Production of polymeric micro- and nanostructures with tunable properties as pharmaceutical delivery systems

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Abstract

The production of novel graft copolymers based on poly-ε-caprolactone (PCL) and polyaspartamide are useful to realize structures for potential biomedical applications. Here, the synthesis of pegylated PCL/polyhydroxyethyl aspartamide (PHEA) graft copolymers (PHEA-g-SUCC-PCL-g-PEG) with tunable composition, was achieved by followpling a synthetic strategy that involved first the grafting of preformed PCL on PHEA backbone, then polyethylen glycol (PEG), by using 1,1'-carbonyldiimidazole (CDI) to speed up the condensation reaction. Graft copolymers with a Derivatization Degree (DD) in PCL ranging between 1.1 and 4.4 mol% were obtained, and processable with different technologies for the realization of micro- and nanostructures for biomedical applications. Pegylated graft copolymers, PHEA-g-SUCC-PCL-g-PEG, with a DD_{PEG} of about 3.9 mol% were synthesized, and starting from these materials, structures such as fibers, micro- and nanoparticles were produced by different methods, such as microfluidics and nanoprecipitation.

Keywords

Graft copolymers, α,β-poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA), poly-ε-caprolactone (PCL), microfluidics, nanoprecipitation, microparticles, nanoparticles.

1. Introduction

Polyesters, such as polylactic acid (PLA) and poly-ε-caprolactone (PCL), represent the most important class of biodegradable polymers for the production pharmaceutical formulations for drug delivery and tissue engineering applications [1–4]. This possibility arises from their physical-chemical properties, such as biodegradability and biocompatibility, and from the possibility to suitably modulate these properties through blending or co-polymerization with other biopolymers, as widely reported in several reviews on this subject [1,2,5,6].

Among polyesters, PCL is the most attractive material in terms of biodegradability, biocompatibility and processability; moreover, it shows high versatility in modulating its chemical, physical and mechanical properties, that makes it a best candidate for biomedical applications [6].

Several PCL-based block and graft copolymers were successfully synthesized by ring opening polymerization of ε–caprolactone initiated with mono- or multifunctional hydrophilic materials such as polyethylene glycols (PEGs), polylysine, chitosan and α,β-poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA) [6–8]. The latter is a protein-like synthetic polymer whose chemical composition in terms of multiplicity of hydroxyl groups available for further conjugation with drugs, targeting ligands and/or labelling portions, gives it great potential for biomedical applications in controlled and targeted drug delivery [9–14].

Miao et al. described the synthesis, characterization and degradation behavior of novel biodegradable graft copolymers based on PHEA and PCL, in which PHEA represents the main polymeric backbone while PCL constitutes the side chains, and the production of micro- and nanoparticles for drug delivery starting from them [8,15]. It was evidenced that the branch lengths of grafted PCL chains could be properly controlled by varying the experimental reaction conditions, thus obtaining a series of PHEA-PCL graft copolymers with different hydrophilicity and crystallinity that

allows affecting the chemical and enzymatic degradation behavior. However, the experimental conditions chosen to allow co-polymerization has given thermal degradation phenomena on PHEA backbone that result in a lower molecular weight of the obtained derivatives than starting material [8].

With the aim of synthesizing new copolymers with increasing performance in the pharmaceutical technology field, our research group has synthesized numerous polymeric amphiphilic derivatives by reacting a polyaspartamide such as PHEA with various functional molecules, such as hydrophobic (fatty acids, polylactide, phospholipids) [10,16], targeting (folic acid, galactose) and solubilizing (PEG) agents [10,17], cationic portions (di-, tri- amines) [14].

Given the wide rise in biomedical and esthetic application of PCL, the excellent chemical versatility of PHEA to react with different polymers to obtain graft copolymers very useful for biomedical and technological applications, both materials seem to be excellent candidates to be combined/grafted in order to obtain novel copolymers with improved chemical and physical-chemical properties. For this reason, in this paper we have performed a systematic study to describe the synthesis of novel graft copolymers by grafting preformed PCL on PHEA backbone by using 1,1'-carbonyldiimidazole (CDI) to make faster the condensation reaction.

We demonstrated that the amounts of grafted PCL could easily modulated by changing experimental reaction conditions and that it is possible to carry further functionalization reactions on the obtained copolymers with PEG. Moreover, we proved that these materials are processable with different methods/technologies for the realization of structures such as fibers, micro- and nanoparticles for biomedical applications. Finally, the surface composition and properties of obtained systems have been modified with processing technologies. Therefore, these new systems possess tunable surface properties, which could be improvable by conjugating more proper ligands, thanks to the presence of numerous potential functionalization sites on the PHEA backbone, giving new openings towards the realization of highly performing drug delivery systems.

2. Experimental

2.1 Materials

Poly-ε-caprolactone (PCL, $\bar{M}_w$=10-18 kDa), succinic anhydride (SA), dimethylaminopyridine (DMAP), anhydrous N,N'-dimethylformamide (a-DMF), 1,1'-carbonyldiimidazole (CDI), N,N'-disuccinimidyl carbonate (DSC), anhydrous dimethylacetamide (a-DMA), Poly(ethylene oxide) standards were purchased from Sigma-Aldrich (Italy). Diethylamine (DEA), triethylamine (TEA), O-(2-Aminoethyl)-O'-methyl poly(ethylene glycol) 2000 (H_2N-PEG_{2000}) (≤ 0.4 mmol NH_2/g), ethyl ether, dichloromethane, tetrahydrofuran (THF) were obtained from Fluka (Italy). α,β-Poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA) was properly synthesized as elsewhere reported [11]. All reagents were of analytic grade, unless otherwise stated.

2.2 Synthesis and characterization of poly-ε-caprolactone-succinate (PCL-SUCC)

Carboxyl-terminated PCL was synthesized accordingly to the following procedure. PCL was dissolved in a-DMF (133.4 mg/ml), and to the obtained solution dimethylamonipyridine (DMAP) and succinic anhydride (SA) were added subsequently, according to R_1=40 and R_2=1.2, being R_1 the molar ratio between SA and PCL and R_2 the molar ratio between DMAP and PCL. The mixture was left to react for 24 h at 60°C under a nitrogen atmosphere and vigorous stirring. Then, the product was precipitated in cold diethylether, vacuum dried and washed several times with twice-distilled water. Finally, the solid product, dissolved in acetone, was dialyzed against twice-distilled water (MWCO 12-14 kDa) and the obtained suspension freeze-dried. Freeze-drying was performed by using a Modulyo freeze-dryer (Labconco Corporation, Kansas City, MO 64132, USA).

The degree of PCL carboxylation was assessed by titration of proper amount of PCL-SUCC (20 mg/ml) in THF with a KOH methanol solution (35.6 mM), using a THF solution of thymol blue (5 mg/ml) as an indicator.

2.3 Synthesis and characterization of PHEA-g-SUCC-PCL

Derivatization of PHEA with PCL-SUCC to obtain the PHEA-g-SUCC-PCL graft copolymers goes on by using CDI as agent to speed up the condensation reaction. A calculated amount of CDI was added to PCL-SUCC solution in a-DMF (66.5 mg/ml), according to R_3=3, that is the mole ratio between CDI and PCL-SUCC. The clear solution proceeds under stirring at 40°C for 5 h under argon atmosphere. Simultaneously, PHEA were dissolved in *a*-DMF (33 mg/ml) at 40°C under argon atmosphere and then DEA, as catalyst, was added according to R_4= 0.3 that is the molar ratio between DEA and those of repeating units of PHEA carrying hydroxyl groups. After activation time, the resulting PHEA solution was added dropwise to CDI-activated PCL-SUCC at R_5= 0.06 or R_5= 0.12, being the molar ratio between PCL-SUCC and those of repeating units of PHEA carrying hydroxyl groups.

The reaction goes on under continuous stirring at 40°C for a chosen time (24, 44 or 68 h). After, the reaction mixture was added dropwise in diethyl ether, the solid product was separated by centrifugation (at 4°C for 15 min, at 10111*g*) with a Coulter Allegra X-22R refrigerated centrifuge (Beckman), and washed three times with a diethylether:dichloromethane mixture (4:1 v/v). The obtained product was dried under reduced pressure by using an evaporation system (Buchi). Then, it was dissolved in DMA, dialyzed against bidistilled water (MWCO 12-14 kDa) and freeze-dried.

PHEA-g-SUCC-$PCL_{(A)}$, PHEA-g-SUCC-$PCL_{(B)}$, and PHEA-g-SUCC-$PCL_{(C)}$ graft copolymers were obtained with R_5= 0.06, and a reaction time equal to 24, 44, and 68 hrs, respectively, while PHEA-g-SUCC-$PCL_{(D)}$, PHEA-g-SUCC-$PCL_{(E)}$, and PHEA-g-SUCC-$PCL_{(F)}$ graft copolymers were obtained with R_5= 0.12 and a reaction time equal to 24, 44, and 68 hrs, respectively.

^{1}H-NMR spectra were registered by a Bruker Avance II-300 spectrometer, working at 300 MHz (Bruker, Milan, Italy).

