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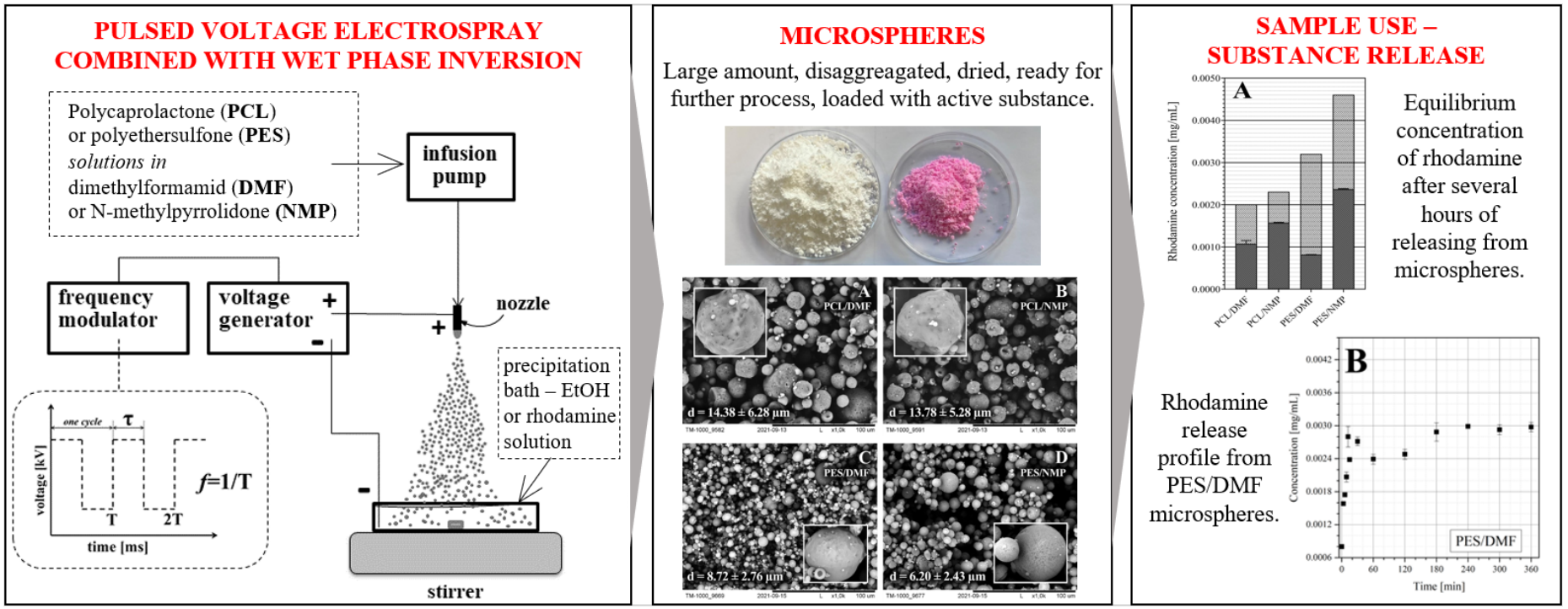
ABSTRACT (200 words)

Polymer microspheres with controlled sizes have attracted interest due to their potential use in pharmaceutical or biomedical fields. Therefore it is of great importance to be able to produce a large number of disaggregated microspheres with a narrow diameter distribution. The paper describes the successful elaboration of the combined methods – electrospray and wet phase inversion – for the manufacturing of such polymer microspheres with mean diameters of 2 – 15 μm. Their recovery and drying are easy and they can be directly applied afterwards. The method is universal enough to produce microspheres from various polymers (polycaprolactone, polyethersulfone) and different solvents (dimethylformamide, N-methylpyrrolidone). It was noticed on the basis of microscopic images and using the nitrogen adsorption–desorption method that the specific surface of the microspheres differs depending on the polymer and solvent used. The work introduces additional parameters resulting from the use of pulsed voltage in the electrospray (pulse frequency f and duration τ) which results in better process control. As an example of the use of microspheres as drug carriers, they were loaded with rhodamine and its release was tested. Such microspheres could be dispersed in polymer solution for the suspension electrospinning process in order to manufacture modified fibers.

GRAPHICAL ABSTRACT



INTRODUCTION

Due to their spherical shape and small size, polymer microspheres are characterized by a large specific surface and as a consequence they have a number of unique properties, for example adsorption, dispersion, mass transfer, permeability, and adhesion behaviors [1, 2, 3]. The size, shape and porosity of the polymer microspheres can be easily controlled by appropriate selection of process for their production and its conditions. The sphericity of such particles reduces their tendency to aggregate compared to non-spherical particles [4]. The use of polymers as the material for microspheres makes it possible to control their physico-chemical properties and to modify their surface.

The unique features of polymer microspheres compared to other polymer materials with a different geometry results in a large field of their applications. They are used in various areas: adsorption [1, 5], catalysis [6, 7], separation [8, 9], microreactors [10, 11], temperature monitoring [12]. They are particularly common in medicine and pharmacy as tissue regeneration scaffolds [13], biosensors [14], vaccines [15], and the most common – controlled drug delivery systems [16]. Research in this area has been carried out for several years, still yielding many interesting results [16, 17]. Various substances can be delivered in this way such as anti-cancer drugs [18], antibiotics [19], growth factors [20], imaging markers [21], anti-inflammatory drugs [22], and others [23, 24]. Sometimes, combinations of different therapeutic scopes can be used in one system [25, 26].

In the case of polymer microspheres used as drug delivery systems, the most important parameters that determine their potential use are their diameters, monodispersity and enhanced specific surface. For example, microspheres with a diameter of 1 – 5 μm can be used to target specific cells, porous ones with a diameter of 5 – 20 μm are effective as drug delivery vehicles for lungs, those with a diameter of 10 – 20 μm can be targeted at tumor tissue by chemoembolization and microspheres of 10 – 100 μm in diameter are small enough to be injected with a syringe and large enough to avoid removal by phagocytic cells, so they can serve as intramuscular or subcutaneous small-scale drug containers [27]. Microspheres have many properties that can be controlled by the method of their preparation and the respective process conditions. These include the possibility of immobilizing relatively high quantities of substances and good control over their release, stability of the preparation after production with a clinically tolerable shelf life, easy control of particle size and their dispersion in solutions, biocompatibility and biodegradability, susceptibility to chemical modification [16].

In recent years, a lot of ideas have arisen on how such microspheres can be obtained. All methods share the same general principle – the polymer solution has to be dispersed into microdroplets and then the polymer droplets have to be solidified to obtain microspheres. The dispersion of the microsphere-forming solution may be based on emulsification, extrusion or microfluidics [28]. The final formation of microspheres can take place by polycondensation, polymerization, coacervation, ionotropic gelation [16], and phase inversion by solvent evaporation or precipitation [29].

An example of a method using the extrusion-based solution dispersion technique is electrohydrodynamic droplet formation with ionotropic gelation used to harden the polymer droplets [30]. This process involves pressing the polymer solution through a metal nozzle to which the electric voltage (direct-current or pulsed one) is applied. As soon as the electrostatic charge repulsion on the surface of the meniscus overcomes the forces of the surface tension, the jet of liquid is interrupted and the droplet breaks off and falls into the collection gelation bath. In the work of Prüsse et al. [30], for instance, the electrohydrodynamic droplet formation with pulsed voltage was used to form alginate microspheres that were gellified in a calcium ion bath. This method was compared to others and it was found to perform best with regard to the size distribution and the shape of the microspheres. However, falling of the relatively large droplets (800 ± 100 μm) into the gelation bath can cause flattening and deformation of the microspheres [31].

Another method for the manufacturing of smaller microspheres is the combination of extrusion-based solution dispersion in the form of electrospray followed by the solvent evaporation to finally form the solid particles [32]. Electrospray is formed when a much higher voltage is applied in an electrohydrodynamic droplet formation system. Then, electrostatic atomization of the liquid stream flowing from the nozzle occurs, resulting in formation of uniform droplets of sizes between tens of nanometers to tens of micrometers which are deposited on a grounded collector (usually aluminum plate) [33]. Due to this, the method is widely used on a laboratory scale, as well as due to good particle size control, low cost, easy and adaptable methodology, low residue generation, and high substance encapsulation efficiency. For example, Tang et al. used the electrospray method to successfully obtain PLGA microspheres with magnetic response and controlled release of vincristine and doxorubicin with target effect under the action of external magnetic field, and with a high concentration and long term sustained release of drugs in the osteosarcoma area. The obtained particles had a two layer structure and diameter distribution of 0.03–1.81 μm [34]. Similarly, the method of

polymer solution electrospraying on a grounded collector in the form of aluminum foil was used by Yao et al. who manufactured the microspheres from an emulsion of PLGA in chloroform and Congo red/albumin in water. The result of their studies was microspheres with a size of 6 – 10 μm which showed the properties of substance release through the degradation of the polymer matrix [35]. Microspheres electrospray can also be used, for example, as coatings for cotton fabrics – Wang et al. manufactures PCL and PEG microspheres with controlled sizes (6 – 12 μm) and tunable surface morphologies by adjusting process parameters. They directly covered the cotton fabrics with microspheres improving the adhesive properties of the fabric surface [36].

