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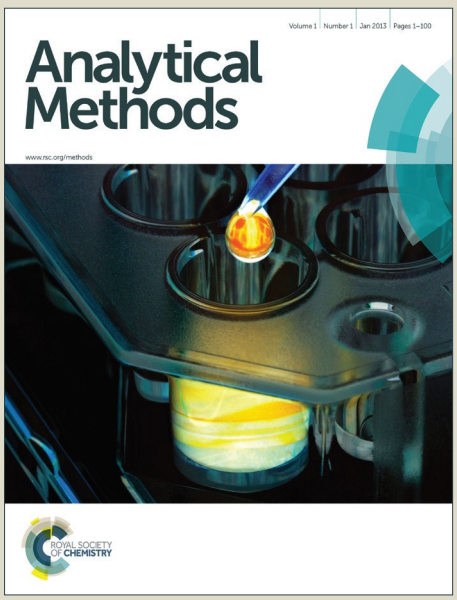

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ARTICLE

Raman Spectroscopy Based Method for the Evaluation of Compositional Consistency of Nanofibrous Layers

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Electrospinning is currently a very popular method used across a number of industries. Electrospinning enables the production of nanofibrous layers of various structures and compositions. The production of a multi-component nanofibrous layer may result in an uneven distribution of the individual components throughout the layer. Confocal Raman spectroscopy combined with statistical methods allows these layers to be analysed by determining their chemical composition and thus provides feedback for the spinning process. This paper presents a method which combines Raman spectroscopy analysis and its subsequent evaluation with singular value decomposition (SVD). Automated measurement of Raman spectra makes it possible to gather extensive spectral data from a particular area selected on a sample; the spectra are measured from a specific volume and not from individual fibres. Samples require no preparation for the analysis and the non-destructive nature of Raman spectroscopy ensures their reusability. When spectra of the individual component materials are included for reference, the subsequent SVD analysis of the spectral data makes it possible to determine the chemical composition of the measured areas, thus providing the content percentages of the individual components, which can be displayed either in the form of a scattered plot or a Raman map.

Introduction

The simplicity, versatility and efficiency of electrospinning (ES) in the production of nanofibrous materials make it a method of choice of many research groups from around the world [1]. ES applies electrostatic forces to produce nanofibrous layers from a polymer solution. The basic application of this method requires no more than a high-voltage power source and two electrodes connected to opposite potentials. One of these electrodes (emitter or jet) administers the polymer solution and forms nanofibres, while the other one (collector) collects the fibres being produced. The standard ES arrangement allows fibres to be spun only from a single material. A number of different modifications of this basic ES arrangement, which enable the production of nanofibrous layers with various structures, are available [2]. These specialized structures enhance the characteristics of the produced nanofibrous materials and thus make them more attractive for a wide range of different applications [3]. Individual applications require corresponding special modifications of product characteristics, including its chemical, physical and biological properties. These modifications serve to improve the physical and/or biological characteristics of the produced materials.

At present, biomedical fields, especially tissue engineering, are stressing the need for the development of composite polymer materials imitating in both structure and function the materials in the natural extracellular matrix. Suitable candidates for their production can be found among natural polymers, including collagen [4, 5], gelatine [6], chitosan [7], hyaluronic acid [8, 9] and more. However, nanofibrous materials made of natural polymers exhibit some weaknesses – above all insufficient mechanical properties and short degradation time. Moreover, transforming a natural biopolymer into nanofibres by electrostatic spinning is usually more difficult than in the case of synthetic polymers. Furthermore, synthetic polymers offer many advantages over natural polymers. They are cheaper, more reliable and can be adapted to a wide variety of properties. By combining natural and synthetic polymers, we can produce materials incorporating the advantages of both: specifically the strength and stability of synthetic polymers and the biocompatibility of natural polymers [10]. One of the crucial elements of such processes is the correct determination of present substances and the estimation of the homogeneity of their distribution in the composite nanomaterial samples.