^{1}H-NMR (300 MHz, [D7].DMF, 25°C, TMS): δ 1.5 and 2.1 (m, $6H_{PCL}$ -[O(O)CCH_2(**CH_2)$_3$**CH_2]$_{122}$-); δ 2.5 (2d, $2H_{PCL}$ -[O(O)CCH_2(CH_2)$_3$**CH_2**]$_{122}$-); δ 2.8 (m, $2H_{PHEA}$ –C(O)CH**CH_2**C(O)NH-); δ 3.2 (t, $2H_{PHEA}$ -NH**CH_2**CH_2O-); δ 3.50 (t, $2H_{PHEA}$ -NHCH_2**CH_2**O-); δ 4.3 (t, $2H_{PCL}$ -[O(O)C**CH_2**(CH_2)$_3$$CH_2$]$_{122}$-), and δ 5.0 (m,

$1H_{PHEA}$ -NH**CH**(CO)CH_2-).

2.4 Synthesis and characterization of PHEA-g-SUCC-PCL-g-PEG graft copolymers

PHEA-g-SUCC-$PCL_{(B)}$ or PHEA-g-SUCC-$PCL_{(F)}$ graft copolymer was dissolved in *a*-DMA (64 mg/ml) and then TEA was added as catalyst. To this solution, a proper amount of DSC was added and the resulting mixture left to react at 40°C for 4 h; then, it was added to a H_2N-PEG solution in a-DMA (12 mg/ml). The amounts of reagents were calculated according to R_6 = 0.075, R_7 = 0.1 and R_8 = 1, being R_6 the molar ratio between H_2N-PEG and moles of repeating units of PHEA-g-SUCC-PCL carrying hydroxyl groups, R_7 the molar ratio between DSC and moles of repeating units of PHEA carrying hydroxyl groups, and R_8 the molar ratio between TEA and moles of DSC, respectively. The mixture was left for 18 h at 25°C, then dialysed (MWCO 12-14 kDa) against distilled water and freeze-dried to recover the obtained copolymer.

PHEA-g-SUCC-$PCL_{(B)}$-g-PEG, and PHEA-g-SUCC-$PCL_{(F)}$-g-PEG graft copolymers were obtained with a yield of 220 and 230 wt% based on the starting PHEA-g-SUCC-$PCL_{(B)}$, and PHEA-g-SUCC-$PCL_{(F)}$, respectively.

^{1}H-NMR (300 MHz, [D7].DMF, 25°C, TMS): δ 1.5 and 2.1 (m, $6H_{PCL}$ -[O(O)CCH_2(**CH_2)$_3$**CH_2]$_{122}$-); δ 2.5 (2d, $2H_{PCL}$ -[O(O)$CCH_2(CH_2)_3$**CH_2**]$_{122}$-); δ 2.8 (m, $2H_{PHEA}$ –C(O)CH**CH_2**C(O)NH-); δ 3.2 (t, $2H_{PHEA}$ -NH**CH_2**CH_2O-); δ 3.50 (t, $2H_{PHEA}$ -NHCH_2**CH_2**O-); δ 3.7 (t, $4H_{PEG}$ -[**CH_2CH_2**O]$_{44}$-); δ 4.3 (t, $2H_{PCL}$ -[O(O)C**CH_2**(CH_2)$_3$$CH_2$]$_{122}$-); and δ 5.0 (m, $1H_{PHEA}$ -NH**CH**(CO)CH_2-).

2.4.1 Size exclusion chromatography (SEC) analysis

SEC analysis was performed by a Waters system (Waters, Mildford, MA), by using Phenogel columns (5 μm particle size, 103 Å and 10^4 Å of pores size) from Phenomenex, and a differential refractometer as detector, at 50°C. The eluent was a 0.01 M LiBr DMF solution with a flow of 0.8 ml/min and PEG standards (range 145−1.5 kDa). Each sample was dispersed in the eluent, filtered (0.2 μm) and

analysed in triplicate.

2.4.2 FT-IR analysis

Each freeze-dried copolymer was analyzed by FT-IR. FT-IR spectra were registered by a Bruker ALPHA FT-IR spectrometer (Bruker, Milan, Italy).

2.5 Microfluidic method

Microfluidic experiment were conducted by using a hydrophilic split-and-recombine micromixer (Dolomite, UK) having 12 mixing stages [18]. PHEA-g-SUCC-$PCL_{(B)}$-g-PEG copolymer was dissolved in dimethylacetamide (DMA) at 0.75, 1.5 and 3% (w/v), then filtered through a 1 µm filter, and placed into a 15 mL tube connected to the chip with PTFE tubes and to the OB1 controller working with a maximum pressure of 2 bar. The polymer solution was inserted into the internal channel and fluxed at 100 $\mu L \cdot min^{-1}$, whereas bidistilled water was fluxed into the external channels into the range comprised between 2000 and 200 $\mu L \cdot min^{-1}$. Two different flow ratios, equal respectively to 0.1, and 0.2 (expressed as the ratio between the flow of the polymer dispersion and of water), were employed. The output tube was inlaid in bidistilled water. Recovered dispersion was dialysed against bidistilled water, and after freeze-drying, stored as dried product.

2.6 Nano-precipitation method

A DMA dispersion of PHEA-g-SUCC-$PCL_{(B)}$-g-PEG graft copolymer (0.75 % w/v) was placed in a burette and added dropwise to twice-distilled water (1:10 v/v), at a flow rate equal to 1ml/min. The mixture was left under stirring for 2 h, then dialysed against twice-distilled water. The obtained dispersion was freeze-dried, collected and stored at -20°C for further characterization. To produce PCL-based nanoparticles to use as control sample in further experiments, the same procedure was followed starting from a DMA dispersion of PCL copolymer (0.75 % w/v).

2.7 Scanning Electron Microscopy (SEM) analyses

For morphological studies, each freeze-dried sample was putted on a double-sided adhesive tape, previously applied on a stainless steel stub, which was then sputter-coated with gold prior to microscopy examination, and then observed by using an ESEM FEI Quanta 200F scanning electron microscope.

2.8 Static contact angle measurements

Static water contact angles of compression-molded films obtained from each freeze dried sample were measured by a FTA 1000 Series (First Ten Angstroms), equipped with 30 Gauge needle (diameter 0.30 mm). Each measurement was conducted in triplicate. The image analysis software was Drop Shape Analysis S.W. 2.0, also from the First Ten Angstroms (Portsmouth, VA).

2.9 X-ray photoelectron spectroscopy (XPS) analysis

The surface composition of each freeze-dried sample was determined by XPS analysis. XPS spectra were recorded using a PHI 5000 VersaProbe II (ULVAC-PHI, Inc.) and monochromatic Al-Kα radiation ($h\nu$ = 1486.6 eV) from an X-ray source operating at a spot size of 200 µm, a power of 50 W and an acceleration voltage of 15 kV.

2.10 Statistical Analysis

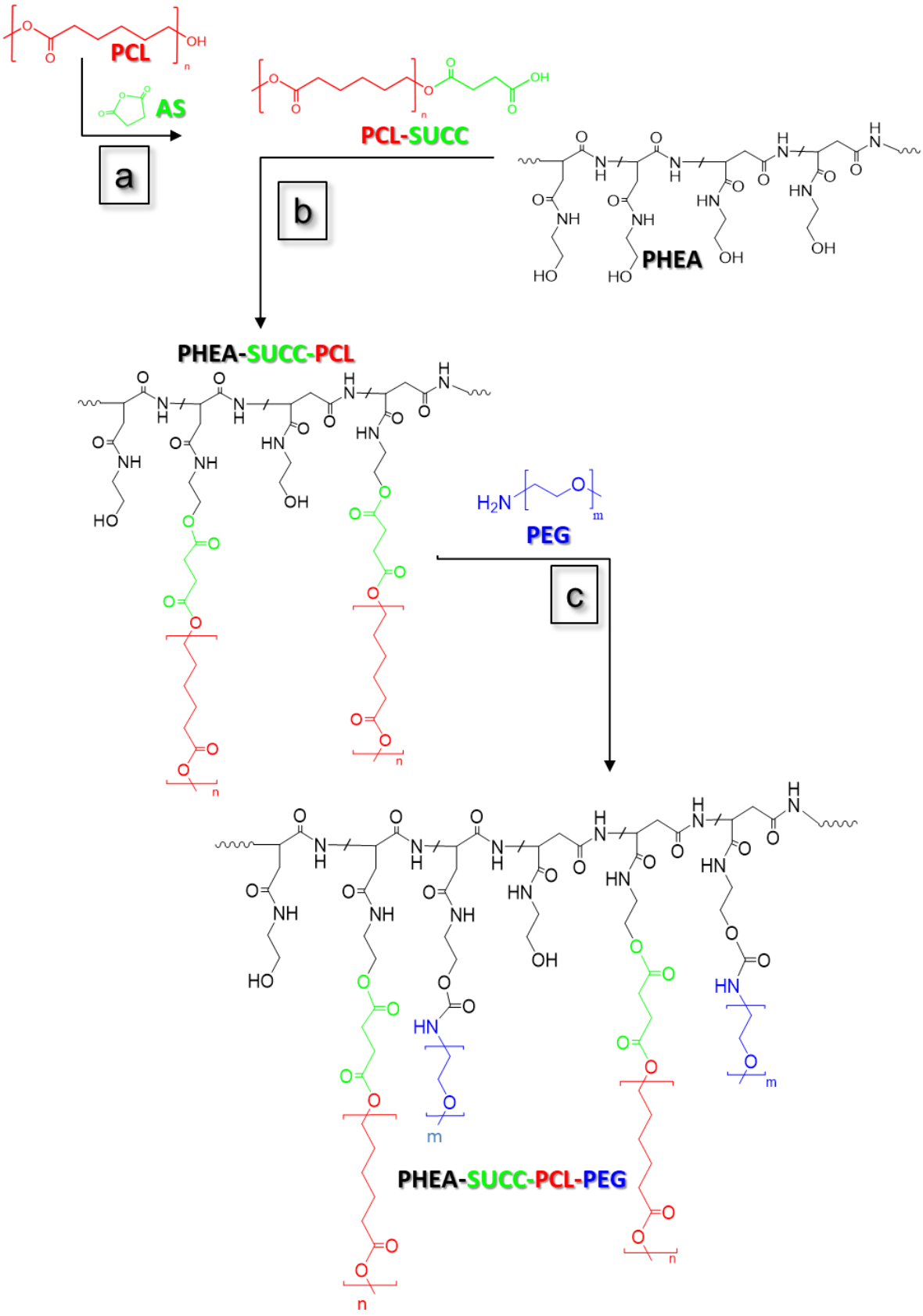
All the experiments were repeated at least three times. All data are expressed as means ± standard deviation. All data were analysed by Student's t test. A p-value < 0.05 was considered as statistically significant, while a p-value < 0.01 was considered as highly significant.