Unfortunately, in most cases of electrospray, the collector for microspheres takes the form of a flat aluminum foil. As a result, collecting their larger amounts, drying them and further processing or characterization are very difficult – the microspheres are collected in layers, they form aggregates and adhere to the collector surface. Moreover, the process efficiency in this case is very low because the process time is limited by the available collector area. The solution to this problem could be to use a different collector – for example a bath filled with a liquid in which polymer droplets suspended in non-miscible phase would be hardened according to the sol–gel mechanism specific for a particular polymer (ionic gelation, UV photopolymerization, precipitation). It could also make it possible to obtain larger amounts of microspheres ready for further use than with two separate methods of electrospray or wet phase inversion described above, which is the starting point for the research presented in this article. Such microspheres with sizes up to 15 μm micrometers, which would not form aggregates and would be easy to collect, dry and store in the form of a powder, could later be used for various purposes. For example, microspheres in this form could be very easily dispersed in solution and the suspension pressed through a narrow nozzle in an electrospinning process in order to obtain modified multi-layer fibers.

Hence, the aim of the work – development of a method of combined electrospray and wet phase inversion techniques for manufacturing the polymer microspheres with a narrow size range (5 - 15 micrometers) ready for further characterization, processing and use. The assumption was that this method was universal enough to be able to produce microspheres from various polymers. Tests were carried out with the use of two types of electric voltage – the one with the electric charge supplied continuously (direct current voltage) and another with the charge in the form of pulses of a specific frequency and duration (pulsed voltage). The influence of different process parameters (the electrical parameters, the concentration of the polymer, the

type of polymer and solvent) on the structure of the obtained microspheres was determined. As an example of use, microspheres loaded with rhodamine (marker) were prepared and the release of it was investigated in dependence on the polymer/solvent composition.

MATERIALS & METHODS

Materials

Polycaprolactone (PCL, M_w = 70 kDa, CAS Number: 24980-41-4) was purchased from Scientific Polymer Products, USA and polyethersulfone (PES, M_w = 42 kDa, Ultrason E2020) from BASF, Germany. Dimethylformamide (99%, DMF, Chempur, Poland, CAS Number: 68-12-2) and N-methyl-2-pyrrolidone (98%, NMP, Chempur, Poland, CAS Number: 872-50-4) were used as solvents for the polymers. Ethanol (95%, EtOH, Polmos, Poland) was used as a non-solvent to induce phase separation in a bath. Rhodamine 640 perchlorate (Exciton, USA, CAS Number: 72102-91-1) was used as a marker.

Experimental setup

The microspheres were manufactured using both direct-current voltage (DCV) and pulsed voltage (PV) and the results were compared. The process was carried out using the setup shown schematically in Figure 1. Polymer solution was placed in a syringe and pressed through a drain to a stainless steel nozzle (inner diameter 0.445 mm) using an infusion pump (Alaris Asena GH). The nozzle was connected to a custom-built high voltage generator of direct-current voltage complemented by a frequency modulator providing voltage impulses. Droplets were formed at the nozzle tip, detach and fall to the grounded precipitation bath below. A Petri dish with a diameter of 7 cm and a height of 1.5 cm fully filled with ethanol was used as the bath. The distance between the nozzle tip and the surface of the bath liquid was set as 5 cm.

Working in a PV mode the generator can supply a high, rectangular wave voltage to the nozzle with the applied voltage value U (in the range 0 – 25 kV, stepwise), frequency of impulse application f (in the range 1 – 100 Hz, stepwise), and duration time of the voltage impulse τ (in the range 1 – 9 ms, stepwise). The time domain waveform signal of the PV supply is schematically presented in Figure 1. The same set-up was used for DC voltage operation (without using a frequency modulator).

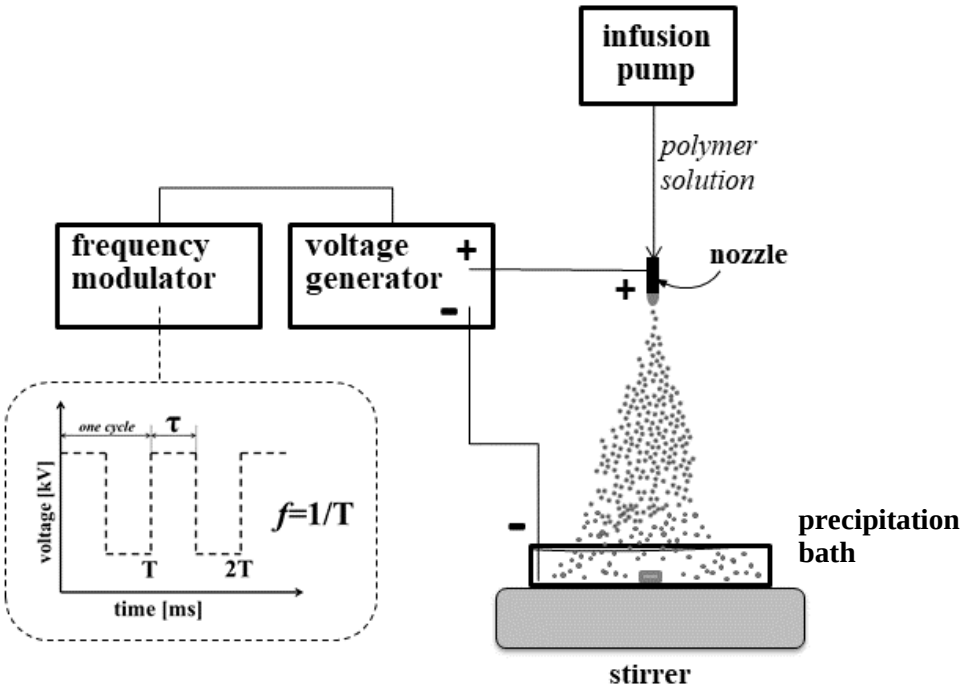


Figure 1. Scheme of the process setup with an operation diagram of the PV supply (dotted line on the graph).

Preparation of microspheres

The following polymer solutions were used to form microspheres:

- 4, 6, 10, 15, 18, 20 % PCL in DMF,
- 15 % PCL in NMP,
- 15 % PES in DMF,
- 15 % PES in NMP.

The values of the electrical parameters investigated in the study are U = 8 kV for DCV and U = 8 kV and 11 kV, f = 20 – 80 Hz, τ = 2 – 8 ms for PV. The polymer solution was delivered to the nozzle at a flow rate of 0.34 ml/h. The resulting product was collected in the stirred ethanol bath for around 90 minutes. After the precipitation process the bath content was transferred to a falcon and centrifuged. Excess ethanol was poured off above the sedimented microspheres. The microspheres were dried at room temperature for several days. All experiments were carried out at a temperature of 25°C and a humidity not exceeding 40%.

Characterization of the microspheres

The meniscus of the polymer solution formed at the nozzle tip during the process was recorded using a monochrome CCD camera. A frame was cut from each film ten minutes after the experiment began.

The morphology of the microspheres was examined on the basis of pictures received by scanning electron microscopy (SEM, Hitachi TM-1000). To prepare the sample for SEM, 100 microliters of microsphere suspension in ethanol were placed directly on the carbon tape on the SEM support. After the ethanol had completely evaporated, the samples were coated with a thin layer (10 nm) of gold and subjected to SEM. The diameters of 200–300 randomly selected microspheres (from several images of one sample) were measured and their mean values and standard deviation were calculated using Statistica software. All the graphs were made using GraphPad Prism software.

Fourier transform infrared spectroscopy (FTIR) was used to determine the chemical structure of the microspheres. The FTIR spectra of each sample was recorded using the NEXUS instrument equipped with an attenuated total reflection (ATR) accessory in the frequency range of 600-4000 cm^{-1} with an average of 64 scans at 2 cm^{-1} resolution. The crystalline phase of the materials was analyzed by X-ray diffraction (XRD), using a PANAlytical Xpert-PRO diffractometer equipped with an X'celerator detector using Ni-filtered Cu-radiation (l = 1.54 A). The scan step size was fixed to 0.0167°/step and the time per step was 55.25 sec/step. The surface area of the microspheres was determined from BET nitrogen adsorption–desorption isotherms at liquid nitrogen temperature using Micromeritics ASAP 2010 equipment (degassing conditions: room temperature – 24 h).