Today, Raman spectroscopy (RS) is a relatively frequently applied analytical method and it is also used in the analysis of

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polymers [11, 12], which are often the source materials for the preparation of nanofibrous layers. One of the major advantages of Raman spectroscopy is its non-destructive nature, which would be especially important, should it be used for online checking of production of nanofibrous layers or other materials. The method allows a sample to be analysed from a safe distance, the value of which depends on the specific construction of the instrument used, and subsequently to be used again in its original condition. The measurement itself usually takes only several seconds as polymers in most cases provide sufficiently strong signals and there is therefore no need for a long exposure. In addition to the measurement of individual spectra, confocal systems also allow locally defined measurements in a selected area of interest on a sample and mapping. These measurements provide information about the local distribution of the individual components and, if a great number of spectra – hundreds or thousands – are obtained from a sample, it is possible to use the results to e.g. determine the homogeneity of distribution of the individual components. Raman spectra can be further analysed with statistical and mathematical methods such as the principal component analysis (PCA) or singular value decomposition (SVD) [13, 14].

The aim of this article is to show the possible applications of a combination of Raman spectroscopy and SVD for the checking of production of simple or composite nanofibrous layers. The article follows our earlier research [15], which outlined the possibility of using RS and SVD to monitor the distribution of polymers in a nanofibrous layer but without the possibility of expressing component percentages. To allow this, results of measurements of a number of reference solutions were included in the SVD analysis, enabling the content proportions of the individual components within the measured area to be expressed in the form of a graph or a Raman map. The primary aim of this article is to describe the method for the analysis of nanofibrous materials. The featured combination of RS and SVD can be used in other areas of material analysis as well.

Materials and Methods

Materials

The nanofibrous layers were prepared using 6% w/w solutions of poly(ethylene) oxide (PEO, 600 kDa, Sigma Aldrich) and hyaluronic acid (HA, 82 kDa, Contipro Pharma a.s.) dissolved in distilled water. The solutions were stirred for 12 h at room temperature before being used for electrospinning.

A calibration curve was plotted using 10 solutions of HA and PEO dissolved in distilled water prepared in the following ratios: HA:PEO – 10:90 w/w, 20:80 w/w, 30:70 w/w, 40:60 w/w, 50:50 w/w, 60:40 w/w, 70:30 w/w, 80:20 w/w, 90:10 w/w.

Methods

Electrospinning: The commercially available laboratory device 4SPIN® LAB1 (www.4spin.info) [16] was used to prepare three nanofibrous materials from HA and PEO.

Nanofibrous layers were prepared from three solutions of HA and PEO with different ratios of the two components – 20:80 (sample 1), 50:50 (sample 2), 80:20 (sample 3). The nanofibrous layers were prepared using a needle-jet emitter and a static continual collector. The solution was dosed at a feed rate of 14 µl/min. The deposition time was 30 min and the voltage was 20 kV. The distance between the electrodes was 18 cm for all processes.

Raman spectroscopy: An in-house developed Raman system consisting of a confocal Raman microscopy probe (CRM, own design) connected via an optical fibre (Thorlabs) to a dispersive spectrograph (Solar TII MS 3504i) equipped with a multichannel CCD detector (Proscan HS101-H; Hamamatsu CCD S9974-1008) was used to collect the Raman spectra. Raman scattering was excited by a 632.8 nm line of a He-Ne laser (Thorlabs HRR170) connected to the CRM probe with an optical fibre (SM 600 Thorlabs). To satisfy optical resolution requirements, the CRM probe was equipped with a microscope objective (Olympus LUCPLFLN 60x, NA 0.7, theoretical laser spot size of ca. 1.1 µm).

The confocality of the system is ensured by focusing the filtered beam with an achromatic doublet (AC127-019-B Thorlabs) to the core of an optical fibre with adjustable output (AFS105/125Y Thorlabs). Micro-Raman results were correlated with SEM images (Zeiss Ultra Plus, Carl Zeiss Group).

Raman characterization of prepared layers and solutions: A total of 56 spectra were measured from the samples. Spectra of the source materials were measured from the bulk for the purpose of the SVD analysis. The exposition time was set to 3 seconds with 20 accumulations. Laser power at the sample was 12.4 mW. The CCD detector was cooled to -40°C.

