components in this particular case, with a clear overestimation of the concentrated cellulose clusters (yellow and white). On the other hand, both the second and third methodologies showed a similar distribution of PLA and cellulose, with PLA forming clusters covering more concentrated cellulosic regions (Fig. 2B). Nevertheless, manual insertion showed less defined structures in the case of more concentrated celluloses (especially in the 2% one, which was interspersed throughout PLA and dry cellulose clusters), while automatic detection clearly displayed defined structures in accordance with those previously observed in the native nanocellulosic aerogels (Fig. 2A).

In the oil-immersed aerogels, the 2430 cm^{-1} filter from the cellulosic background remained continuous along the analysed areas according to its low intensity. However, both the 1100 cm^{-1} and 2900 cm^{-1} filters were affected by the overlapping of the spectrum of the oil with that of the cellulose, having the former a much higher intensity (Fig. 1). This hindered the proper identification of cellulose-rich regions by the univariate analysis. As a consequence, the images obtained using these filters showed a very similar distribution to that from the oil filter (1660 cm^{-1}), revealing an interference of the components (Fig. 2C). In contrast, PLA could still be clearly identified by the 2955 cm^{-1} filter in spite of a minor overlapping with the oil spectrum as seen in Fig 2C. By overlapping the distribution of the different filters in the combined image, it could be observed that both oil (red) and PLA (blue) were completely overlapped with concentrated cellulose, hindering a proper identification of every component. However,

with the multivariate methodologies, the oil was seen to be distributed over a continuous layer that, in some regions, could penetrate to the inner parts of the aerogel. Moreover, the oil seemed to be preferentially located in regions of the aerogel covered with PLA, which is reasonable due to its higher hydrophobicity in comparison with cellulose. As it has been already mentioned, the combined image might differ in the manual method as concentrated cellulose (2%) represents a minority component in comparison with the total area. In this context, when this spectrum is manually included in the analysis, it can be mistakenly assigned to areas which are actually covered by PLA or oil due to the similarities between their spectra (Fig. 2B and 2C). Furthermore, more intense and defined spectra (like 100% cellulose, in yellow) might be overestimated in systems like this one, where the peaks from different components are overlapped (becoming the oil the most relevant component); hence displaying poorly defined regions in areas covered by PLA or oil, which can lead to confusion (Fig. 2C).

Overall, the methodology based on univariate analysis could provide information on the microstructure of the native (nano)cellulosic aerogels (i.e. the simplest systems). However, as the number of components in the materials increased, there was rather extensive band overlapping, which resulted in uncertainties about the distribution of individual components in the samples and even masked the presence of components with less intense Raman spectra. This represents a limitation of this methodology of analysis in more complex systems with many components and, thus, it should not be considered as the unique methodology followed for image analysis as it has been typically reported in the literature^{22,25,26}.

On the other hand, multivariate analysis by comparison with reference materials seemed to provide less precise or, in the best case, similar information than the method based on automatic component detection, as the former needs preliminary knowledge of the sample

components and forcing the software to detect certain components may lead to their overestimation. This became especially relevant in the case of components with similar spectra such as cellulose and oil (strongest peak at $2800\text{-}3000\text{ cm}^{-1}$), which led to misinterpretation of the signals. This is shown in Figures 2B and 2C, where a minority component such as cellulose (2% and/or even 100%) were detected in areas that belonged to PLA and/or oil due to the relative similarities in their spectra. As observed in the existing literature, multivariate analysis is starting to be the method of preference for more complex systems in order to allow a more accurate mapping of component distribution, although integration of single filters is still being used as the main analysis methodology in many works ^{23,24}. In this context, automatic detection of the components was selected as the most optimum methodology for analysing this kind of aerogels.