Immobilization of rhodamine in microspheres

The possibility of using microspheres as carriers of biologically active substances, in this case the marker – rhodamine, was investigated. The marker-loaded microspheres were prepared directly from a polymer solution with the addition of rhodamine. For this purpose, the method of producing microspheres described above with minor modifications was used. At the stage of preparation of the polymer solutions (concentration 15%), rhodamine was also added to them, so that its concentration was 0.1 mg/mL (resulting microspheres: PCL/DMF+Rod, PCL/NMP+Rod, PES/DMF+Rod and PES/NMP+Rod). To avoid the diffusion of rhodamine from the microsphere-forming solution to the precipitation bath, a 0.1 mg/mL solution of rhodamine in ethanol was used as a bath instead of pure ethanol. Then the electrospray/wet

phase inversion process was carried out. The resulting suspensions were centrifuged, the supernate was collected, and the microspheres were dried.

Rhodamine release tests

For the rhodamine release tests, 70 mg of each type of microspheres (PCL/DMF+Rod, PCL/NMP+Rod, PES/DMF+Rod and PES/NMP+Rod) were weighed, placed in test tubes which were then filled with 5 mL of pure ethanol each. The suspensions were stirred to remove excess rhodamine from the outer surface of the materials and centrifuged after 2 minutes. The concentration of the supernate was tested, allowing to determine the amount of rhodamine adsorbed on the surface of the microspheres. The washed microspheres were then suspended in 3.5 mL fresh pure ethanol each. The suspensions of the test tubes were mixed, and at specified time intervals, they were centrifuged and the absorbance of the rhodamine solution was measured spectrophotometrically (light wavelength: 574 nm). The experiment results are presented in the graphs showing the change of the released rhodamine concentration profile over time.

Statistical analysis

The normality of the distribution of the measured diameters of microspheres in all samples was tested using the graphical method of the normality plots complemented by the Shapiro-Wilk test. Due to the high sample sizes, the mean diameter and median for individual samples had almost the same value, therefore the mean diameter was used as a parameter characterizing the microspheres. Since in most cases the distributions were not normal, the statistical differences between the mean diameters of microspheres obtained in different sets of experiments were determined using the non-parametric Kruskal-Wallis one-way analysis of variance test on ranks (instead of the parametric ANOVA test) followed by post-hoc test for multiple comparisons. The significance level (α) was set at 0.05 and the probability of data was considered statistically significant for p-values < 0.05. The results were marked on charts in the form of asterisks: (*) for $p<0.05$, (**) for $p<0.01$ and (***) for $p<0.001$ or (ns) when the differences were non-significant. All the calculations were performed with Statistica software.

RESULTS & DISCUSSION

The most important result of the described research was a successful elaboration of an electrostatic method for manufacturing microspheres in the form of a powder in every case (PCL/DMF, PCL/NMP, PES/DMF, PES/NMP). Figure 2A shows a macroscopic image of

dried PCL/DMF microspheres ready for further use and characterization. Moreover, it has been shown that such microspheres can be used, for example, as substance carriers. Figure 2B shows dried rhodamine-loaded microspheres.

Figure 2. **(A)** dried PCL/DMF microspheres in the form of a powder without any addition, **(B)** dried rhodamine-loaded PES/NMP microsphere powder.

Direct-current voltage electrospray

The influence of the concentration of the PCL solution (4 – 20 %) on the geometric shape of the liquid at the nozzle tip as well as on the shape and diameter of the microspheres obtained in the electrospray process using DCV with a precipitation bath was investigated.

Recordings showing the solution shape at the nozzle tip during the process were compared to determine the above-mentioned effect. Frames from the films are shown in Figure 3. In all cases, it can be seen that the solution takes an elongated spike-like shape. During the process, the liquid at the nozzle tip vibrates vertically, causing the polymer droplets to break up in the form of a spray of the tip of the jet. The liquid vibrations are visible in the photos in the form of striped blur. For more concentrated solutions (15% and 20%) it can be seen that the liquid drips up on the outside of the nozzle (Figure 3D and E).

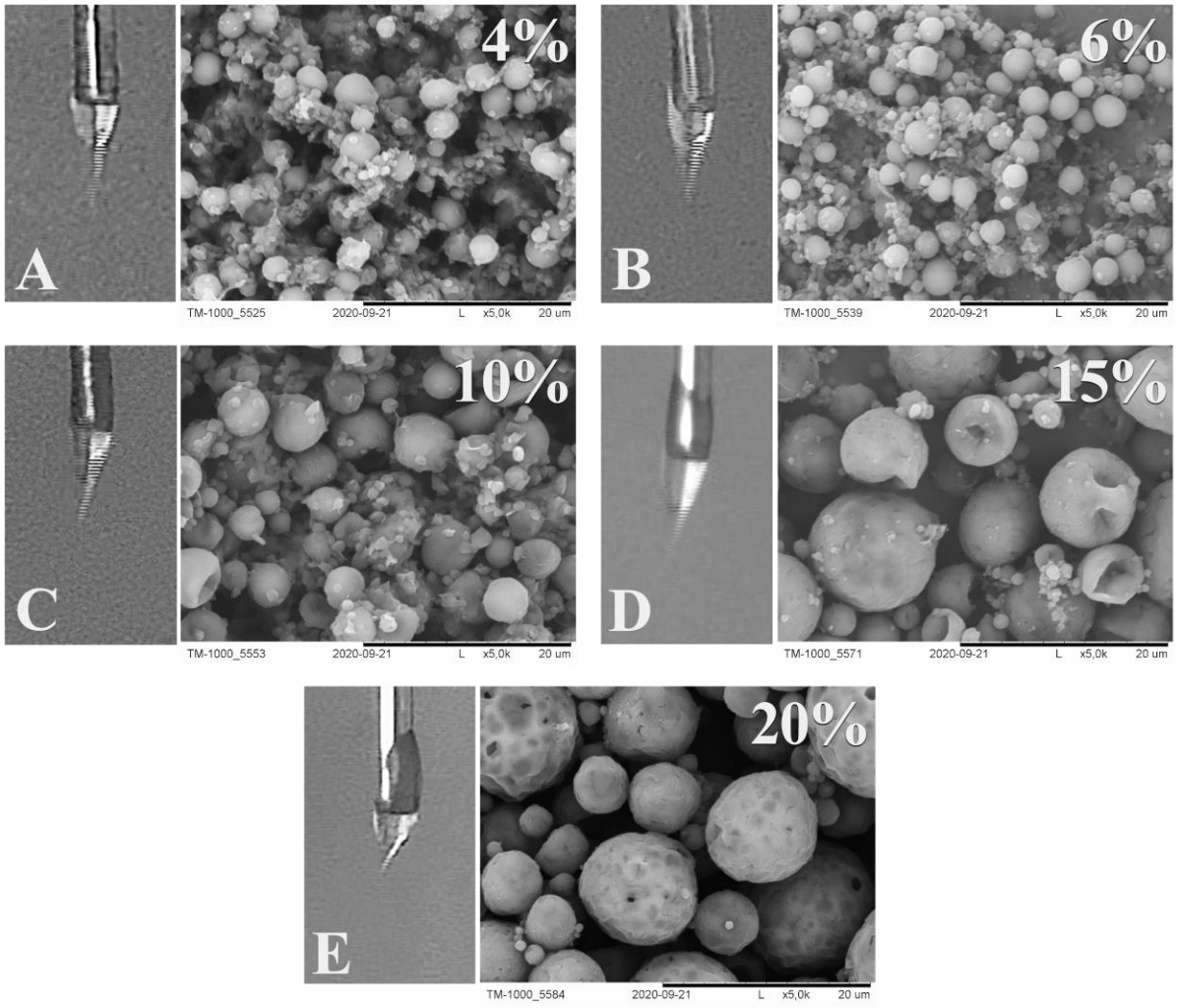


Figure 3. Liquid cone and microdroplet jet formation at the tip of the nozzle during electrospray process under DCV of 8 kV with the PCL solutions of different concentrations: **(A)** 4%, **(B)** 6%, (**C)** 10%, **(D)** 15%, and **(E)** 20%. Scanning electron microscope images of the PCL microspheres obtained from the process with the mentioned solutions.

Figure 3 also features the SEM images of the electrosprayed microspheres manufactured from all the experiments with DCV. These images illustrate that the solution concentration has a great impact on the obtained product. For example the polymer non-spherical artifacts can be formed and their quantity is reduced as the polymer concentration increases. Moreover, the higher the concentration of the polymer, the more spheroidal the microparticle shape. Additionally, it is evident that the increase in polymer concentration causes an increase in the diameter of the microspheres, which is also confirmed by the measurements presented in the plot in Figure 4 below.