After drying on glass slides, the solutions for the plotting of the calibration curve were measured under the same conditions as the nanofibrous layers. At least ten spectra were measured from each sample and the averages were used for the calibration curve.

All measured spectra were processed after measurement – spikes were removed, background was subtracted and an SVD-based normalization was carried out.

Calibration: An SVD analysis was used to prepare the calibration curve according to the procedure described in [15]. Spectra of all the solutions, prepared with increasing proportions of HA, were collected in a single data file including the spectra of pure HA and PEO. The SVD analysis coefficients were then saved and recalculated to component percentages. A graph showing the relation between the real HA percentage in the solution and the percentage obtained with SVD was then plotted. The trends in both cases indicated a non-linear development of the

ratio of band intensities of HA and PEO with growing proportions of HA. A cubic function was used to recalculate the results to real percentages according to the calibration set. The model equation of the function used is as follows:

$$C = a \times A^3 + b \times A^2 + c \times A + d$$

Where C is the unknown concentration, A is the concentration estimated by SVD and a, b, c and d are the coefficients of the cubic function. The coefficient of determination R^2 was equal to 0.99893.

Results and Discussion

Raman spectroscopy

Spectra were measured and analysed in the fingerprint region, i.e. approximately from 700 cm^{-1} to 1800 cm^{-1} (Fig. 1). This region was chosen for the presence of a greater number of

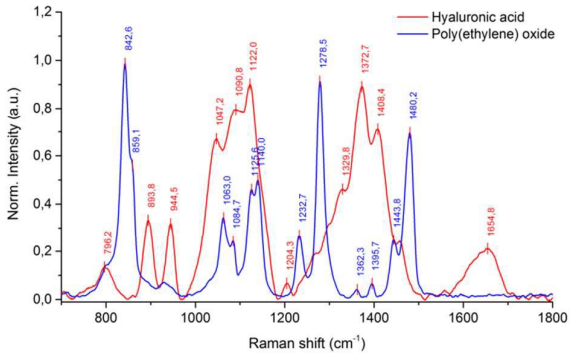

Fig. 1 A comparison of Raman spectra of HA and PEO.

bands of HA and PEO. The fingerprint region of PEO consists mainly of several well-defined peaks. There are peaks of CH_2 vibrations at 1233, 1279, 1444 and 1480 cm^{-1} and a vibrational band of CH_2 and CC at 1396 cm^{-1}. There are also bands of CC-

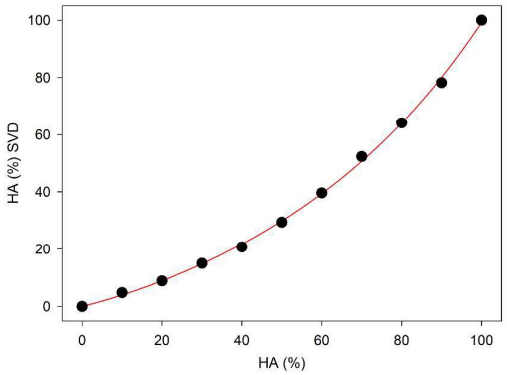

Fig. 3 The relation between the content of hyaluronic acid in the solutions and the content estimated using singular value decomposition; each spot shows the average value from at least ten spectra. The errors in the HA wt. % after SVD analysis are: 10 wt. % = 0.40, 20 wt. % = 0.17, 30 wt. % = 0.55, 40 wt. % = 0.31, 50 wt. % = 0.39, 60 wt. % = 0.22, 70 wt. % = 0.16, 80 wt. % = 0.95, 90 wt. % = 1.53.

COC group vibrations (1140 cm^{-1}), CC-CH_2 (1126 cm^{-1}), COC and CH_2 (1063 cm^{-1}), and finally there are two significant bands of the CH_2-COC group at 859 cm^{-1} and rocking vibrations of CH_2 at 843 cm^{-1} [17].