INSERT FIGURE 2 ABOUT HERE

3.2.2. Impact of aerogel density/composition on component distribution

Each aerogel was observed by 2D depth scans in triplicate using the most optimum methodology tested (automatic detection of the components). Representative images of two aerogels with significantly different densities (i.e. NANO F2A 0.5% with 13 mg/cm^3 , and NANO F3 2% with 114 mg/cm^3 ¹⁹) are shown in Figure 3 at each stage (native nanocellulosic aerogels, PLA-coated aerogels and PLA-coated aerogels after oil immersion and subsequent release). The denser and more compact aerogels from pure nanocellulose displayed regions with greater cellulose concentration, which were not detected in the lighter and more porous aerogels obtained from less purified (nano)cellulosic fractions ¹⁷. This was even more evident in Fig. 3, due to the greater

concentration of the dispersion used to prepare the NANO F3 aerogel as compared with NANO F2A (2% vs 0.5%).

The three cross-sections of each type of aerogel were analysed using the histogram of the single components of the image. A complete compilation of the components' relative abundance is shown in Tables 2-5. As expected, more porous and expanded structures, such as NANO F2 and NANO F2A, displayed the greatest cellulose background values (due to their higher air volume), while F3 and NANO F3 showed denser cellulosic areas (both of lower and higher cellulose concentration spectra, coloured in white and yellow respectively). These results demonstrate significant differences on how cellulose forms the internal aerogels' skeleton. While the hemicelluloses present in the least purified fractions resulted in a decrease in the density of the aerogels (from 42 mg/cm³ for F3 0.5% to 13 mg/cm³ for NANO F2A 0.5%), stronger hydrogen-bonded networks were formed in the more purified cellulose and nanocellulose fractions ¹⁹, leading to the formation of denser cellulose regions (referred to as 2% (white) and 100% (yellow) regions). Indeed, denser aerogels (such as F3 2%, with 125 mg/cm³ ¹⁹) displayed higher percentages of white and yellow areas (Table 2).

2D images of PLA-coated aerogels were also analysed and the results are shown in Table 3. It was previously demonstrated that more porous aerogels were able to incorporate greater amounts of PLA into their structures ¹⁹. Thus, the PLA relative abundance was expected to become much higher in lighter aerogels, such as NANO F2A and NANO F2, since these presented the highest background values (related to the greater porosity) (cf. Table 2). The results from our previous work suggested that aerogel densities below 20 mg/cm³ could promote the incorporation of PLA into the inner pores of the aerogel, as opposed to the denser aerogels where PLA was preferentially present on the surface ¹⁹. Since it was not feasible to obtain micrographs of the whole cross-sectional areas of the

aerogels and imaging was only performed close to the surface, standard deviations were higher in those particular cases where PLA could penetrate deeper; as a result, the differences observed amongst the most expanded aerogels (NANO F2 and NANO F2A) at different concentrations were not statistically significant. Nevertheless, for the same concentration, the incorporation of PLA into the denser aerogels, such as F3 and NANO F3, remained significantly lower in comparison with their NANO F2 and NANO F2A counterparts. Interestingly, these denser aerogels (2% F3 and NANO F3, with 126 and 114 mg/cm³, respectively) became the only ones where more compacted cellulose areas (yellow) were still seen, even in the presence of PLA. These results are in accordance with the higher amounts of PLA incorporation reported for the less purified aerogels (NANO F2 and NANO F2A), according to their lower density ¹⁹. Regarding spatial distribution, PLA was preferentially distributed near the surface in all cases (Fig. 3B). Interestingly, in the denser aerogels (NANO F3 2% in Fig. 3B) PLA seemed to be preferentially located around cellulose-rich regions (in white), suggesting that these clots hindered a proper PLA diffusion along the porous (nano)cellulosic network.

INSERT FIGURE 3 ABOUT HERE

Oil distribution along the aerogels was also analysed and the results are shown in Table 4. Again, the density and porosity played a crucial role in the incorporation and visualization of new components. Although remarkable standard deviations prevented from extracting clear conclusions, there seemed to be a tendency correlating less dense aerogels with higher amounts of oil incorporated. This hypothesis is consistent with the gravimetric results from the oil sorption experiments carried out in our previous work ¹⁹.