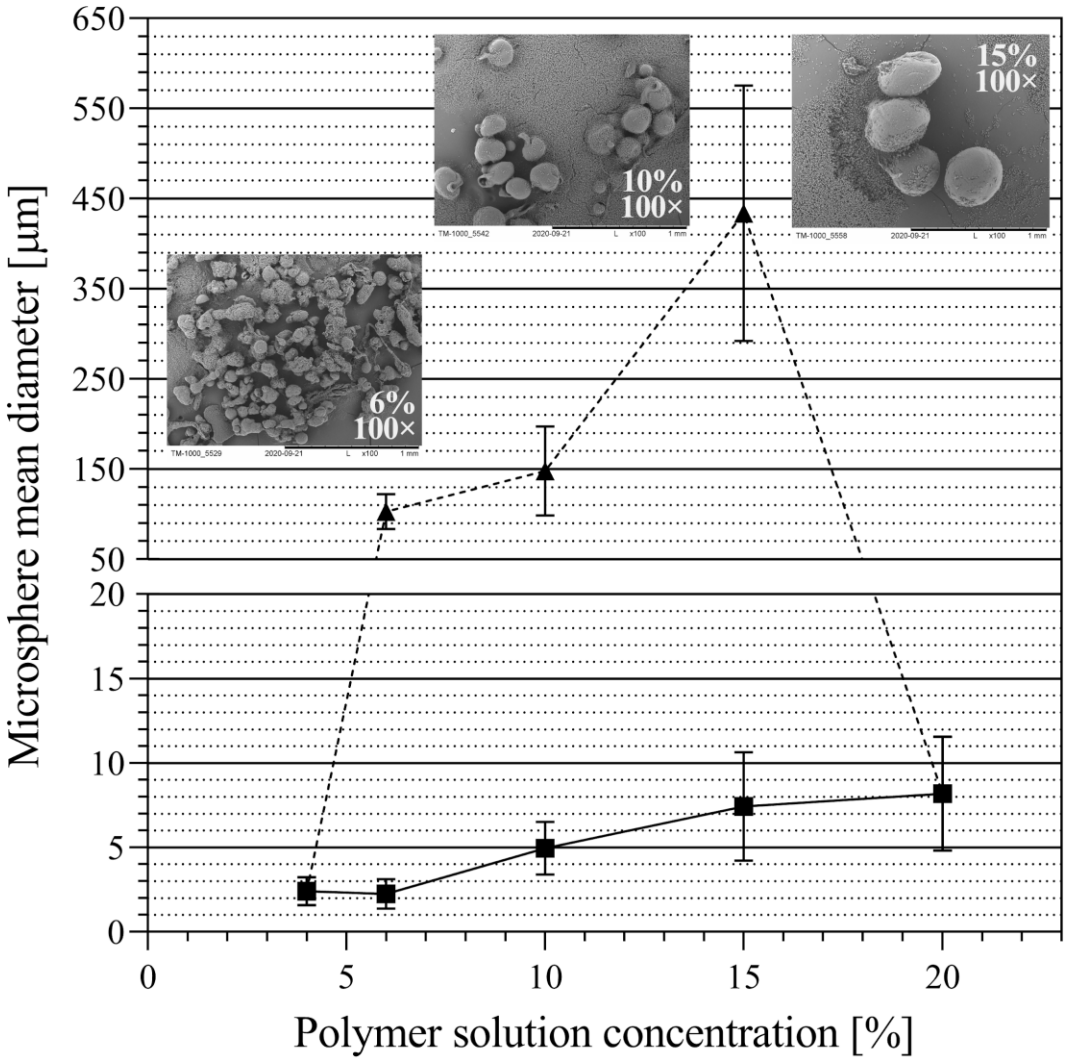


Figure 4. The relationship between the concentration of the PCL solution and the diameter of the microspheres obtained in the electrospray process (squares). In some cases, two microsphere fractions (marked with a triangle) are visible. The SEM pictures show these fractions.

The plot in Figure 4 shows the average diameters of the obtained microspheres depending on the polymer concentration. The lower curve (square points) shows the basic fraction of microspheres obtained in each case. It confirms the increase in the diameter of the microspheres with the increase of the polymer concentration. The average diameter of the microspheres in the basic fraction is less than 20 micrometers. However, in some cases (for concentrations of 5, 10 and 15%), an additional fraction of much larger microspheres was also obtained, with sizes even exceeding 500 micrometers. The SEM images showing these fractions are presented in Figure 4. The extra fraction in the case of the 6% solution has irregularly shaped particles, they cannot be considered as spheres. With increasing concentration, the spheroidicity of the particles increases, at 15% they take the shape of slightly flattened spheres.

Use of pulsed voltage to eliminate the extra fraction of large microspheres

The SEM photos in Figure 5A-C show that the use of pulsed voltage can eliminate the formation of an extra fraction of large microspheres for the solutions of the polymer concentration less than 20%. Such solutions were selected for the study, the use of which in the case of DCV would lead to obtaining two fractions of microspheres - small (1 – 17 μm) and large (150 – 850 μm). Histograms presenting the distribution of the measured diameters of the microspheres in the samples were marked on the photos (Figure 5).

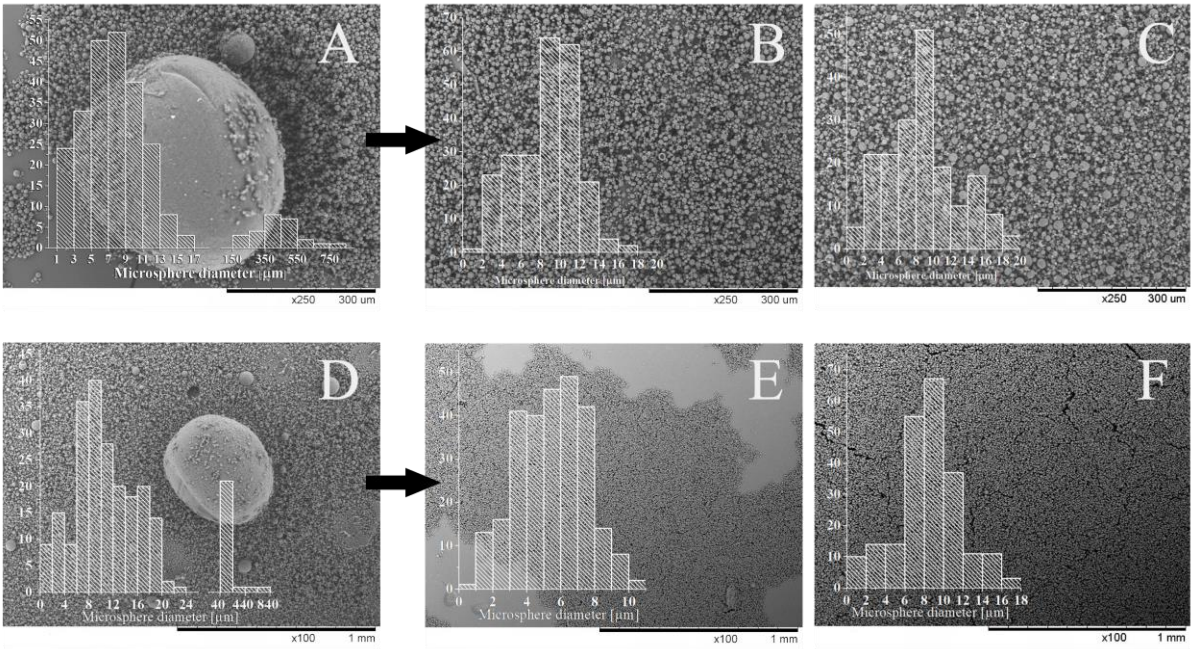


Figure 5. Scanning electron microscope images of the PCL microspheres obtained with different PCL/DMF solutions under various electrical conditions: **(A)** 15%, DCV of 8 kV, **(B)** 15%, PV of 8 kV, 70 Hz, 8 ms, **(C)** 15%, PV of 8 kV, 50 Hz, 9 ms; **(D)** 18%, PV of 8 kV, 60 Hz, 6 ms, **(E)** 18%, PV of 11 kV, 60 Hz, 6 ms, and **(F)** 18%, PV of 8 kV, 80 Hz, 5 ms. Histograms showing the distribution of the measured microsphere diameters for a given sample were marked on the photos.

Figure 5A shows a sphere of the extra fraction surrounded by multiple microspheres of the basic fraction as well as the histogram that confirms the distribution of microspheres diameters is bimodal in this sample – the basic fraction is within 1 – 17 μm, the extra fraction is an order of magnitude larger, its range is 150 – 850 μm. Among the microspheres shown in the photos in Figure 5B and C, no large microspheres of the additional fraction are observed. This is also confirmed by the histograms showing the unimodal distribution of the diameters of the measured microspheres, and in both cases their range is 0–20 μm. These pictures show microspheres obtained using PV.