3. Results and Discussion

3.1 Synthesis and characterization of copolymers

Given the well-known possibility to combine/graft different materials to obtain copolymers with improved chemical and physical-chemical properties, this paper is focused on the synthesis and characterization of new amphiphilic PCL-based graft copolymers, having chemical-physical characteristics that make them suitable for the realization of drug carriers such as fibers, micro- and / or nanoparticles, potentially exploitable such as Drug Delivery Systems (DDS).

In detail, PCL, a hydrophobic linear polyester, was grafted onto the PHEA backbone by using 1,1'-carbonyldiimidazole (CDI) as agent to speed up the condensation reaction. It was chosen because its biodegradability and the acceptability for its approval by Food and Drug Administration (FDA). To increase the reactivity of PCL towards the coupling reaction with PHEA hydroxyl groups, the end-chain PCL hydroxyl group was previously succinylated with succinic anhydride (SA), obtaining the PCL-SUCC (Scheme 1, step a).



Scheme 1. The synthetic route of PHEA-g-SUCC-PCL-g-PEG graft copolymers (n =122, m = 44). Reagents and conditions: (**a**) *α*-DMF, DMAP,7/24h at 60°C; (**b**) *α*-DMF, CDI, DEA, 5h at 40°C, 24, 44, or 68 h at 40°C; (**c**) *α*-DMA, DSC, TEA, 4h at 40°C, 18h rt.

In particular, the step a was optimized by putting the terminal -OH of PCL to react for different times with SA allowing the introduction of terminal carboxylic groups. The highest percentage of PCL succinylation was obtained by fixing the mole ratio between SUCC and PCL equal to 40, operating at 60°C for 24 h and by using dimethylaminopyridine (DMAP) as catalyst.

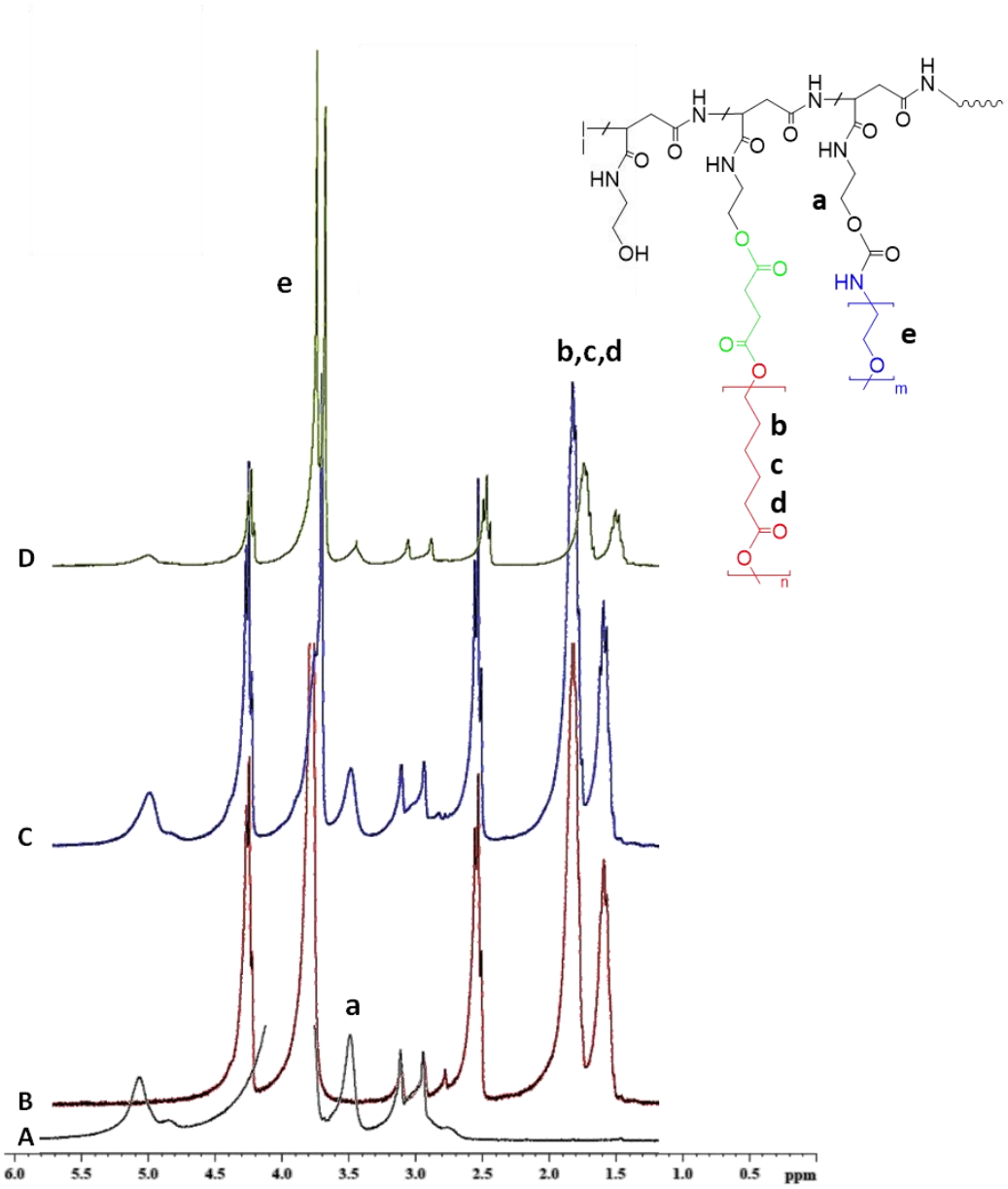
The step b (Scheme 1,b), to obtain the PCL-SUCC grafting reaction on PHEA backbone, was carried out in organic anhydrous solvent at 40°C by previously activating the end-chain carboxylic groups of PCL-SUCC with CDI, in the presence of diethylamine (DEA) as catalyst. Moreover, the amount of PCL grafted onto the PHEA backbone was opportunely tuned by maintaining the time and temperature of the activation reaction (t_{ACT} and T_{ACT}, respectively) of PCL-SUCC with CDI equal to 5 h and 40°C, and by varying the other experimental conditions in terms of reaction times (t_{REACT}) and the mole ratios of PCL-SUCC, PHEA, CDI and DEA reagents. In this way, several copolymers with different Derivatization Degrees in PCL chains (DD_{PCL}%) were easily obtained. The experimental conditions and molar ratios of reagents chosen for the grafting reaction of PCL-SUCC on PHEA backbone have been summarized in Table 1.

Table 1. Experimental conditions, molar ratios, weight-average molecular weight ($\bar{M}_w$) and polydispersity index ($\bar{M}_w/\bar{M}_n$) values of obtained PHEA-g-SUCC-PCL graft copolymers.

Graft copolymers	**Experimental conditions**	**Molar ratios**			**Molecular weight**		**Yield**
	t_{tREACT}/T_{REACT} (hrs/°C)	**R_3**	**R_4**	**R_5**	**($\bar{M}_w$) (kDa)**	**$\bar{M}_w/\bar{M}_n$**	**wt/wt %** based on all starting materials
PHEA-g-SUCC-PCL$_{(A)}$	24/40	3	0.3	0.06	114.0	1.6	30.6
PHEA-g-SUCC-PCL$_{(B)}$	44/40	3	0.3	0.06	134.0	1.5	39.2
PHEA-g-SUCC-PCL$_{(C)}$	68/40	3	0.3	0.06	158.5	1.2	41.4
PHEA-g-SUCC-PCL$_{(D)}$	24/40	3	0.3	0.12	108.5	1.4	22.9
PHEA-g-SUCC-PCL$_{(E)}$	44/40	3	0.3	0.12	153.0	1.3	28.0
PHEA-g-SUCC-PCL$_{(F)}$	68/40	3	0.3	0.12	187.0	1.2	37.5

R_3 = CDI / PCL-SUCC molar ratio
R_4 = DEA/ hydroxyl-carrying PHEA repeating unit molar ratio
R_5 = PCL-SUCC/ hydroxyl-carrying PHEA repeating unit molar ratio

The amount of PCL chains linked to PHEA, defined as Degree of Derivatization with PCL (DD_{PCL}%) was calculated from the ^{1}H-NMR spectra in deuterated DMF. **Fig. 1** and report a typical ^{1}H-NMR spectrum and integral values of PHEA-g-SUCC-$PCL_{(B)}$, in comparison with PHEA and PCL-SUCC polymers.