As expected, all the aerogels showed a higher cellulose background value at their lowest (nano)cellulosic concentration (0.5%) with respect to their 2% counterpart. Moreover, the most compacted aerogels (i.e. F3 and NANO F3) showed lower percentages of both PLA and oil. In the particular case of NANO F2A 0.5% and NANO F2 0.5%, PLA values might be expected to be higher according to their low density ($\sim 13 \text{ mg/cm}^3$). However, this lower density may also imply a greater incorporation of both oil and PLA into the internal pores; thus, these components might not be only present in the external $200 \mu\text{m}$ layers examined for the analyses and deeper screenings should be carried out in forthcoming research to enrich the analyses. After the oil sorption process for the PLA-coated aerogels, the white areas (2%) significantly decreased their abundance, being only present in NANO F2A 2%, while the most intense spectra corresponding to dry cellulose (yellow) were still present in the more compacted aerogels. A masking or overlapping of minor components whose spectra cannot be easily differentiated (such as 2% cellulose) was suggested in the presence of more intense spectra with similar or overlapped representative peaks (such as oil), and could be considered as a possible limitation of this method for a complete detection of the components in complex systems. In this context, Raman analysis may omit some information due to the mentioned overlapping; thus, the relative intensity of the spectra of the different components analysed is a crucial factor to be considered. Regarding the spatial distribution of oil, this component seemed to penetrate across the (nano)cellulosic matrix with a progressive decrease of its presence towards the inner parts of the cross-sections. Interestingly, the oil seemed to be preferentially located in close contact with PLA, since the PLA-rich regions were overlapped with the oil, leading to a purple colouration. On the other hand, the excess oil also covered an extensive region of the (nano)cellulosic matrix (Fig. 3C).

One of the advantages of confocal Raman microscopy is the possibility of mapping several sections within the materials. Z-stacks were also carried out in order to obtain a three-dimensional visualization of all the components in the aerogel. Accordingly, the distribution of the different components in the hybrid aerogels was confirmed using a larger sample area. Figure S4 (supporting information) shows a representative example where, in accordance with the 2D images, a gradual decrease of sorbed oil when penetrating deeper along the (nano)cellulosic matrix was observed. Moreover, the preferential location of PLA on the surface of the aerogels was clearly demonstrated. A video showing the 3D image from different angles is also available as part of the Supporting Information.

The micrographs obtained after oil release were also analysed and the results are shown in Table 5. Firstly, all the values for the relative abundance of oil were significantly lower in comparison with those obtained right after the oil sorption process (cf. Table 4). Unfortunately, a clear correlation between the density of the aerogels and the amount of oil retained could not be extracted from Table 5. According to previous results, the oil was shown to be more easily retained in the more compacted structures of F3 and NANO F3¹⁹. However, since the analysed area was 200 μm , only the most superficial layers of the aerogels were studied and a bigger scan of the aerogels might have been more representative for this particular case (similarly to the case of PLA incorporation, as already mentioned when discussing the results from Table 3). Nevertheless, the study of larger areas may cause a significant decrease on the image's quality, as laser intensity must be lowered when increasing the exposition time in order to avoid the overexposure and burning of the aerogels. This could also be considered as one of the possible

limitations of Raman imaging when analysing low density materials such as these (nano)cellulosic aerogels. With regards to the (nano)cellulosic clusters, the less dense clusters (white ones) that were not detected in the presence of a higher amount of oil, were detected again as the oil was released, being present in three different aerogels. The yellow clusters were again more representative for denser aerogels with a relative abundance higher than 12% in the case of NANO F3 2% aerogel. This confirms that the automatic detection of components, although selected as the optimal approach, was not completely accurate in the most complex systems. Regarding spatial distribution, the oil remaining after the release process seemed to be preferentially located in close contact with PLA, as suggested by the purple tonality. Moreover, most of the initially sorbed oil was released after 7 days, as also demonstrated by the BF images in Figure S3 (Supporting Information), being these results consistent with previous gravimetric analyses for the oil sorption-desorption processes ¹⁹.