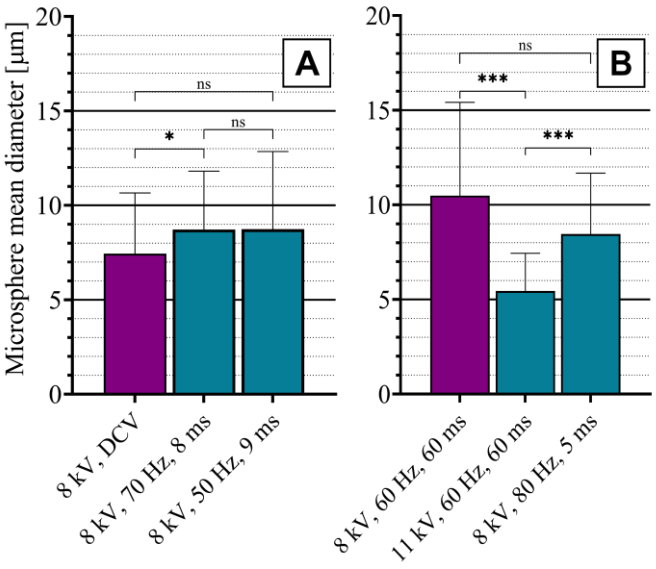


Figure 6. The mean diameters of microspheres obtained from **(A)** the 15% PCL solution, **(B)** the 18% PCL solution with DCV and PV.

On the basis of the graphs presented in Figure 6A, which shows the mean diameters of the microspheres for the above-described conditions for the polymer solution concentration of 15%, it can be seen that properly selected PV conditions (*f, τ*) do not significantly change the diameter of the microspheres. In the case of DCV, the mean diameter was 7.44 ± 3.22 µm, which is smaller than the mean diameter obtained for PV (8.72 ± 3.10 µm for 70 Hz, 8 ms and 8.73 ± 4.12 µm for 50 Hz, 9 ms), but only in the first of these cases the difference is significant statistically, but yet at a low confidence level.

In the previous paragraph it was mentioned that the PV conditions must be properly selected. The crucial parameter that characterizes the PV is the effective voltage U_{eff}. For a PV of amplitude U, impulse frequency f, duration τ and a period of one cycle T (see the operational diagram in (Figure 2.) U_{eff} is specified by the Eq. (1):

$$U_{eff} = \sqrt{\tau/T} \cdot U = \sqrt{\tau \cdot f} \cdot U \qquad (1)$$

Thus, effective voltage is always lower than the voltage U used in the case of DCV so a smaller total amount of electric charge is supplied to the nozzle.

The above remark explains why Figure 5D shows a sphere belonging to the extra fraction of large spheres even though the sample was made with PV. This photo presents microspheres electrosprayed with the voltage value of 8 kV, frequency of 60 Hz, and pulse time of 6 ms from the 18% PCL solution. The photo is complimented by a histogram showing the bimodal distribution of the measured microsphere dimeters. The basic fraction is in the range 0 – 24 µm

and the extra fraction 40 – 840 μm. Increasing the voltage during the process to 11 kV while maintaining the same values of parameters *f* and *τ*, eliminates the additional fraction (Figure 5E) and the diameter distribution becomes unimodal in the range of 0 – 11 μm. Figure 5F shows a sample obtained from an 18% PCL solution while maintaining the voltage value of 8 kV, but with changed values of parameters *f* and *τ*, respectively 80 Hz and 5 ms. There is only the basic fraction with a unimodal diameter distribution in the range of 0 – 18 μm. In both mentioned cases (Figure 5E and F) the effective voltage value was increased in relation to the initial case (Figure 5D), which resulted in the elimination of the extra fraction of large microspheres.

The change in the mean diameter of the microspheres in the above-mentioned variants of the experiment is shown in the graph in Figure 6B. The average diameter of the microspheres in the initial case (8 kV, 60 Hz, 6 ms) is 10.50 ± 4.92 μm and is slightly larger than the diameter of the microspheres in the case with changed *f* and *τ* values (8 kV, 80 Hz, 5 ms) – 8.45 ± 3.22 μm, however, this difference is statistically insignificant. In the case with increased voltage (11 kV, 60 Hz, 6 ms), the diameter of the microspheres is significantly smaller – 5.45 ± 1.99 μm, which confirms that with increasing voltage, the diameter of the microspheres in the electrospray process decreases [37].

Influence of impulse duration τ and frequency f on microsphere size

The previous section presents examples of such selection of PV parameters for which the diameters of microspheres obtained in the electrospray process with the precipitation bath do not differ significantly. More detailed research has been carried out to see if this is the case under all conditions.

The analysis of the SEM images shown in Figure 7 leads to the observation that the PCL microspheres obtained with a 20% polymer solution in DMF for the voltage value of 11 kV and various parameters *f* and *τ* show no differences in structure. They all have regular spherical shapes, some of them with small pores. These microspheres resemble those obtained from the same solution for DC voltage (Figure 3E). The difference can be seen at the outlet of the nozzle, where for the PV a much smaller meniscus with a shorter tip is observed in comparison to the one formed with DCV (Figure 3). For the PV, the nozzle does not clog and the polymer solution does not leak upward on its outer side as in the case of the use of DCV (Figure 3D–E).

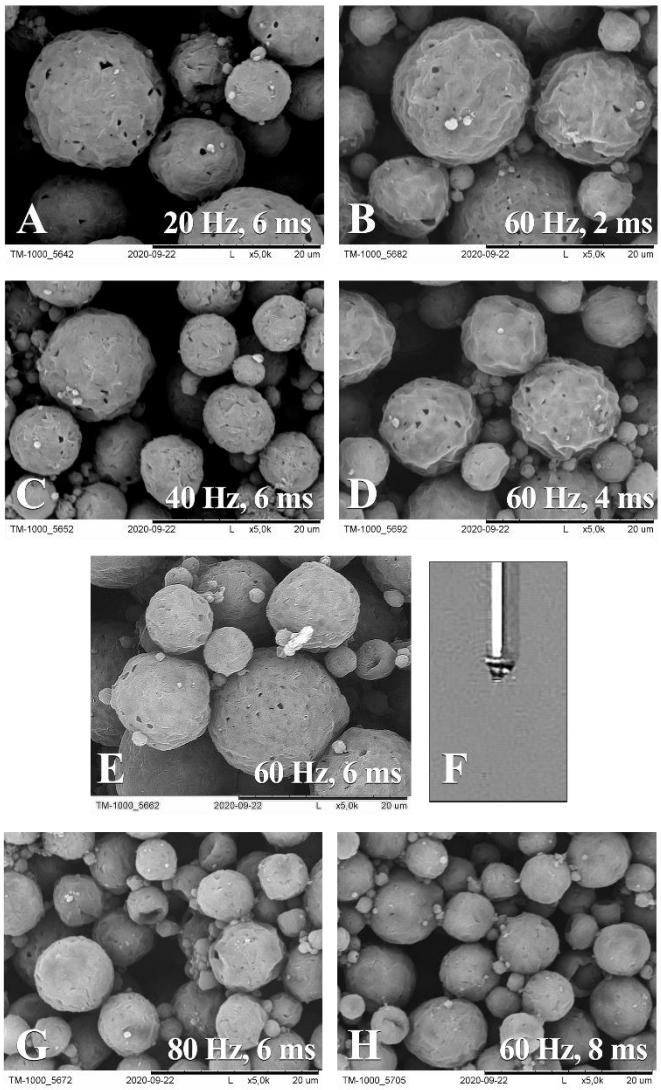


Figure 7. Scanning electron microscope images of the PCL/DMF microspheres obtained with 20% polymer solution under different electrical conditions (f, τ) for PV of 11 kV: **(A)** 20 Hz, 6 ms; **(B)** 60 Hz, 2 ms; **(C)** 40 Hz, 6 ms; **(D)** 60 Hz, 4 ms; **(E)** 60 Hz, 6 ms; **(G)** 80 Hz, 6 ms; **(H)** 60 Hz, 8 ms; **(F)** a frame from the recording of the nozzle outlet showing the geometric shape of the liquid at the conditions [11 kV, 60 Hz, 6 ms].

The plot in Figure 8A shows the change in the diameter of the microspheres obtained in the electrospray process using 20% PCL solution for a voltage of 11 kV and a pulse duration of 6 ms for various values of the pulse frequency from 20 to 80 Hz. The diameters of the obtained microspheres differ significantly in each case except for two pairs [20 Hz and 60 Hz] and [40 Hz and 80 Hz]. The diagram has a specific sawtooth shape but based on the results, the relation between the diameter of the microspheres and the pulse frequency cannot be explicitly determined although the tendency may appear to be slightly declining.