	Integral values		
Peak in the spectrum	e	**a**	**b,c,d**
Attribution	**$4H_{PEG}$ $-[CH_2CH_2O]_{44}-$**	**$2H_{PHEA}$ $-C(O)CHCH_2C(O)NH-$**	**$6H_{PCL}$ $-[O(O)CCH_2(CH_2)_3CH_2]_{122}-$**
Spectrum C	---	1	7.5 ± 0.5
Spectrum D	3.5 ± 0.2	1	7.5 ± 0.5

Fig. 1. ^{1}H-NMR spectra of: (A) PHEA, (B) PCL-SUCC, (C) PHEA-g-SUCC-PCL, and (D) PHEA-g-SUCC-PCL-g-PEG copolymers in DMF-d_7.

In particular, the integral of the peaks related to protons between δ 1.5 and 2.1 (H= 732) as well as to protons at δ 2.6 (H=244) and at δ 4.3 (H=244) belonging to linked PCL chains, were compared to the integral related to protons at δ 3.6 (H=2) attributed to protons on PHEA backbone.

The DD_{PCL} resulted to be 1.2 ± 0.3, 2.0 ± 0.2 and 2.2 ± 0.3 mol%, respectively, for PHEA-g-SUCC-$PCL_{(A)}$, PHEA-g-SUCC-$PCL_{(B)}$ and PHEA-g-SUCC-$PCL_{(C)}$ graft copolymers. For PHEA-g-SUCC-$PCL_{(D)}$, PHEA-g-SUCC-$PCL_{(E)}$ and PHEA-g-SUCC-$PCL_{(F)}$ copolymers, DD_{PCL} resulted to be equal to 1.1 ± 0.2, 2.2 ± 0.2, 4.4 ± 0.3 mol%, respectively.

These results are reported in **Fig. 2**. As can be seen, at lower t_{REACT}, the DD_{PCL}% of obtained copolymers do not change by increasing R_5 (i.e. the PCL-SUCC/hydroxyl-carrying PHEA repeating unit molar ratio), whereas at higher t_{REACT} such as 68 h, the DD_{PCL}% was strongly increased, going from 2.2 to 4.4 at R_5 = 0.06 and 0.12, respectively.

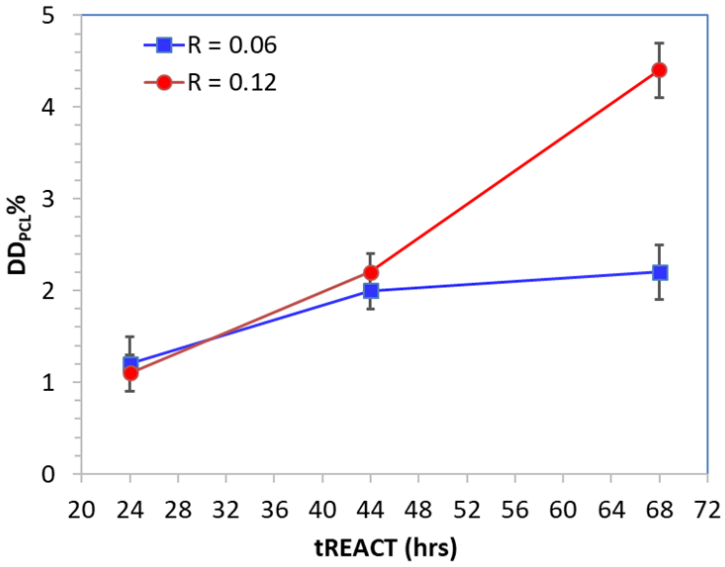


Fig. 2. DD_{PCL}% of obtained PHEA-g-SUCC-$PCL_{(A)-(F)}$ graft copolymers as a function of reaction time (t_{REACT}) (hrs). Each reaction was conducted in triplicate.

All obtained copolymers were characterized by the determination of the $\overline{M}_w$ and the $\overline{M}_w/\overline{M_n}$ values by SEC analysis in organic solvent. Results are also reported in Table 1. Obtained data are in good

agreement with theoretical $\bar{M}_w$ calculated considering the DD values. Considering all data, the number of PCL chains grafted on a single starting PHEA chain (with a mean molecular weight of 43.7 kDa) can varied from 3 to about 12, respectively, from PHEA-g-SUCC-$PCL_{(A)}$ to PHEA-g-SUCC-$PCL_{(F)}$ derivatives.

The functionalization reactions was confirmed by the FT-IR analysis of either starting or obtained copolymers (**Fig. 3**, range 1800-1000 cm^{-1}), in which the FT-IR spectra overlay highlights the presence in the spectrum of the PHEA-g-SUCC-PCL copolymer of typical bands of both PHEA and PCL-SUCC polymers.

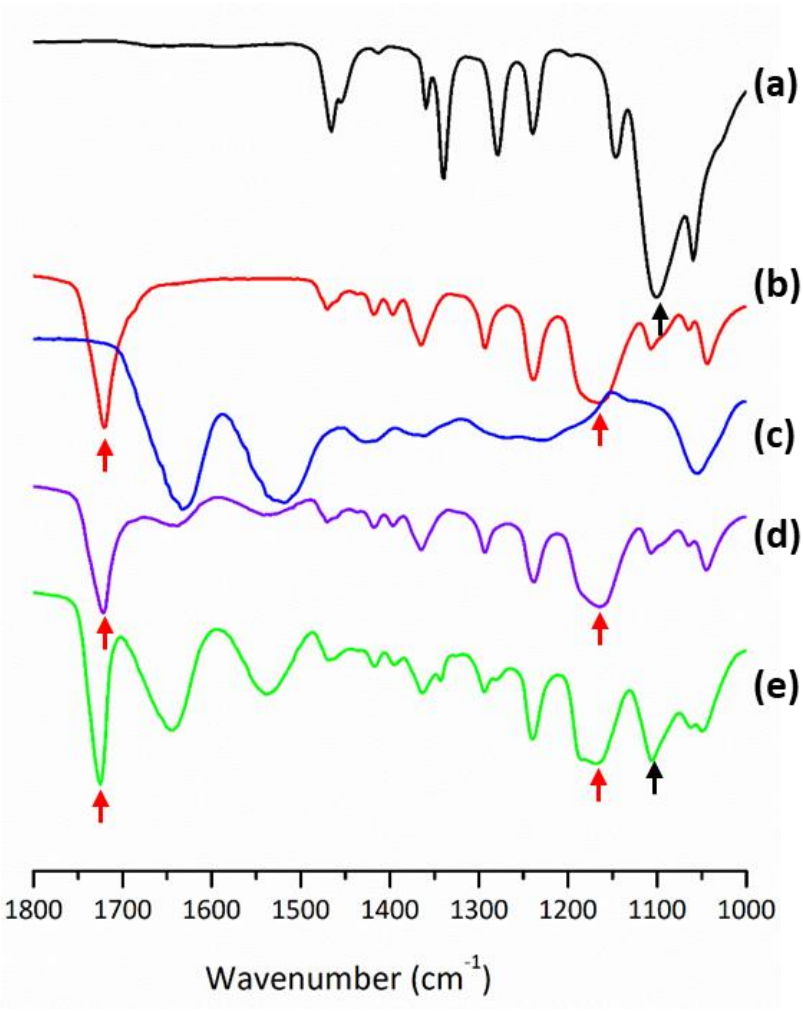


Fig. 3. FT-IR overlay spectra (in the range 1800-1000 cm^{-1}) of (a) PEG, (b) PCL-SUCC, (c) PHEA, (d) PHEA-g-SUCC-PCL and (d) PHEA-g-SUCC-PCL-g-PEG graft copolymers.

In particular, the FT-IR spectrum of PHEA-g-SUCC-PCL graft copolymer (spectrum **d**, violet line) showed the characteristic strong bands at 1645-1633 cm^{-1} (amide I) and 1533-1518 cm^{-1} (amide II), corresponding respectively to the asymmetric stretching of C=O and bending of N-H on the aspartamide backbone (spectrum **c**, blue line), and two bands at at 1721 and 1169 cm^{-1} attributed, respectively, to the asymmetric stretching of C=O and C-O of the grafted PCL structure (spectrum **b**, red line), indicated as red arrows in the **Fig. 3**.

Thanks to the introduction of hydrophobic PCL chains to the water-soluble PHEA copolymer, all the obtained copolymers were insoluble in water, but soluble in organic solvents such as dimethylsulfoxide, dimethylformamide and dimethylacetamide.

Therefore, we have obtained a class of novel graft copolymers with PCL as hydrophobic portions which biodegradability could be modulated by the conjugation with other biopolymers such as PHEA. The synthetic strategy was properly optimised in order to obtain graft copolymers with suitable weight average molecular weights and polydispersity, resulting from little or no degradation due to experimental conditions. This property, together with the fact that PHEA possesses in their backbone a great multiplicity of hydroxyl groups available for further conjugation with several class of ligands, allows to confers to the resulting copolymer proper structural and functional properties suitable for the production of advanced drug delivery systems.