The Raman spectrum of HA consists of several wider bands. There are bands of C-C and C-O stretching vibrations at 1047 cm^{-1}, a C-OH bending vibration of an acetyl group (1091 cm^{-1}) and a peak formed by bending vibrations of C-OH and C-H (1122 cm^{-1}). There is also a small band of CH_2 twisting vibrations at 1205 cm^{-1}. The second, wider band consists mainly of deformation modes of CH and C-OH. There is an Amid III band (1330 cm^{-1}), which is caused by the *cis* arrangement of the C=O and N-H groups with respect to the C-N bond, and asymmetric and symmetric bending vibrations of $C-H_3$ groups (1373 cm^{-1} and 1455 cm^{-1}), where the former band also includes contributions from a CH_2 scissoring vibration. There is also a symmetric stretching vibration of COO^- at 1408 cm^{-1}, which is a vibration sensitive to hydrogen bonding [18].

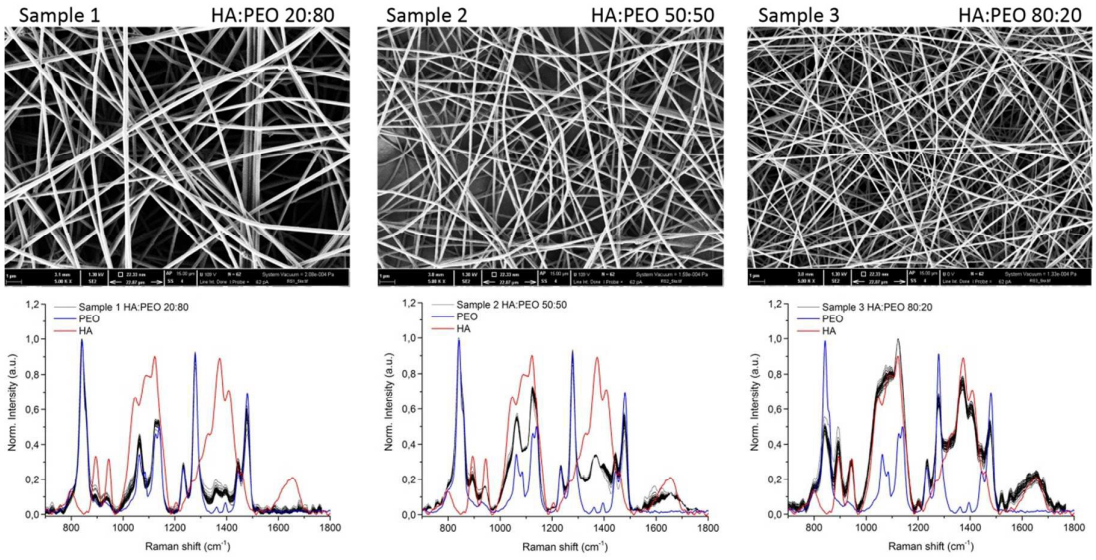

Fig. 2 SEM images of the prepared samples and their Raman spectra together with the spectra of HA and PEO powders: (left) sample 1, the ratio between HA and PEO equals 20:80 in solution, (centre) sample 2, the ratio between HA and PEO equals 50:50 in solution, (right) sample 3, the ratio between HA and PEO equals 80:20 in solution.

The last band that consists of several overlapping bands is the region of carbonyl stretching. It is formed by the vibrations of C=C groups and Amid I C=O groups (1655 cm^{-1}) and two shoulders around 1630 cm^{-1} and 1600 cm^{-1}, which could be assigned to the asymmetric stretching of COO^- [18, 19]. The spectra of the measured samples are shown along with their SEM images in the figure below (Fig. 2).

As shown in Fig. 1, it was possible to discern the bands of both PEO and HA in all the spectra. With increasing content of HA, the intensity of HA bands also increases while the intensity of PEO bands decreases. This experiment together with previous experiments have revealed that the dependence between the contents of HA and PEO and the intensities of their bands is not linear (Fig. 3), which is caused among other things by a partial overlap of the bands of both components. This non-linearity affects the SVD analysis and causes an error in the subsequent recalculation to percentages which is the most user-friendly and suitable form of expression for routine analyses. The trend of gradually changing band intensities in dependence on the ratios of the components was identified with the help of spectra measured from reference samples with different but clearly defined proportions of HA and PEO.