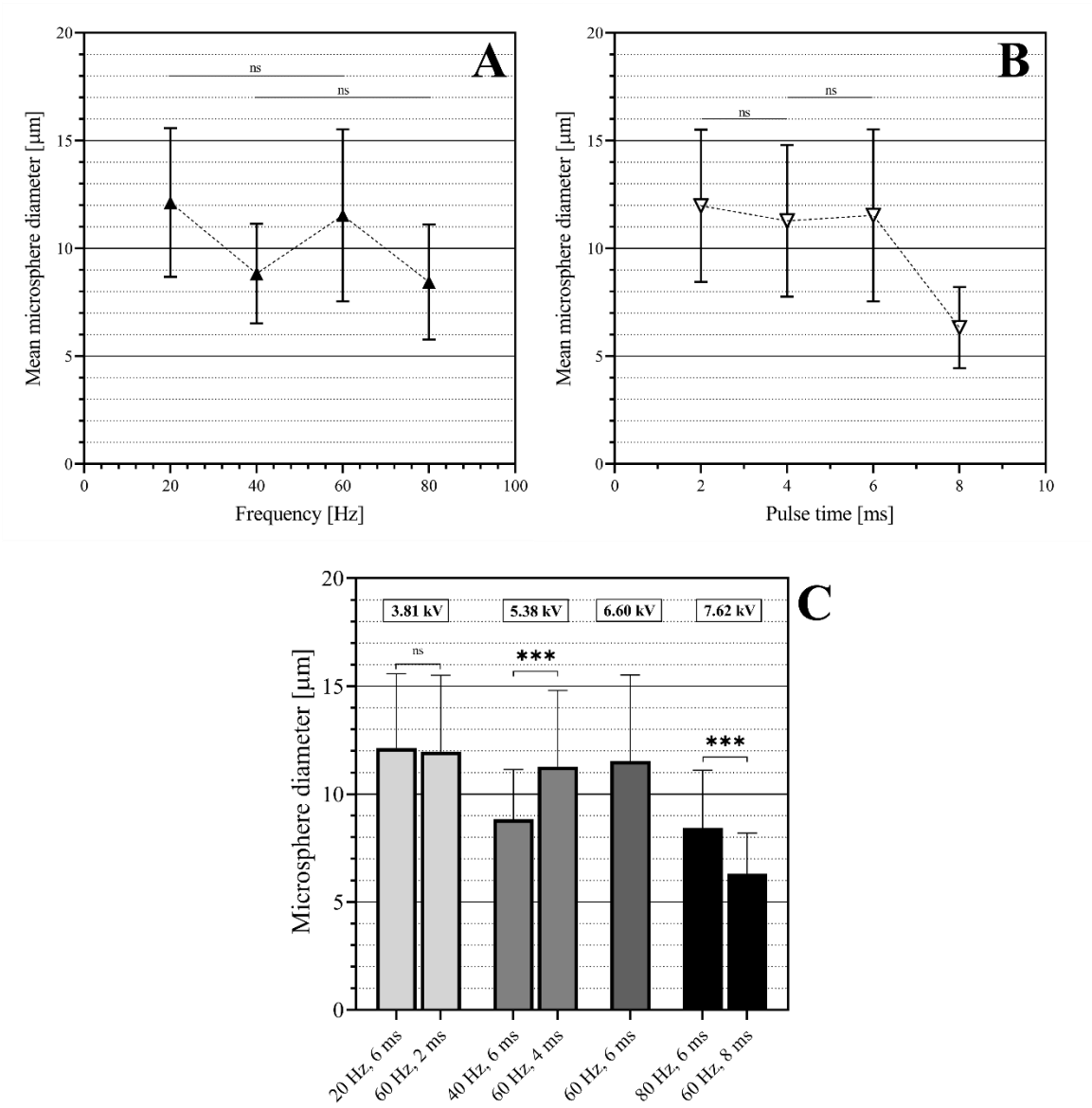


Figure 8. The mean diameter of the microspheres obtained in the electrospray process from the 20% PCL/DMF solution with PV of 11 kV: **(A)** in the function of frequency of impulse application f for τ = 6 ms; **(B)** in the function of the duration time of the voltage impulse τ for f = 60 Hz. **(C)** Influence of the change of effective voltage U_{eff} on the diameter of PCL/DMF microspheres electrosprayed with PV.

The graph in Figure 8B shows the change in the diameter of the microspheres obtained by the electrospray process using a 20% PCL solution for a voltage of 11 kV and a pulse frequency of 60 Hz for various values of pulse duration from 2 to 8 ms. In this case it can be seen that for the pulse time values lower than 8 ms the diameter remains almost unchanged. Only at the pulse duration of 8 ms, a significant decrease in the diameter of the microspheres is noticeable.

An analysis of the influence of effective voltage (U_{eff}) on the diameter of the microspheres was carried out. It was determined whether for given pairs of variants f and τ, for which U_{eff} has the same value, the diameters show significant differences. U_{eff} values were determined for

all cases on the basis of Eq. (1) and the results are shown in the graph in Figure *8*C. For a low value of the effective voltage, no differences were noticed between the two variants [20 Hz, 6 ms] and [60 Hz, 2 ms]. In the remaining cases, the mean diameters of the microspheres do not differ much from each other (about 2 μm), but these differences are statistically significant. As U_{eff} increases, the diameter of the microspheres decreases.

Influence of the used polymer/solvent on the structure of microspheres

Figure 9. shows SEM pictures of microspheres made with solutions with different polymer/solvent combinations. The pictures also show the mean diameter of the microspheres along with the standard deviation.

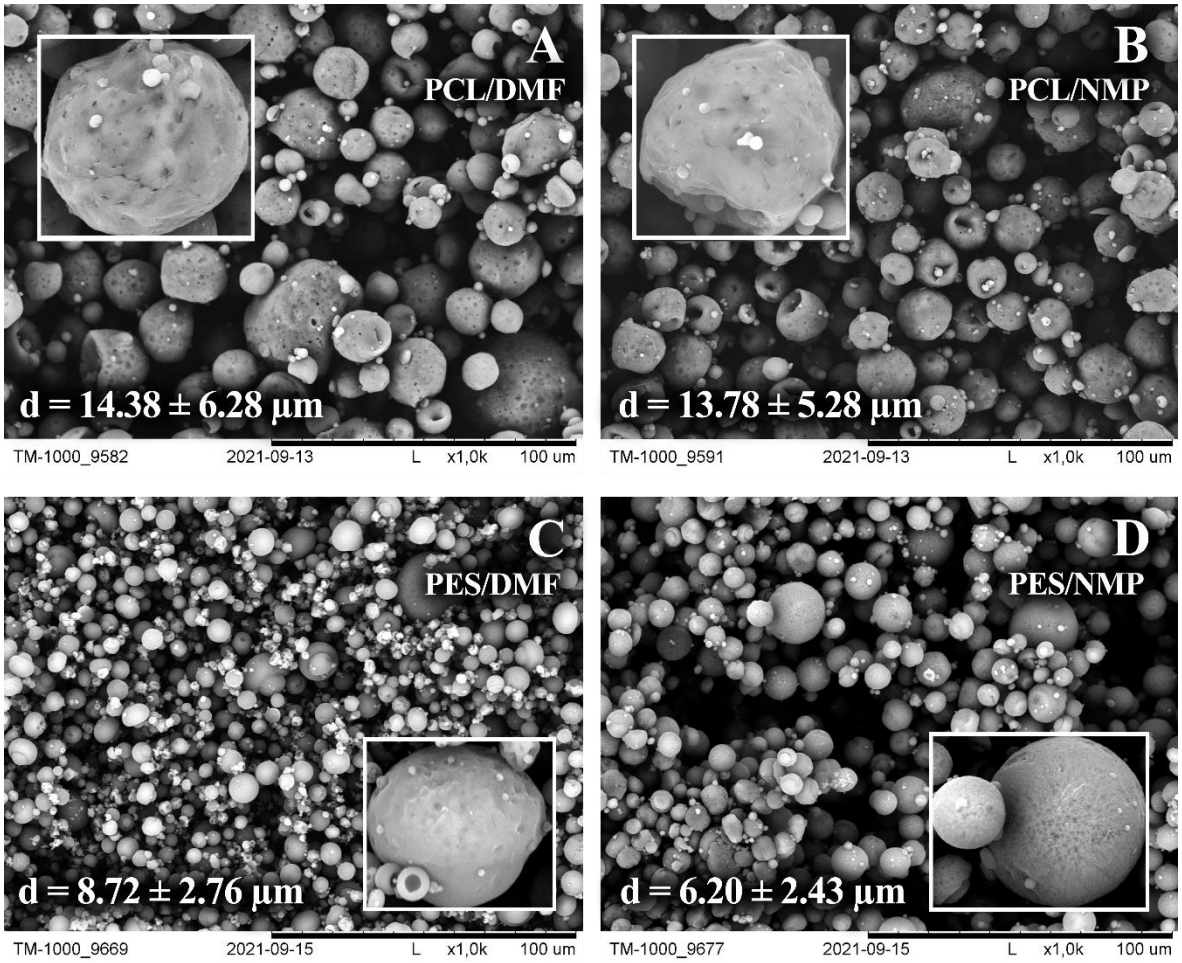


Figure 9. Scanning electron microscope images of the microspheres obtained with 15% polymer solution with the microsphere average diameter *d* and standard deviation: **(A)** PCL in DMF [11 kV, 40 Hz, 4 ms], **(B)** PCL in NMP [11 kV, 40 Hz, 4 ms], **(C)** PES in DMF [8 kV, 60 Hz, 6 ms], **(D)** PES in DMF [8 kV, 60 Hz, 6 ms].