3.3. Confocal laser scanning microscopy (CLSM)

One of the limitations of confocal Raman microscopy is that scattering of the Raman photons and interaction with interfaces in the sample can reduce the resolution with respect to the theoretical optical resolution. As a complementary technique for evaluating the different aerogel morphology and compound distribution, CLSM images of the materials surface, able to provide a better resolution, are shown in Figure 4. Greater porosity could be evidenced in the native (nano)cellulosic aerogels, especially in the most expanded structure of NANO F2A 0.5% (Fig. 3A). After PLA coating and, as this process led to a ~2:1 ratio of PLA:cellulose it can be inferred that PLA was not only covering the

(nano)cellulose fibres, but also filling in some of the pores, thus substantially reducing the porosity of the materials.

INSERT FIGURE 4 ABOUT HERE

After immersion, the oil was distributed across the material surface, not only being adsorbed to the cellulose-rich regions but also filling the pores. After the oil release process, the remaining oil stayed attached to the PLA-coated (nano)cellulosic skeleton. Interestingly, a greater amount of oil seemed to remain in the denser aerogel (Fig. 5B vs Fig. 5A), which was expected according to its greater oil retention capacity (2.4 g oil/g aerogel for NANO F3 2% vs 0.7 g oil/g aerogel for NANO F2A 0.5%)¹⁹ and was in agreement with the tendency previously seen in Tables 4 and 5.

4. Conclusions

(Nano)cellulosic aerogels have been prepared from several (nano)cellulosic fractions obtained from *Posidonia oceanica* waste biomass with different purification degrees (NANO F3 > F3 > NANO F2 > NANO F2A) and at two different concentrations (0.5% and 2%). These aerogels were subsequently coated with PLA to improve their hydrophobicity and oil-affinity. Confocal Raman Imaging has been used to characterize the internal microstructure of these biopolymeric aerogels with different degrees of complexity. Both univariate and multivariate analyses were used and compared in order to determine the most optimum methodology for analysing the structure of multicomponent aerogels.

Univariate analysis only provided accurate information in the simplest systems (native (nano)cellulosic aerogels). However, in the case of the more complex hybrid aerogels, the more intense spectra from PLA and, especially, oil, masked the characteristic bands of cellulose and precluded its identification. In contrast, multivariate analyses provided images showing the distribution of every component simultaneously. The data processing approach based on the automatic identification of components was selected as the optimal methodology, since the method based on comparison with reference materials resulted in unrealistic images for the most complex systems. However, this method may also underestimate the presence of less intense components in the most complex systems due to peak overlapping.

On the other hand, micron-sized regions with more concentrated cellulose were more abundant in denser aerogels, while the spectrum of the cellulose background was significantly more abundant in the most porous aerogels. The results also confirmed that PLA was preferentially located close to the surface, while the oil could penetrate deeper along the matrix. Regarding the amount of oil being retained after the release process, although the results from Confocal Raman Imaging were not conclusive due to the relatively short depths analysed, CLSM images confirmed a higher oil retention in the denser aerogels.

Overall, this manuscript shows the potential of Raman imaging for the characterization of the microstructure of complex biopolymeric aerogels, and highlights some of its limitations.

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Table 1. Characteristic spectral ranges of cellulose, PLA and oil identified in the samples

Component	Spectral range (cm ⁻¹)	Band assignment		Reference
Cellulose	2970-2840	C—H	stretching vibrations	29
	1450-1200	C—C—H bending, CH ₂ wagging and C—O—H bending		29
	1150-950	C—O	Stretching vibrations	29
	600-300	Ring torsion and bending vibrations		29
PLA	3000-2900	CH ₃	asymmetric stretching	31
Oil	3020-2980	=C—H	symmetric stretching	32
	2980-2850	C—H stretching of methyl and methylene groups		32
	1680-1640	<i>cis</i> C=C stretching		32

Table 2. Cellulose distribution in the different native (nano)cellulosic aerogels. Different letters show significant differences between samples in the same column. All values were displayed as mean $\pm$ standard deviation.