SVD analysis and recalculation

One advantage of the SVD method, besides the relative simplicity of its execution and the subsequent interpretation of its results, is that it is not necessary to extract information, such as e.g. the areas or intensities of individual bands, from the measured spectra in order to determine the proportions of the individual components. Nor is it necessary to know any input data or assignments of individual bands. It is possible to analyse raw spectra, which can be modified as needed (e.g. by subtracting background, smoothing, removing spikes or selecting a suitable area), and the method can thus be standardized according to one's requirements and adapted to specific applications.

By applying the mathematical procedure of SVD, the datasets consisting of Raman spectra are decomposed into a set of orthonormal abstract functions, weights factors expressing the succession of their importance and coefficients of linear combinations. These coefficients, or rather factor scores, are used for the determination of the present content of polymer expressed in percentage. The factor scores of the most distinct differences in the chemical composition are used for this purpose [14, 15]. However, the values of these factor scores are mostly both negative and positive and moreover they are always in different value intervals, which may cause complications when evaluating the results and especially when comparing them. But if properly rescaled, they can represent the percentage proportion of a polymer in a spectrum. However, the dependence between the real percentage content of a polymer and the percentage obtained with an SVD analysis is non-linear (Fig. 3) and it is necessary to subsequently recalculate the obtained results.

Tab. 1 Differences in HA content percentages with their standard errors after the SVD analysis and recalculation.

Solution		Nanofibrous layer	
HA (wt. %)	HA (wt. %) after SVD analysis	HA (wt. %) after SVD analysis	HA (wt. %) after recalculation
20	12.3 ± 0.17	12.1 ± 0.11	20.4 ± 0.45
50	32.7 ± 0.39	34.0 ± 0.10	50.8 ± 0.61
80	65.1 ± 0.95	66.1 ± 0.08	79.5 ± 1.09

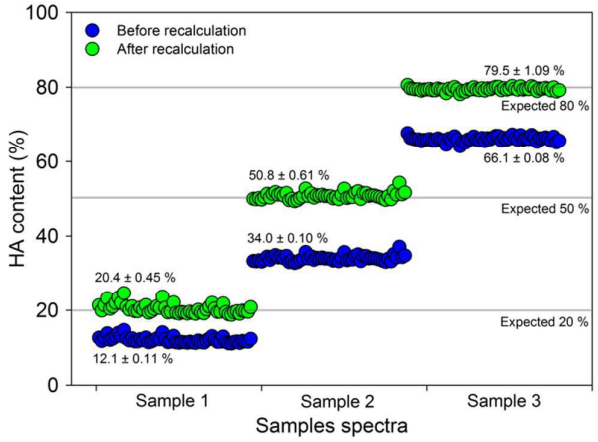

Fig. 4 Content percentages of hyaluronic acid in the measured samples with their standard errors – blue dots represent results obtained only by the SVD analysis (rescaled factor scores), green dots represent values recalculated using a reversed cubic curve fit.

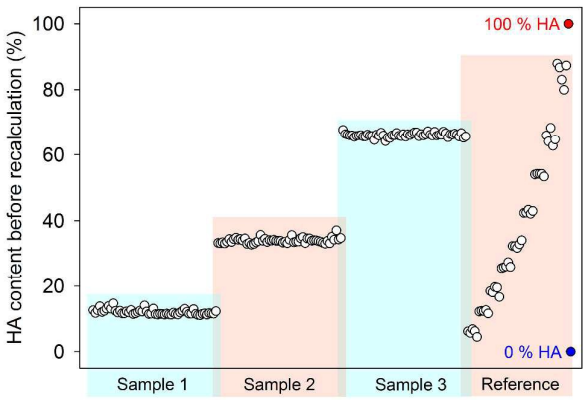

Fig. 5 The distribution of HA percentages in the samples after the SVD analysis and before recalculation according to the calibration curve together with the values of the reference solutions and of the pure HA and PEO powders.