The analysis of the SEM pictures presented in Figure 9 leads to a clear observation that the selection of the polymer/solvent composition does have an impact on the structure of the microspheres obtained in the electrospray/phase inversion process while maintaining other

process conditions. In the case of PCL, it can be seen that the microspheres obtained from the polymer solution in DMF are more spherical and their average diameter is larger, but this difference is not statistically significant. In the case of PES, the change of the solvent from DMF to NMP resulted in an improvement in the sphericality of the microspheres and a decrease in their mean diameter. The following fact is also noteworthy - the PCL microspheres were made at a voltage higher (11 kV) than the PES microspheres (8 kV), which would suggest that the latter will be larger, if based on the previously presented results for PCL (e.g. in Figure 7B). However, the PES microspheres are significantly smaller in diameter. This is further evidence that the selection of the polymer/solvent composition is of great importance to the overall process.

Based on the microscopic observation of PES microspheres, it was noticed that their surface structure differed depending on whether DMF or NMP was used to dissolve the polymer. In the case of microspheres obtained from the PES/NMP solution, their surface seems to be more porous, uneven, and slightly reminiscent of an orange peel. A similar case was not observed for the PCL microspheres. In order to verify the validity of the above observations, the specific surface area of the microspheres was measured using BET nitrogen adsorption. The results of the study are presented in Table 1.

Table 1. Specific surface area of microspheres determined by BET nitrogen adsorption method.

Sample	Specific surface area [m^2/g]
PCL/DMF	1.566 ± 0.044
PCL/NMP	0.607 ± 0.040
PES/DMF	52.926 ± 0.260
PES/NMP	148.199 ± 1.189

The results of measurements of the specific surface of the microspheres confirm the observations made on the basis of SEM pictures. They additionally allow to determine measurably the difference in the surface morphology of the microspheres. In the case of PES microspheres, the specific surface area is several ten times larger than in the case of PCL. In addition, the use of NMP in the formation of PES microspheres increased their specific surface almost threefold, which was also noticed in the microscopic pictures.

Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) tests were performed to check the influence of the use of different polymer/solvent compositions on the

chemical structure and crystallinity of the obtained microspheres. Figure (supporting information) presents the results.

The IR spectra shows that the chemical structure of the polymers (both PCL and PES) did not change after the microspheres production process, because in both cases the peaks appearing on the black curve (pure polymer) have their counterparts on the red (microspheres from a polymer solution in DMF) and blue curves (microspheres from a polymer solution in NMP). However, there were some additional peaks present in the spectra. One of them is slender and occurs around 1662 – 1677 cm^{-1}, which corresponds to C=O stretching in the tertiary amide. These bonds are characteristic for the solvents used in the process (both DMF and NMP) and they are not present in the structure of any of the polymers (neither PCL nor PES). The second, double peak appears around 2880 – 2972 cm^{-1}. In the case of the PCL spectrum, it is derived from C–H stretching alkane. It appears to a small extent in the PES/DMF and PES/NMP spectra too, where it identifies the hydrocarbons present in the chemical structure of both solvents. Probably this stretch would also occur for PCL/DMF and PCL/NMP microsphere samples, however, the accumulation of alkane bonds in PCL overrides the ones in the solvents. The third peak is much wider. The IR spectrum shows a low intensity stretch at around 3426 – 3444 cm^{-1} corresponding to O–H, most likely for absorbed moisture or ethanol residues for all microspheres. Taking into account the above observations, it can be undoubtedly stated that the process of producing microspheres did not change the chemical structure of the polymers. However, there are some residual solvents in the samples, which should be removed by increasing the DMF (or NMP) leaching time with ethanol, additional rinsing after the end of the process with fresh portion of pure ethanol and more thorough drying.

The X-ray diffraction patterns of the PES microspheres have the same shape and additionally it is the shape of a pure PES pattern [38]. Despite the method of producing microspheres, the amorphous nature of the PES remains intact. The case of PCL microspheres is similar – XRD diffraction patterns are the same for both types of material (after solution in DMF and NMP) and reveal the presence of significant crystallinity of PCL – the same as in the case of pure polymer [39]. Regardless of the method of producing microspheres, PCL shows a sharp peak at 2θ of 21° and a relatively low intensity peak at 23.5° corresponding to the (110) and (200) planes of the orthorhombic crystal structure [40]. The XRD results suggest that cristal nature of PCL does not change due to its dissolution in different solvents and subsequent preparation of the microspheres.

Rhodamine immobilization and release

Rhodamine was added to the microsphere-forming solution as well as to the precipitation bath and the electrospray was conducted. The microspheres obtained in this system were dried. A change in their color compared to pure microspheres was noticed – they were slightly pink (Figure 2B). In order to determine the amount of rhodamine that was deposited on the surface of the microspheres due to the evaporation of residual ethanol from the bath solution, the microspheres were rinsed with the pure alcohol. The rhodamine concentration of the solutions obtained in this way is shown in Figure 10A on the bar chart (filled bottom bar). The washed microspheres were placed in a fresh ethanol and the concentration of the solution was measured at specified time intervals and the charts showing the rhodamine release profiles from the microspheres (varying in the polymer/solvent combination) were plotted (Figure 10B-E). Additionally, the bar chart (Figure 10A) shows the equilibrium concentration of rhodamine reached at the end of the release test from the microspheres (striped top bar).

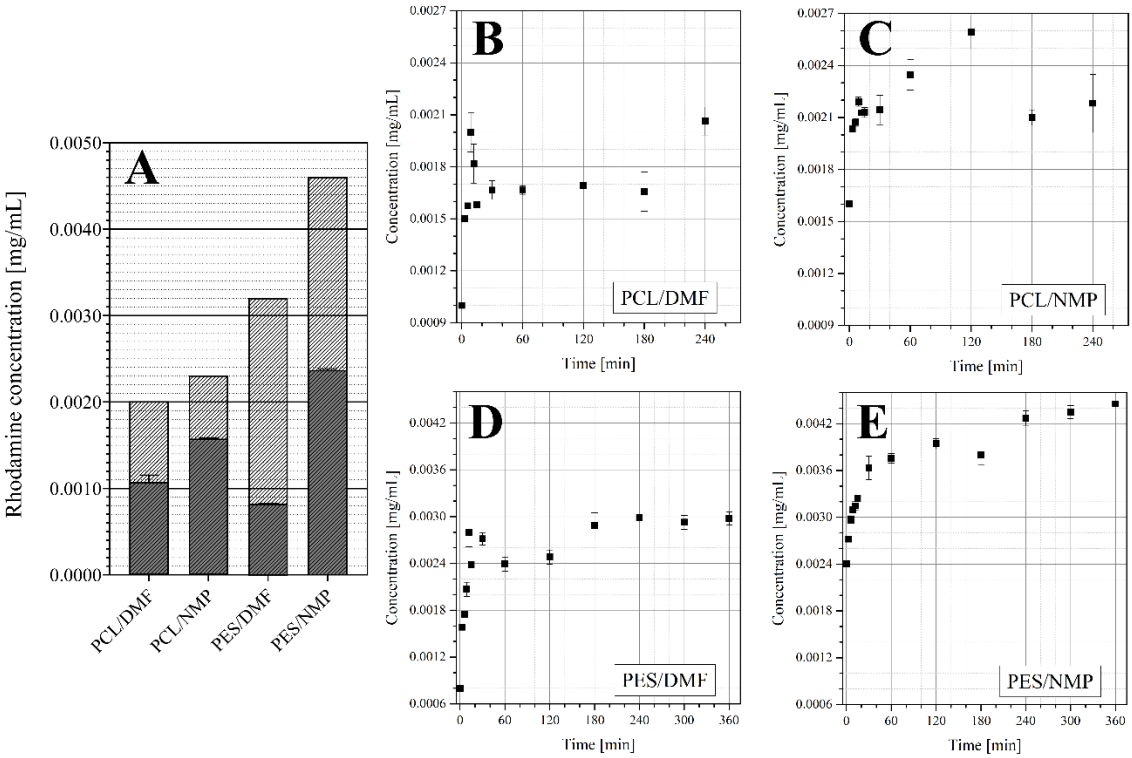


Figure 10. (A) The concentration of the rhodamine solution in ethanol obtained by pre-rinsing the rhodamine-loaded microspheres (bottom bar) and the equilibrium concentration after several hours of releasing (top bar). (B-E) Rhodamine release profile from the rinsed microspheres varying in the polymer/solvent combination: (B) PCL/DMF, (C) PCL/NMP, (D) PES/DMF, (E) PES/NMP.