Therefore, in order to explore the possibility to conjugate to these copolymers hydrophilic chains such as polyethylene glycol (PEG) to obtain copolymers with amphiphilic properties and able to give, almost potentially, copolymers useful for biomedical and pharmaceutical applications, a pegylated derivative of PHEA-SUCC-PCL graft copolymers was synthesized, by following a synthetic strategy already developed for PLA graft derivatives based on PHEA [17].

In particular, taking into consideration either the obtained yield values or the two polymers with a statistically significant difference in the DD_{PCL}, PHEA-g-SUCC-$PCL_{(B)}$ and PHEA-g-SUCC-$PCL_{(F)}$ graft copolymers were chosen as starting copolymers, while N,N'-disuccinimdyl carbonate (DSC) was used

to activate the hydroxyl groups of PHEA-g-SUCC-PCL toward O-(2-Aminoethyl)-O'-methyl poly(ethylene glycol) (H_2N-PEG_{2000}) with $\bar{M}_w$ of 2,000 Da. The synthetic procedure, reaction conditions and general chemical structure of PHEA-g-SUCC-PCL-g-PEG graft copolymers are reported in the Scheme 1c. Yield values for obtained pegylated copolymers were comparable and results of about 75 wt% on the total starting materials.

The degree of derivatization in PEG_{2000} chains (DD_{PEG}%) of obtained copolymers was calculated from the ^{1}H-NMR spectra, by the ratio between the integral of the peak related to protons (H=176) at 3.7 δ attributed to protons on PEG and the integral related to protons (H=2) a δ 3.50 attributed to protons on PHEA backbone. The DD_{PEG}%, expressed as mean of three values, resulted to be 3.9 ± 0.3 and 4.3 ± 0.4 mol%, respectively for PHEA-g-SUCC-$PCL_{(B)}$-g-PEG and PHEA-g-SUCC-$PCL_{(F)}$-g-PEG graft copolymers, therefore not significantly different from each other.

In other words, regardless of the initial amount of grafted PCL, a comparable amount of PEG can be covalently linked on the PHEA backbone, being the same the experimental conditions and reaction parameters of the two pegylation reactions. $\bar{M}_w$ values of both copolymers result to be higher respect to the starting PHEA-g-SUCC-PCL copolymers, in accordance with the expected increase considering the obtained DD_{PEG}, being respectively equal to 151.0 kDa ($\bar{M}_w/\bar{M}_n$= 1.5) and 201.6 kDa ($\bar{M}_w/\bar{M}_n$= 1.3) for PHEA-g-SUCC-$PCL_{(B)}$-g-PEG and PHEA-g-SUCC-$PCL_{(F)}$-g-PEG graft copolymers.

Moreover, FT-IR analysis confirmed the introduction of PEG in the starting copolymer, showing the FT-IR spectrum of the obtained copolymer the typical bands of both PHEA-g-SUCC-PCL and PEG chains. In particular, the FT-IR spectrum of PHEA-g-SUCC-PCL-g-PEG graft copolymer (spectrum **e**, green line) showed the bands of PHEA-g-SUCC-PCL (spectrum **d**, violet line) and band at 1106 cm^{-1} attributed to the grafted PEG chains (spectrum **a**, black line), indicated as a black arrow in the **Fig. 3**.

3.2 Production and characterization of micro- and nanostructures

The PHEA-g-SUCC-$PCL_{(B)}$-g-PEG graft copolymer was chosen to evaluate the potential of such graft copolymer to obtain micro- and/or nanostructures to be used for drug delivery, by applying methods such as microfluidics and nanoprecipitation. In particular, among the two pegylated copolymers, it was selected having a fairer balance between hydrophilic and hydrophobic portion as the PCL amount corresponds to about 54 and 72 wt% of the total weight respectively for the PHEA-g-SUCC-$PCL_{(B)}$-g-PEG and PHEA-g-SUCC-$PCL_{(F)}$-g-PEG graft copolymers.

The microfluidic process was carried out by choosing DMA and bidistilled water as internal and external phase, respectively. Moreover, the polymeric concentration in DMA was varied from 3.0 to 0.75% w/v, and the volume ratio between internal and external ratio from 0.2 to 0.1. In **Fig. 4,** SEM images of freeze-dried samples obtained by microfluidics are reported.

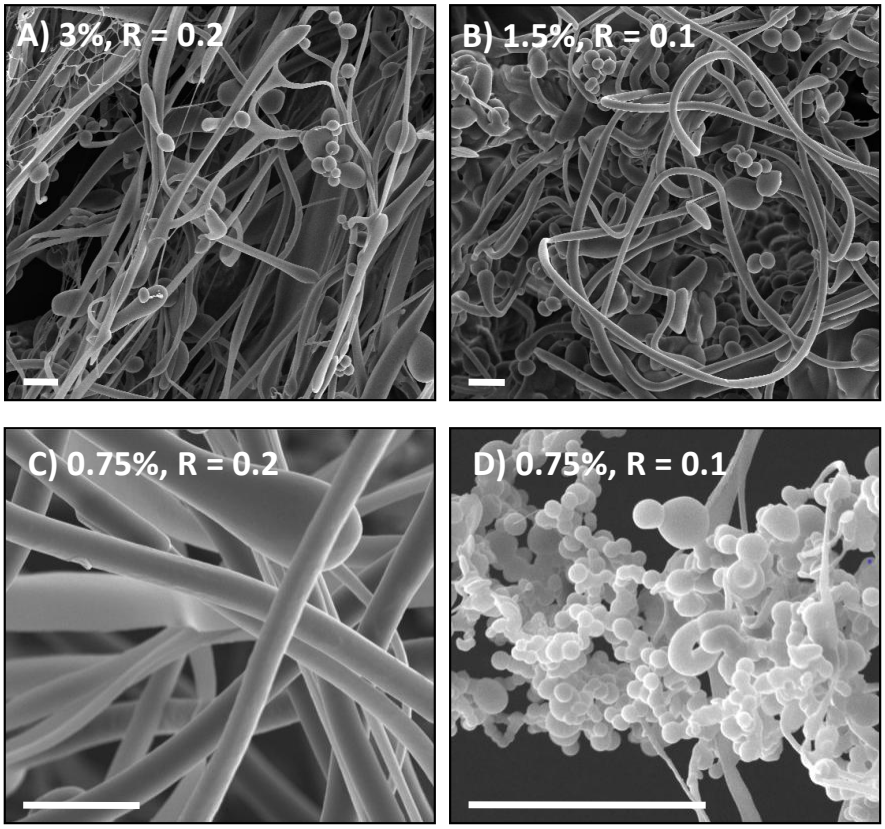


Fig. 4. SEM images of freeze-dried samples obtained by microfluidics at copolymer concentration in DMA solution and DMA/H_2O volume ratio equal to: A) 3% w/v, R = 0.2; B) 1.5% w/v, R= 0.1; C) 0.75% w/v, R= 0.2; D) 0.75% w/v, R=0.1. The scale bar represents 10 μm in all images.

As can be seen, at the highest concentration values (3 and 1.5% w/v), mixed structures including fibers and microparticles were obtained. At the lowest chosen polymeric concentration (0.75% w/v) and by varying the volume ratio between internal/external phases, different and better controlled structures can be obtained, from fibers (**Fig. 4C**, mean diameter equal to 2.6 μm, S.D. = 1.0) to microparticles (**Fig. 4D**, mean size equal to 0.9 μm, S.D. = 0.73).

Finally, by using a different aggregation method such as nanoprecipitation, a copolymer concentration such as 0.75% w/v and an internal/external phase volume ratio of 0.1, submicronic structures are obtained. In **Fig. 5**, SEM images of freeze-dried sample obtained by nanoprecipitation showed particles of 0.59 μm (S.D.= 0.11) as mean diameter. This value was confirmed by Dynamic Light Scattering, that gives an average mean diameter of 56.6 nm and a PDI of 0.27.

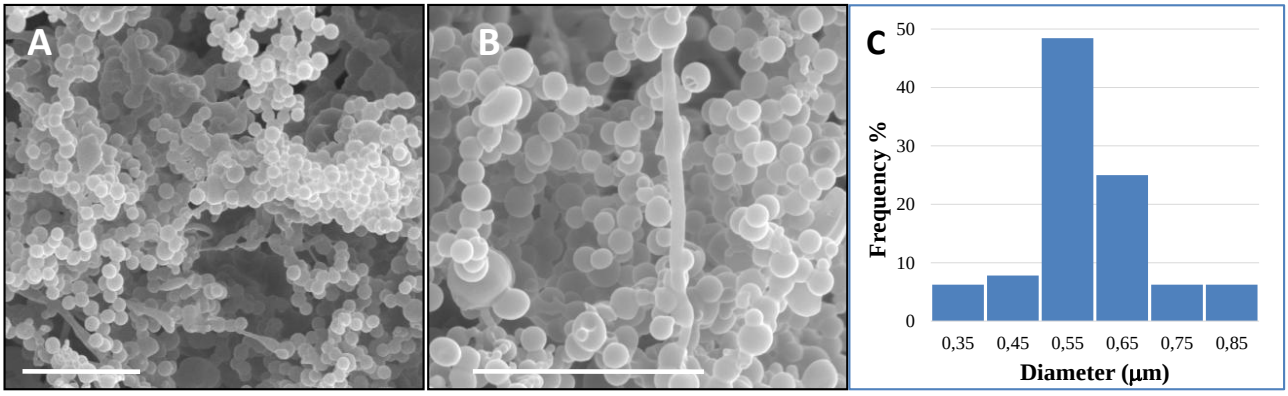


Fig. 5. SEM images (A-B) and size distribution (C) of freeze-dried sample obtained by nanoprecipitation at a copolymer concentration equal to 0.75% w/v in DMA and a DMA/H_2O volume ratio equal to 0.1. The scale bar represents 5 μm.