The differences in HA content percentages in our samples caused by this non-linearity are shown in Fig. 4. As evident, the percentages of HA obtained directly from the SVD coefficients are lower than they should be, both for the solution and the nanofibrous layers. The difference increases with decreasing content of HA. Percentage results are visually represented in Fig. 4 – their values before recalculation by curve fit are shown in blue colour: sample 1 (expected 20% w/w of HA), sample 2

(expected 50% w/w of HA) and sample 3 (expected 80% w/w of HA). These values represent results obtained by only rescaling the factor scores.

The recalculation of SVD coefficients to percentages requires the inclusion of spectra of reference solutions and pure components. The spectra of pure components (in this case of HA and PEO powders) allow us to set the negative and positive SVD coefficients, which, after recalculation, serve as a zero point (0% of HA) and a point designating a 100% content of HA. The SVD coefficients – or their pre-recalculation percentage representations – of the reference solutions and samples themselves are then between these two points (Fig. 5).

In order for this method to be usable for fast and accurate analysis of prepared materials, and in our case primarily for the checking of the homogeneity of distribution of the individual polymers in the prepared nanofibrous layers, it was necessary to determine a method for the recalculation of the rescaled values obtained with the SVD analysis. Considering the trend exhibited by the measured reference data (Fig. 3), a reverse cubic curve fit based on a cubic function was chosen. The recalculation was performed in Excel. The values of HA percentages after recalculation are also indicated in Fig. 4.

After recalculation, the HA content percentages obtained with SVD are quite close to the expected values – sample 1 20.4 ± 0.45 % w/w, sample 2 50.8 ± 0.61 % w/w and 79.5 ± 1.09 % w/w (Tab. 1).

The HA content results shown in Fig. 4 indicate good homogeneity of the prepared nanofibrous layers as standard deviations are 1.3 for sample 1, 0.9 for sample 2 and 0.4 for sample 3, although in this case measurements were taken from a mere 56 locations on each sample. The results can also be expressed in the form of Raman maps (Fig. 6), where the varying colours and hues represent different percentages of HA in the samples. As the standard deviations were low, these maps show areas of the samples with homogeneous distribution of HA.

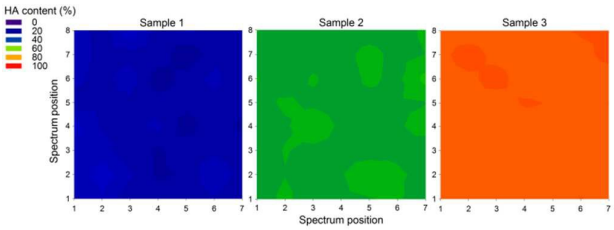

Fig. 6 Raman maps showing the content of hyaluronic acid in nanofibrous layers.

To demonstrate the validity of this method, we prepared another set of solutions with component ratios identical to those used in the preparation of the calibration curve. These solutions were spun and then analysed only in the form of nanofibrous layers – samples 4 to 12. The results are given in Tab. 2. Again, a gradual increase in the content of HA is seen, as expected. The only exceptions were samples 4 and 8 where the identified HA contents were around 15 wt. % instead of the expected 10 wt. % and around 55 wt. % instead of 50 wt. %, respectively. This was probably caused by incorrect weighing during solution preparation.

We apply the method in routine qualitative analysis of produced nanofibrous materials. The analysed products are nanofibrous layers spun from a solution of HA and PEO – always in the ratio of 80% w/w of HA to 20% w/w of PEO. The analysis is carried out on several samples randomly selected from each batch and the final result is their average value. Several spectra are measured from each sample, their SVD analysis is carried out, the coefficients are recalculated to percentages using a calibration curve, and an average value is calculated from the obtained HA content percentages. In these routine product measurements, we allow for an HA content percentage determination error of ± 5%. In this case, in addition to the errors of this method allowed for above, errors that may occur anywhere within the whole process, e.g. as early as in weighing, are taken into consideration.