The graph in Figure 10A shows that the amount of rhodamine deposited on the surface of the microspheres varies depending on the polymer/solvent combination of the microsphere-forming solution used. It is especially visible in the case of using NMP instead of DMF (both for PCL and NMP) – the amount of rhodamine adsorbed on the surface is significantly higher. Of all four types of microspheres, the PES/NMP microspheres showed the largest surface area for rhodamine deposition, which corresponds well with the results of surface area studies and SEM observations. The PES microspheres in general were able to immobilize (and release) more rhodamine than the PCL microspheres. This is mainly due to the smaller diameter of the PES microspheres (see microsphere diameter in Fig. Figure *9*) and therefore the larger total specific surface area (Table 1). In the case of the latter, for both types of PCL / DMF and PCL/NMP microspheres, the amount of rhodamine deposited on the surface after drying is greater than that which was later released from their interior. For PES/DMF microspheres, the initial amount rinsed out from the rhodamine surface is much smaller than that released in the further part of the test, for PES/NMP microspheres these two values are almost the same.

Differences in the properties of the substance release kinetics from microspheres made from different solutions can also be seen by analyzing the graphs showing the profiles of changes in the released rhodamine concentration over time. In the case of PCL microspheres (regardless of the solvent used), the release profiles of the substances are chaotic and the burst-release effect occurs very quickly (Figure 10B-C). The release from the PES microspheres (Figure 10D-E) is much smoother, and in the case of the burst-release PES/NMP microspheres, the burst-release effect is delayed.

Advantages of the proposed method

An attempt to combine the electrospray process and the use of a bath to collect polymer microspheres was undertaken by some researchers before. For instance, Malik et al. [41] manufactured microspheres from PLGA using the electrospray coupled with a novel thermally induced phase separation process with a liquid nitrogen collection bath. The particles had diameters of 16.77 – 122.91 μm, and the method of obtaining PLGA microspheres proposed by them turned out to be chemical-free, sustainable and scalable. Another work combining electrospray with a gelation bath has been reported by Lee et al. [42]. They presented a coaxial tri-capillary electrostatic technique that can directly produce PLGA microspheres with multiple drug compounds incorporated in various layers. As a collector they used the ring immersed in a plastic dish containing a conductive mixture of olive oil and tributylphosphine. However,

more than the method itself, they focused on controlling the thickness of the individual microsphere layers by changing the flow rate of the solution flowing through the nozzle.

Each of these methods described above uses a bath liquid that may cause difficulties to work with (liquid nitrogen, oil), whereas the authors of this work elaborated a method with ethanol as an anti-solvent that is easily available and simple to operate. None of the methods is universal for many polymers, nor do they use pulsed voltage, which undoubtedly would increase the possibility of control over the obtained product by increasing the number of process parameters that can be adjusted.

The presented method has an easy and adaptable methodology and does not require any additional substances (apart from the polymer, its solvent and antisolvent) which could leave residues or change the structure of the polymer (e.g. crosslinking agents). Biological products can be encapsulated inside the microspheres. The process is conducted in mild conditions such as ambient temperature and gentle stirring. Additionally, by conducting the experiments for many weeks, the authors of the study proved that there is an excellent reproducibility of the microsphere end product. A relatively large number of microspheres with a narrow diameter distribution of several micrometers is obtained. They do not form aggregates (neither in the bath nor after drying) and are simple to further process and use. The recovery of the product is easy and it can be directly applied afterwards. Such microspheres, for instance, could be dispersed in the polymer solution to form an emulsion which can then be pressed through a nozzle in electrospinning or 3D bioprinting process.

CONCLUSIONS

In the presented work, a method for manufacturing ready-to-use polymer microspheres was developed using combined pulsed voltage electrospray and wet phase inversion techniques. The method is simple and universal and it can be modified by using different polymers and solvents, and by changing the process parameters leading to the production of microspheres of various sizes and structures.

The paper indicates the advantages of using a pulsed electric field instead of direct-current voltage. First of all, using PV removes the unwanted extra fraction of large microspheres making the microspheres monodispersed. It stabilizes the liquid at the nozzle tip (keeping its geometric shape the same throughout the process). Finally, properly selected PV parameters (pulse frequency f, pulse duration τ) result in obtaining the desired structures. The microspheres were made of two polymers (PCL and PES) dissolved in two solvents (DMF and NMP). It has been shown that the selection of the composition of the microsphere-forming solution has an impact on their size, porosity and specific surface area, but does not change their chemical or crystalline structure (compared to pure polymers).

As an example of the use of microspheres, the microspheres with rhodamine for the controlled release of substances were suggested. The substance-loaded microspheres were successfully produced and the differences in marker release profiles resulting from the microsphere specific surface area of the microspheres were indicated.

The work described above has a very promising outcome as it provides support for our hypothesis that the combination of pulsed voltage electrospray and wet phase inversion techniques is a suitable method for the production of monodispersed ready-to-use polymer microspheres. Such microspheres, for example, can be used as drug delivery systems but their application will certainly not be limited to biomedical engineering as it is easily transferable to other sectors.

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Supporting information

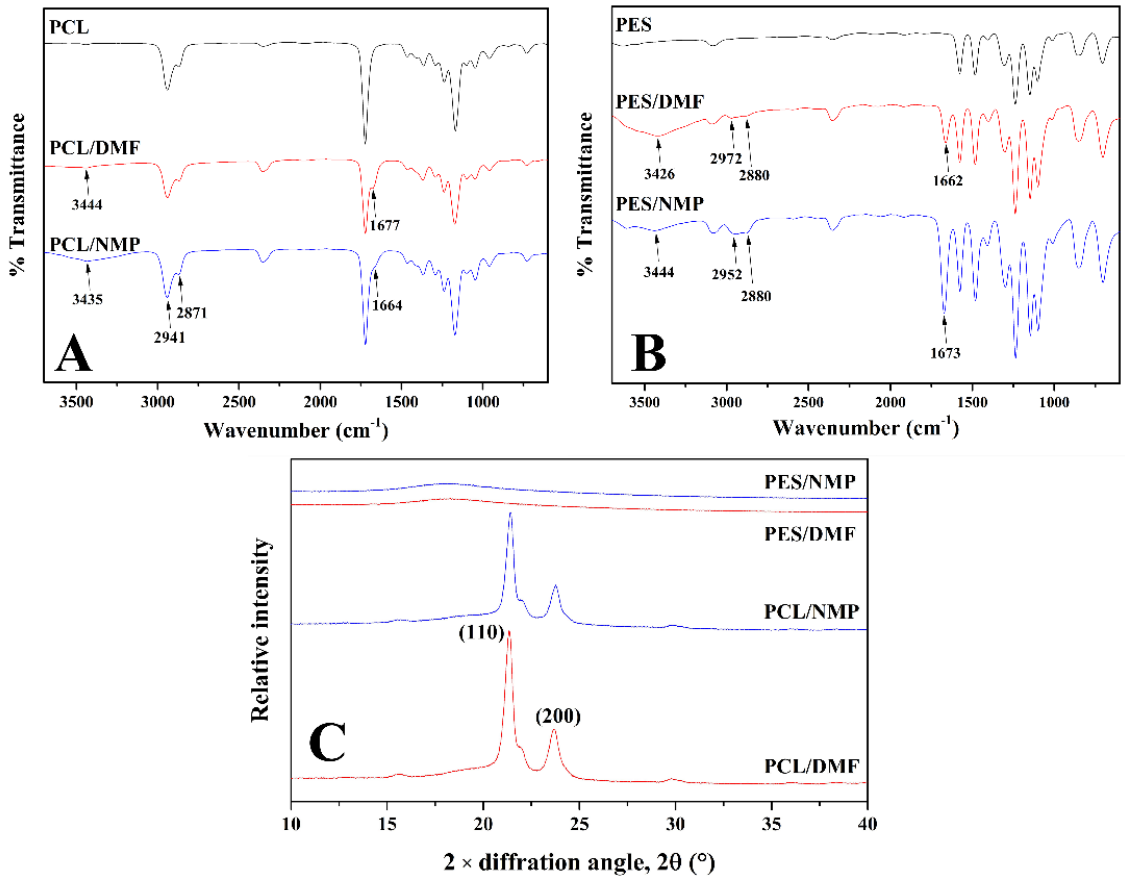

Figure SI1. **(A-B)** FTIR spectra of pure PCL and PES as well as the microspheres obtained from a polymer solution – in DMF (PCL/DMF and PES/DMF) and in NMP (PCL/NMP and PES/NMP). **(C)** XRD patterns of the microspheres.