Therefore, by using a PHEA-g-SUCC-$PCL_{(B)}$-g-PEG solution in DMA at a concentration equal to 0.75% w/v and a DMA/H_2O volume ratio equal to 0.1, by microfluidics microparticles were produced, while by nanoprecipitation nanoparticles are obtained.

To evaluate the surface properties of obtained PHEA-g-SUCC-PCL-g-PEG based particles, the static water contact angle was determined. In particular, measurements were carried out on compression-molded films, which were obtained from freeze-dried PHEA-g-SUCC-PCL-g-PEG - based microparticles and PHEA-g-SUCC-PCL-g-PEG - based nanoparticles. For comparison, the water contact angle was also measured on a compression molded film of nanoparticles, which were obtained from PCL alone by nanoprecipitation.

Fig. 6 shows the exhibited water contact angles of compressed films obtained from the PCL nanoparticle – based (A), PHEA-g-SUCC-PCL-g-PEG microparticle – based (B) and nanoparticle - based (C) samples, are showed, which resulted to be respectively, equal to 68.8°, 37.5° and 0°.

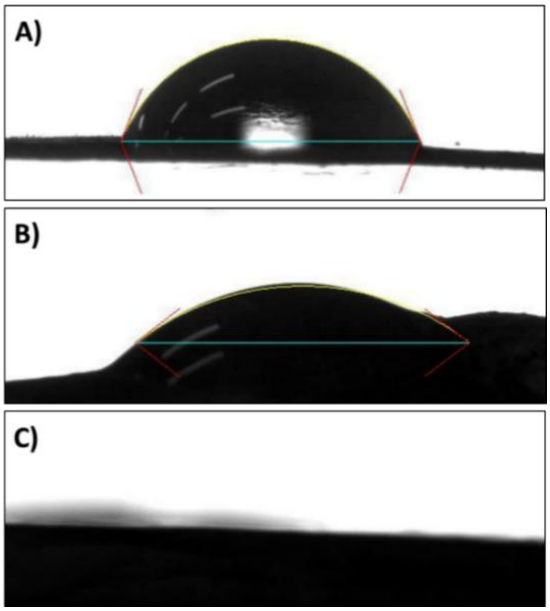


Fig. 6. Optical images of PCL nanoparticle – based (A), PHEA-g-SUCC-PCL-g-PEG microparticle – based (B) and PHEA-g-SUCC-PCL-g-PEG nanoparticle - based (C) compact surfaces.

This analysis demonstrates that respect to PCL alone, the presence of hydrophilic portions in the copolymer, i.e. PHEA and PEG into the PHEA-g-SUCC-PCL-g-PEG graft copolymer, decreased the static contact angle value, thus increasing the wettability. It also demonstrates that starting from the same material, the PHEA-g-SUCC-PCL-g-PEG copolymer, the production method influences the exposition

of the hydrophilic portions, being the particle surfaces highly or completely wettable if obtained, respectively, by microfluidic or nanoprecipitation.

To confirm that the production method influences the composition of the surface layer, a XPS analysis was carried out on PHEA-g-SUCC-PCL-g-PEG - based microparticles and nanoparticles. Moreover, the investigation was also carried out on PCL-based nanoparticles and all obtained results were compared.

In Table 2, results of XPS analyses of PHEA-g-SUCC-PCL-g-PEG - based micro- and nanoparticles, and of PCL - based nanoparticles, are reported as the relative distribution of the carbon, oxygen and nitrogen species on the particle surface, determined by a curve-fitting procedure of the photoelectron peaks.

Table 2. XPS Surface chemical composition of obtained particles[a].

Sample	C 1s	O 1s	N 1s
PCL nanoparticles[b]	74.49	25.51	---
PHEA-g-SUCC-PCL-g-PEG microparticle[c]	71.08	25.69	3.23
PHEA-g-SUCC-PCL-g-PEG nanoparticles[b]	72.60	25.32	2.08

[a]Relative distribution of the carbon, nitrogen, oxygen and phosphorus species on the FNP surface determined by a curve-fitting procedure of the photoelectron peaks.
[b]Obtained by nanoprecipitation.
[c]Obtained by microfluidics.

As showed, the C 1s relative atomic percentage on both PHEA-g-SUCC-PCL-g-PEG - based particles is lower than that found on PCL-based sample, while in both samples is present nitrogen, at a binding energy equal to 399.7 eV, it should be attributed to N-C/N-C(O) linkage, that is attributed to PHEA. As expected, in the PCL-based sample this peak is absent. Moreover, the C 1s relative atomic percentage is less abundant on the PHEA-g-SUCC-PCL-g-PEG-based microparticle surface than that found on the PHEA-g-SUCC-PCL-g-PEG-based nanoparticle surface. On the contrary, the N 1s relative atomic percentage is higher on the PHEA-g-SUCC-PCL-g-PEG-based microparticle surface, demonstrating that the surface of the microparticles is more enriched of the PHEA backbone.

As reported elsewhere, the introduction of PEG on the particle surface caused a decrease of the detected nitrogen atomic percentage [16]. In this case, being the same starting material, it could mean that there are more PEG chains exposed on the nanoparticles obtained by nanoprecipitation than on the microparticles obtained by microfluidics. To support this, the relative distributions of the carbon species on the particle surface were determined by a curve - fitting procedure, and results are reported in **Table 3**.

It is evident that in both the samples based on PHEA-g-SUCC-PCL-g-PEG graft copolymer, the C 1s core level spectrum showed species at the binding energy (BE) of 286.0 and 287.6 eV, attributed respectively to C-N and N-C=O bonds (belonging to PHEA backbone), that are absent on the PCL-based sample. Moreover, the C 1s core level spectrum at the BE of 286.2 eV, assigned to C–O–C bonds belonging to PEG chains is more pronounced in the nanoparticles than in microparticles (42.96 vs 31.66%).

Table 3. XPS Surface chemical composition of obtained particles[a].

	C-C/C-H	C-N	C-O	N-C=O	O-C=O
Sample	**284.8**	**286.0**	**286.2**	**287.6**	**288.6**
PCL nanoparticles[b]	66.65	---	16.66	---	16.69
PHEA-g-SUCC-PCL-g-PEG microparticle[c]	46.83	5.26	31.66	5.23	11.05
PHEA-g-SUCC-PCL-g-PEG nanoparticles[b]	39.09	4.46	42.96	4.36	9.23

[a]Relative distribution of the carbon species on the FNP surface determined by a curve-fitting procedure of the photoelectron peaks.
[b]Obtained by nanoprecipitation.
[c]Obtained by microfluidics.

This results demonstrates that although are prepared starting from the same material, a higher amount of PEG is exposed on the nanoparticle surface than in the microparticle surface. In particular, considering the chemical composition of the bulk material, the amount of PEG on the nanoparticle surface resulted to be 2.8 times higher than expected, while it results to be 1.7 times higher than expected when the microfluidics was used to obtain particles. It can be assumed that the different

surface composition of the obtained particles is due to a different arrangement of the copolymer at the interface between the organic and the aqueous phase according to the followed method. In both cases, however, the particle surfaces are highly pegylated. In **Fig. 7a** and **7b**, the curve fittings of the C 1s spectra of PHEA-g-SUCC-PCL-g-PEG micro- and nanoparticles are reported, respectively.

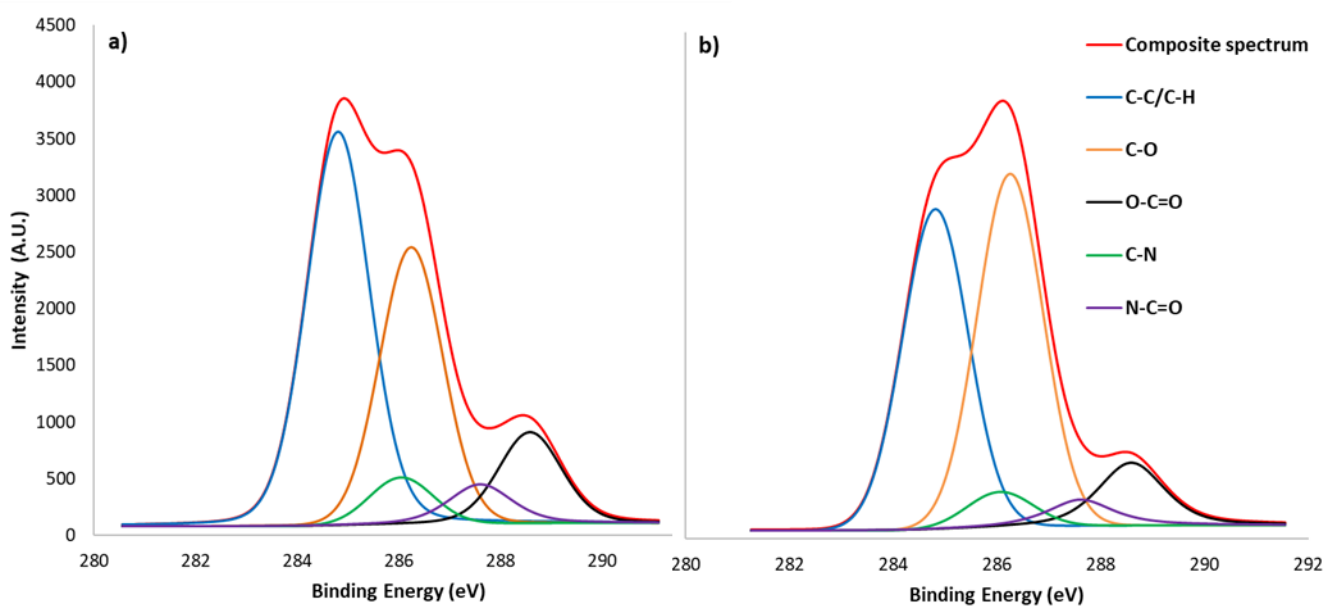


Fig. 7. Curve-fitting of the C 1s spectrum of PHEA-g-SUCC-PCL-g-PEG- based: a) microparticles (obtained by microfluidics) and b) nanoparticles (obtained by nanoprecipitation).