Tab. 2 Results of measurements of nanofibrous layers prepared from the second set of solutions with increasing contents of HA (from 10 wt. % to 90 wt. %). Std. dev. = standard deviation.

Nanofibrous layers spun from the second set of solutions				
Sample number	HA (wt. %) expected	HA (wt. %) after SVD analysis	HA (wt. %) after recalculation	Std. dev.
4	10	9.1 ± 0.85	15.4 ± 1.25	1.21
5	20	14.0 ± 0.80	23.4 ± 0.97	1.09
6	30	19.1 ± 0.79	31.3 ± 1.34	1.05
7	40	25.3 ± 0.73	40.0 ± 1.04	0.92
8	50	37.4 ± 0.33	54.6 ± 0.72	0.39
9	60	46.0 ± 0.81	63.3 ± 1.02	0.88
10	70	57.2 ± 0.54	72.9 ± 0.70	0.54
11	80	70.1 ± 0.53	82.2 ± 1.48	0.47
12	90	86.6 ± 0.34	92.1 ± 1.86	0.27

Satisfactory results, i.e. HA content percentages with which products are considered suitable for sale (that is where no quantitative changes have occurred and the HA content percentages in the products are identical to those in the solutions), are from 75 wt. % to 85 wt. %. The standard deviation for values obtained from one sample indicates its homogeneity. Selected results of these routine measurements (of the last 10 batches) are given in a table below (Tab. 3), the samples are labelled sample 13 - 22. As indicated, the HA content percentages in the prepared batches were around 80 wt. % with the standard deviation being allowed for. The standard deviations were from 0.19 to 1.40; the most homogeneous sample was thus sample 9, while sample 1 was the least homogeneous. Standard deviation is an important value especially when dealing with samples made of several solutions as these samples are spun by several emitters operating simultaneously. In these cases – in the production of composites – substantial variations in the distribution of the individual components in the sample may occur. The required homogeneity is usually obtained only within a small area of the

prepared sample. This analysis is thus suitable not only for a routine analysis of samples and their quality assessment but also for the study of changes in nanofibrous layers dependent on spinning parameters.

If this procedure is to be used for routine analysis of nanofibrous materials or other products the expected composition of which needs to be verified, several factors must always be taken into consideration. We should know what material we will be working with and adjust the measurement process accordingly, including e.g. sample preparation, sample handling and in the case of Raman spectroscopy laser wavelength and power as well. Furthermore, it is necessary to determine the optimal measurement conditions, i.e. to optimally set up the Raman system in order to obtain results of sufficient quality. It is also necessary to determine the optimal method for the subsequent processing of the obtained data, since even a small variation between different background subtractions can lead to differences in the results in the range of several percent; the same effect can be caused by e.g. noise, excessive smoothing of spectra or insufficient spike removal.

Tab. 3 Results of routine measurements and analyses of nanofibrous samples with 80 wt. % of HA and 20 wt. % of PEO. The error of the method was determined to be ± 5 %. The standard deviations between the content of HA (wt. %) at several spots within each sample are added to indicate homogeneity of each sample.

Nanofibrous layers, expected 80% of HA			
Sample number	HA (wt. %) after SVD analysis	HA (wt. %) after recalculation	Standard deviations
13	71.7 ± 1.91	83.2 ± 5.0	1.40
14	72.4 ± 1.25	83.7 ± 5.0	0.43
15	70.7 ± 1.59	82.6 ± 5.0	0.94
16	71.4 ± 1.19	83.1 ± 5.0	0.35
17	72.2 ± 1.23	83.6 ± 5.0	0.41
18	71.5 ± 1.32	83.1 ± 5.0	0.45
19	71.6 ± 1.28	83.2 ± 5.0	0.48
20	71.7 ± 1.43	83.3 ± 5.0	0.69
21	72.3 ± 1.08	83.6 ± 5.0	0.19
22	72.2 ± 1.26	83.6 ± 5.0	0.44