CELLULOSE			
Sample	Background (green, %)	Cellulose aerogel (2%) (white, %)	Dense cellulose (100%, dry) (yellow, %)
F3 0.5%	86.1 $\pm$ 3.3 ^b	13.9 $\pm$ 3.3 ^a	0
F3 2%	81.9 $\pm$ 3.1 ^b	3.4 $\pm$ 1.6 ^b	14.7 $\pm$ 2.3 ^a
NANO F3 0.5%	89.6 $\pm$ 3.3 ^b	6.7 $\pm$ 3.7 ^{ab}	3.7 $\pm$ 2.9 ^b
NANO F3 2%	80.5 $\pm$ 2.4 ^b	3.6 $\pm$ 0.6 ^b	15.9 $\pm$ 1.8 ^a
NANO F2A 0.5%	96.1 $\pm$ 2.2 ^a	3.9 $\pm$ 2.2 ^b	0
NANO F2A 2%	92.6 $\pm$ 4.2 ^{ab}	6.0 $\pm$ 4.2 ^{ab}	1.4 $\pm$ 1.1 ^b
NANO F2 0.5%	100 ^a	0	0
NANO F2 2%	96.4 $\pm$ 1.2 ^a	3.6 $\pm$ 1.2 ^b	0

Table 3. Cellulose and PLA distribution of the different PLA-coated (nano)cellulosic aerogels. Different letters show significant differences between samples in the same column. All values were displayed as mean $\pm$ standard deviation.

Sample	CELLULOSE			PLA
	Background (green, %)	2% aerogel (white, %)	100% (dry) (yellow, %)	Blue (%)
F3 0.5%	83.9 $\pm$ 1.9 ^a	5.6 $\pm$ 0.8 ^a	0	10.5 $\pm$ 2.2 ^b
F3 2%	88.2 $\pm$ 3.5 ^a	5.2 $\pm$ 2.2 ^{ab}	1.8 $\pm$ 1.6 ^b	4.8 $\pm$ 1.9 ^c
NANO F3 0.5%	86.3 $\pm$ 2.1 ^a	5.1 $\pm$ 1.3 ^a	0	8.6 $\pm$ 1.1 ^{bc}
NANO F3 2%	86.7 $\pm$ 2.7 ^a	1.6 $\pm$ 1.4 ^b	7.4 $\pm$ 3.7 ^a	4.3 $\pm$ 1.6 ^c
NANO F2A 0.5%	76.5 $\pm$ 1.9 ^b	6.3 $\pm$ 1.5 ^a	0	17.2 $\pm$ 7.7 ^{ab}
NANO F2A 2%	72.0 $\pm$ 4.6 ^a	6.9 $\pm$ 3.3 ^{ab}	0	21.1 $\pm$ 1.4 ^a
NANO F2 0.5%	81.7 $\pm$ 3.5 ^b	0	0	18.3 $\pm$ 9.9 ^{ab}
NANO F2 2%	67.2 $\pm$ 3.6 ^a	7.4 $\pm$ 2.9 ^a	0	25.4 $\pm$ 3.3 ^a

Table 4. Cellulose, PLA and oil distribution of the different (nano)cellulosic PLA-coated aerogels after oil immersion. Different letters show significant differences between samples in the same column. All values were displayed as mean $\pm$ standard deviation.

Sample	CELLULOSE			PLA	OIL (day 1)
	Background (green, %)	2% aerogel (white, %)	100% (dry) (yellow, %)	Blue (%)	Red (%)
F3 0.5%	38.2 $\pm$ 6.8 ^b	0	2.6 $\pm$ 1.4 ^b	18.5 $\pm$ 0.2 ^b	40.7 $\pm$ 7.6 ^{ab}
F3 2%	38.0 $\pm$ 9.9 ^{ab}	0	6.2 $\pm$ 2.4 ^{ab}	13.3 $\pm$ 1.6 ^c	35.5 $\pm$ 2.4 ^b
NANO F3 0.5%	36.1 $\pm$ 5.2 ^b	0	2.9 $\pm$ 0.6 ^b	16.3 $\pm$ 5.1 ^{bc}	44.7 $\pm$ 5.1 ^a
NANO F3 2%	47.8 $\pm$ 2.7 ^a	0	8.6 $\pm$ 1.3 ^a	12.1 $\pm$ 1.2 ^c	31.5 $\pm$ 0.4 ^b
NANO F2A 0.5%	35.4 $\pm$ 10.6 ^b	0	0	23.9 $\pm$ 1.8 ^a	40.7 $\pm$ 1.7 ^a
NANO F2A 2%	39.3 $\pm$ 4.9 ^b	1.6 $\pm$ 1.9 ^a	0	19.3 $\pm$ 5.5 ^{ab}	39.8 $\pm$ 3.7 ^{ab}
NANO F2 0.5%	39.6 $\pm$ 3.5 ^b	0	0	13.2 $\pm$ 1.1 ^c	47.2 $\pm$ 6.4 ^a
NANO F2 2%	44.4 $\pm$ 8.7 ^{ab}	0	0	18.5 $\pm$ 1.0 ^b	37.1 $\pm$ 5.8 ^{ab}