As expected, in the PCL-based sample the C 1s core level spectrum at the BE of 286.2 eV was significantly lower than that found on PHEA-g-SUCC-PCL-g-PEG- based particles, while the C 1s core level spectra at BE of 284.8 and 288.6 eV, attributed to C–C/C-H and O-C=O bonds (belonging mainly from the PCL polyester), are significantly higher and mainly represented.

4. Conclusion

Here, we have reported the synthetic procedure to easily obtain novel copolymers, containing a polyaspartamide backbone and grafted PCL chains. To do that, a two-step synthetic strategy was performed by conjugating PHEA to a succinylated derivative of PCL, by varying reaction times and

amount of PCL-SUCC. Moreover, the presence of PHEA backbone allows further functionalizations, i.e. with PEG chains. Compared to similar graft copolymers, these copolymers has high and controlled molecular weights which could give high performing behaviour. It was demonstrated that the obtained PHEA-g-SUCC-$PCL_{(B)}$-g-PEG graft copolymer (with DD_{PCL} and DD_{PEG} equal to 2.2 and 3.9 mol%, respectively) can be processable by different methods to obtain various types of systems potentially useful in the biomedical field, such as fibers and particles. In particular, at the copolymer concentration of 0.75% w/v and at a volume ratio between the organic and the aqueous phases of 1/10, micro- and nanoparticles were obtained respectively by microfluidics ad nanoprecipitation. By static water contact angle and XPS analyses, it was demonstrated that by varying the method to produce particles, i.e. microfluidics versus nanoprecipitation, the amount of exposed PEG on the surface can vary, thus the surface characteristics could be modulated, such as the wettability.

Therefore, a novel material with tunable properties was produced by scalable synthetic steps, which can be used as starting material to produce systems with different shape, dimensions and surface characteristics, with high potential for biomedical applications.

Declaration of competing interest

The authors declare no competing financial interests.

Acknowledgments

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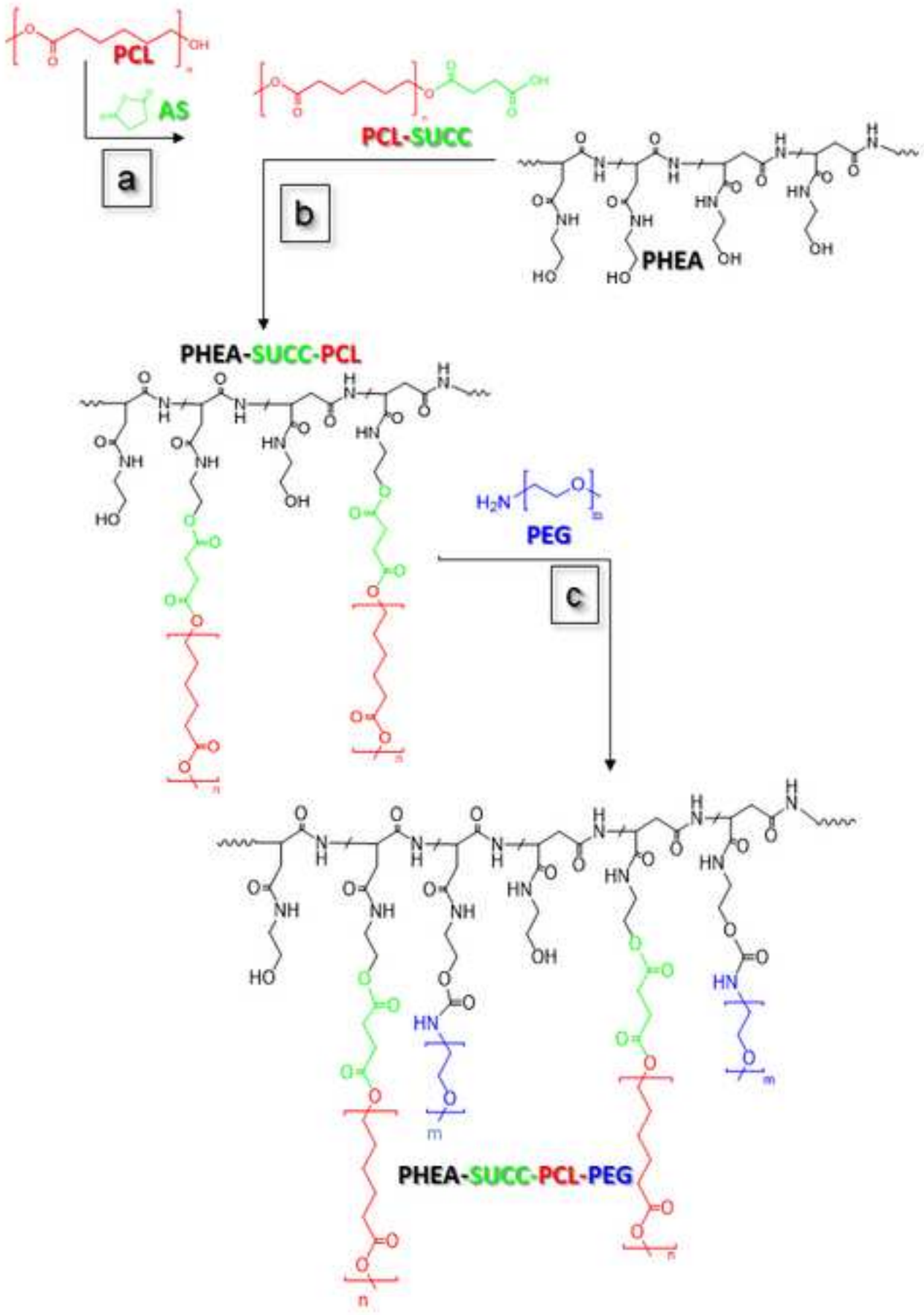

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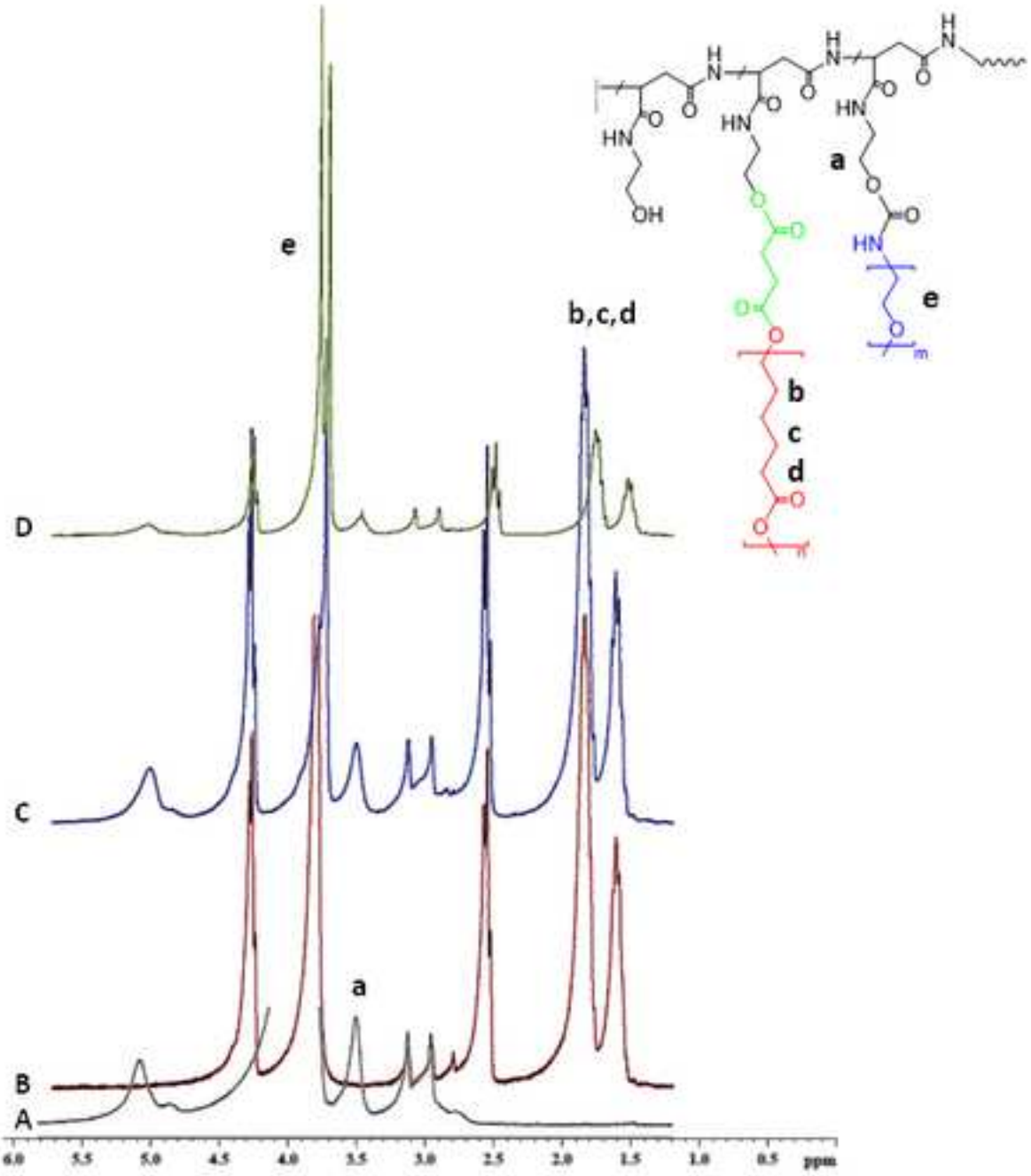