Conclusions

When preparing composite nanofibrous materials with the electrospinning method, inhomogeneities may occur in the distribution of the individual polymers in the layer due to a large number of parameters (e.g. mixing of the solution components, inappropriate solvent system, processing voltage, electrodes geometry, feed rate of polymer solutions, ambient air condition). Combining the analytical method of confocal Raman spectroscopy with the mathematical method of singular value decomposition makes it possible to perform a relatively fast, non-destructive analysis of nanofibrous products and other materials, allowing us to determine the content of the individual components within the selected area with a relatively high accuracy. The application of a calibration curve then makes it possible to express the results in the form of percentages of the individual components and to display the results in the form of Raman maps. This recalculation to percentages makes the method easy to use on a daily basis.

Acknowledgements

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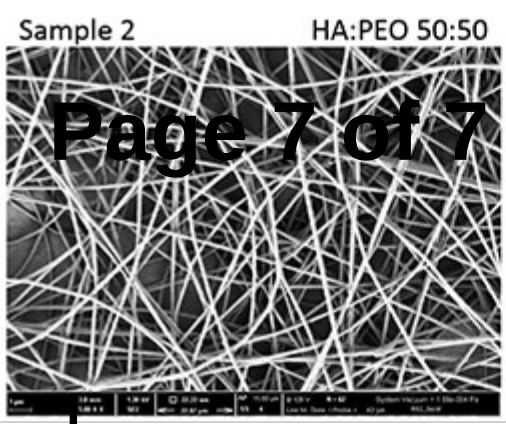
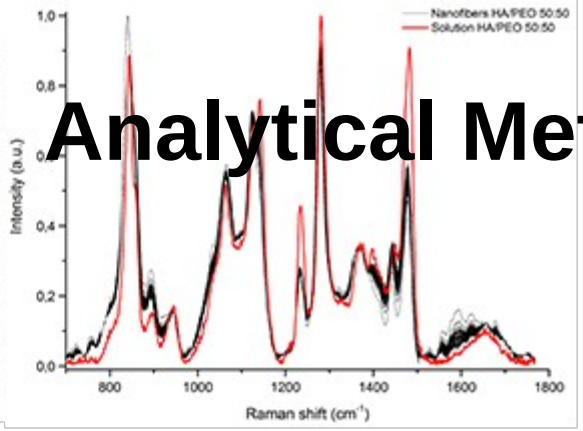
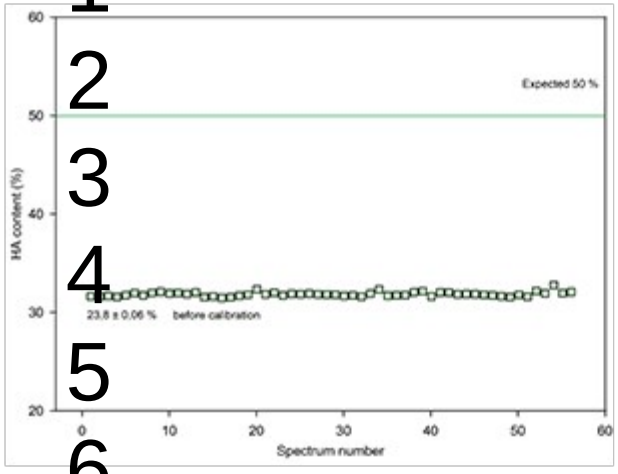
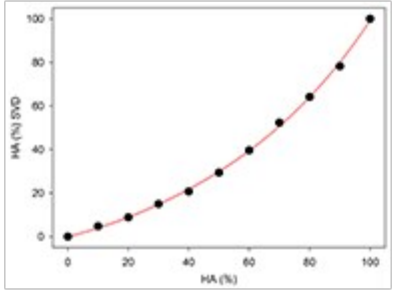




Analysis of the nanofibers based on Raman spectroscopy and singular value decomposition is presented. The calibration of results is included and local homogeneity of samples is shown through Raman maps.





SVD results after and before calibration curve application and recalculation to percents.