Table 5. Cellulose, PLA and oil distribution of the different (nano)cellulosic PLA-coated aerogels after oil release (7 days). Different letters show significant differences between samples at the same column. All values were displayed as mean $\pm$ standard deviation.

	CELLULOSE			PLA	OIL (day 7)
Sample	Background (green, %)	2% aerogel (white, %)	100% (dry) (yellow, %)	Blue (%)	Red (%)
F3 0.5%	64.5 $\pm$ 4.9 ^{bc}	0	0	5.9 $\pm$ 4.4 ^a	27.6 $\pm$ 5.3 ^a
F3 2%	72.3 $\pm$ 4.0 ^b	4.8 $\pm$ 3.9 ^a	0	7.2 $\pm$ 2.8 ^a	15.7 $\pm$ 6.5 ^{ab}
NANO F3 0.5%	69.5 $\pm$ 5.1 ^{bc}	3.3 $\pm$ 2.8 ^a	4.6 $\pm$ 2.4 ^b	9.4 $\pm$ 1.9 ^a	13.2 $\pm$ 2.7 ^b
NANO F3 2%	58.6 $\pm$ 2.1 ^c	0	16.6 $\pm$ 3.6 ^a	8.9 $\pm$ 0.3 ^a	15.9 $\pm$ 3.7 ^{ab}
NANO F2A 0.5%	81.5 $\pm$ 1.4 ^a	0	0	12.0 $\pm$ 2.9 ^a	6.5 $\pm$ 1.0 ^c
NANO F2A 2%	81.9 $\pm$ 5.5 ^{ab}	2.6 $\pm$ 2.0 ^a	0	9.4 $\pm$ 1.5 ^a	6.1 $\pm$ 1.5 ^c
NANO F2 0.5%	86.4 $\pm$ 5.2 ^a	0	0	8.3 $\pm$ 2.2 ^a	5.3 $\pm$ 4.6 ^c
NANO F2 2%	77.6 $\pm$ 2.9 ^b	0	0	12.5 $\pm$ 4.4 ^a	9.9 $\pm$ 5.6 ^{ab}

Figure 1. Average Raman spectra of the reference components: cellulose background, 2% aerogels, dry cellulose (100%), PLA and oil. Blue bands indicate the spectral ranges used for the image analysis based on the integration of single bands.

Figure 2. Monochromatic images of (A) pure nanocellulosic aerogel (2% NANO F2A), (B) PLA-coated aerogel and (C) PLA-coated oil-immersed aerogel. The central wavelength of each filter is annotated in the legend. Combined images are represented by cellulose background (green), 2% cellulose (white), 100% cellulose (yellow), PLA (blue) and oil (red).

242x281mm (120 x 120 DPI)

Figure 3. 2D Raman images of the cross-section (X, Z) of (A) native nanocellulosic aerogels, (B) PLA-coated aerogels, (C) PLA-coated aerogels just after oil immersion and (D) PLA-coated aerogels after oil release (7 days).

Figure 4. CLSM images of (A) NANO F2A 0.5% and (B) NANO F3 2% aerogels at the four analysed stages ((1) native (nano)cellulosic aerogel, (2) PLA-coated aerogel, (3) PLA-coated aerogel + OIL and (4) PLA-coated aerogel + OIL (f)). (Nano)cellulosic fractions and PLA are shown in green, while oil is shown in red.