	Integral values		
Peak in the spectrum	e	a	b,c,d
Attribution	$4H_{PEG}$ $-[CH_2CH_2O]_{44}-$	$2H_{PHEA}$ $-C(O)CHCH_2C(O)NH-$	$6H_{PCL}$ $-[O(O)CCH_2(CH_2)_3CH_2]_{122}-$
Spectrum C	---	1	7.5 ± 0.5
Spectrum D	3.5 ± 0.2	1	7.5 ± 0.5

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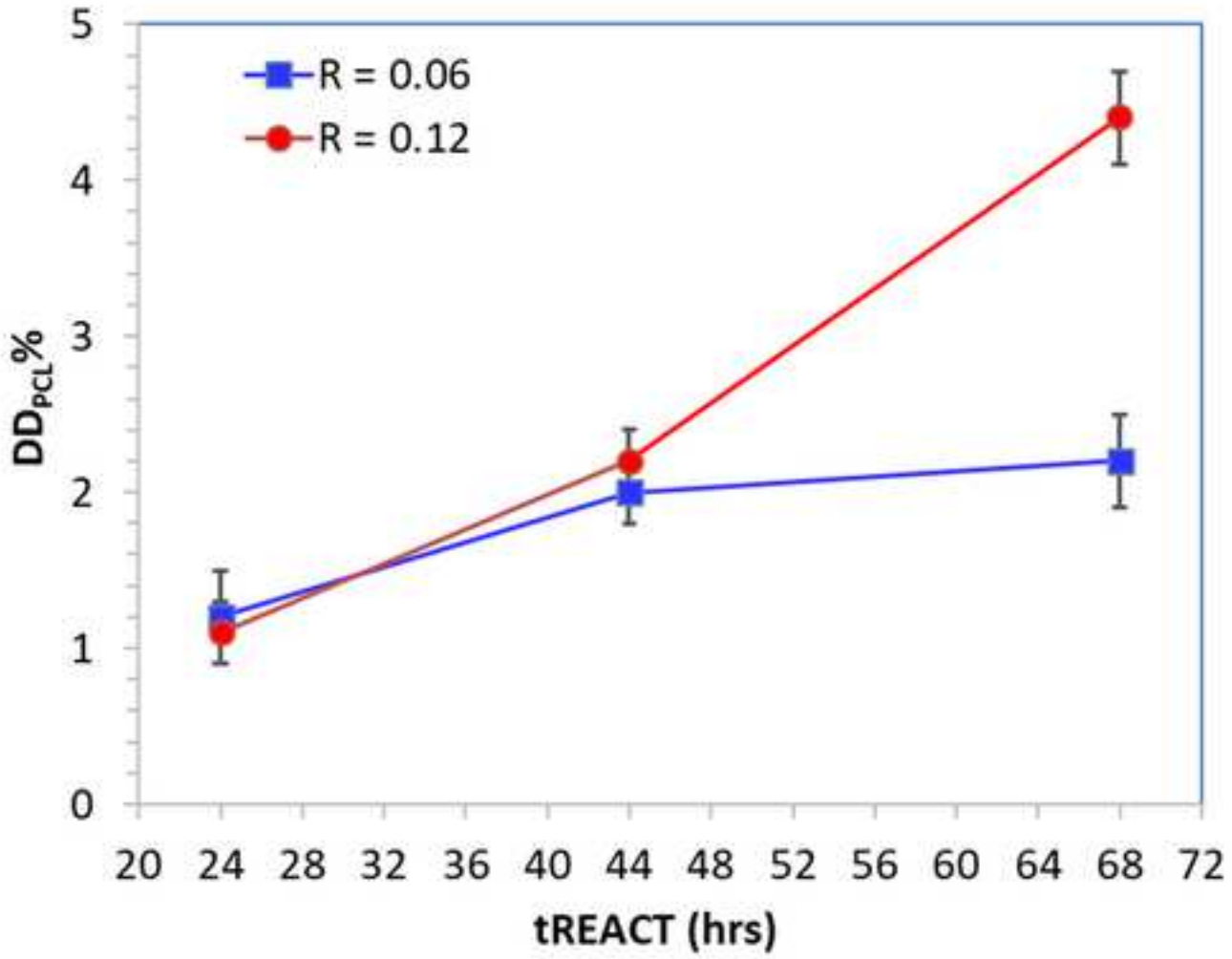

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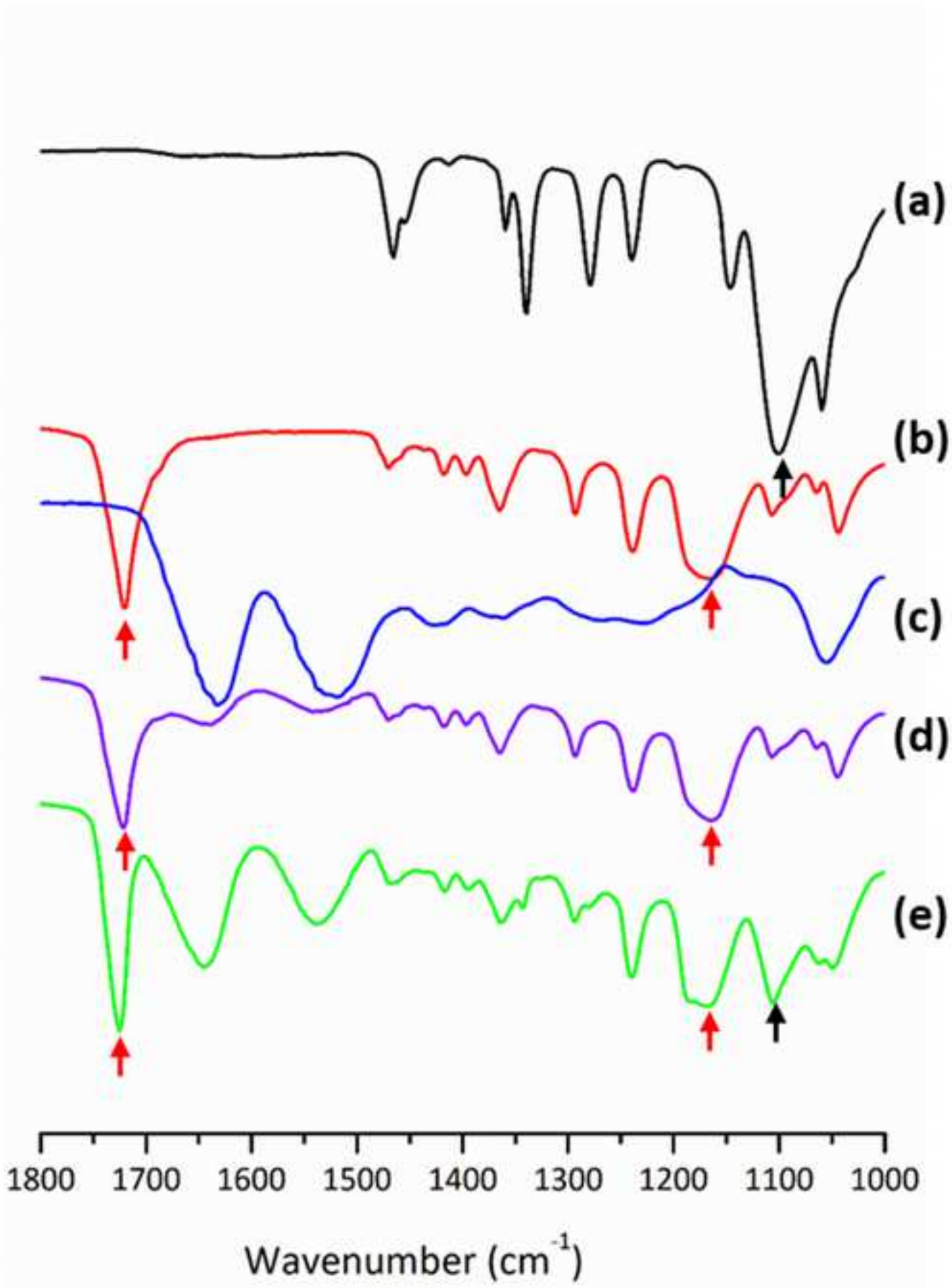

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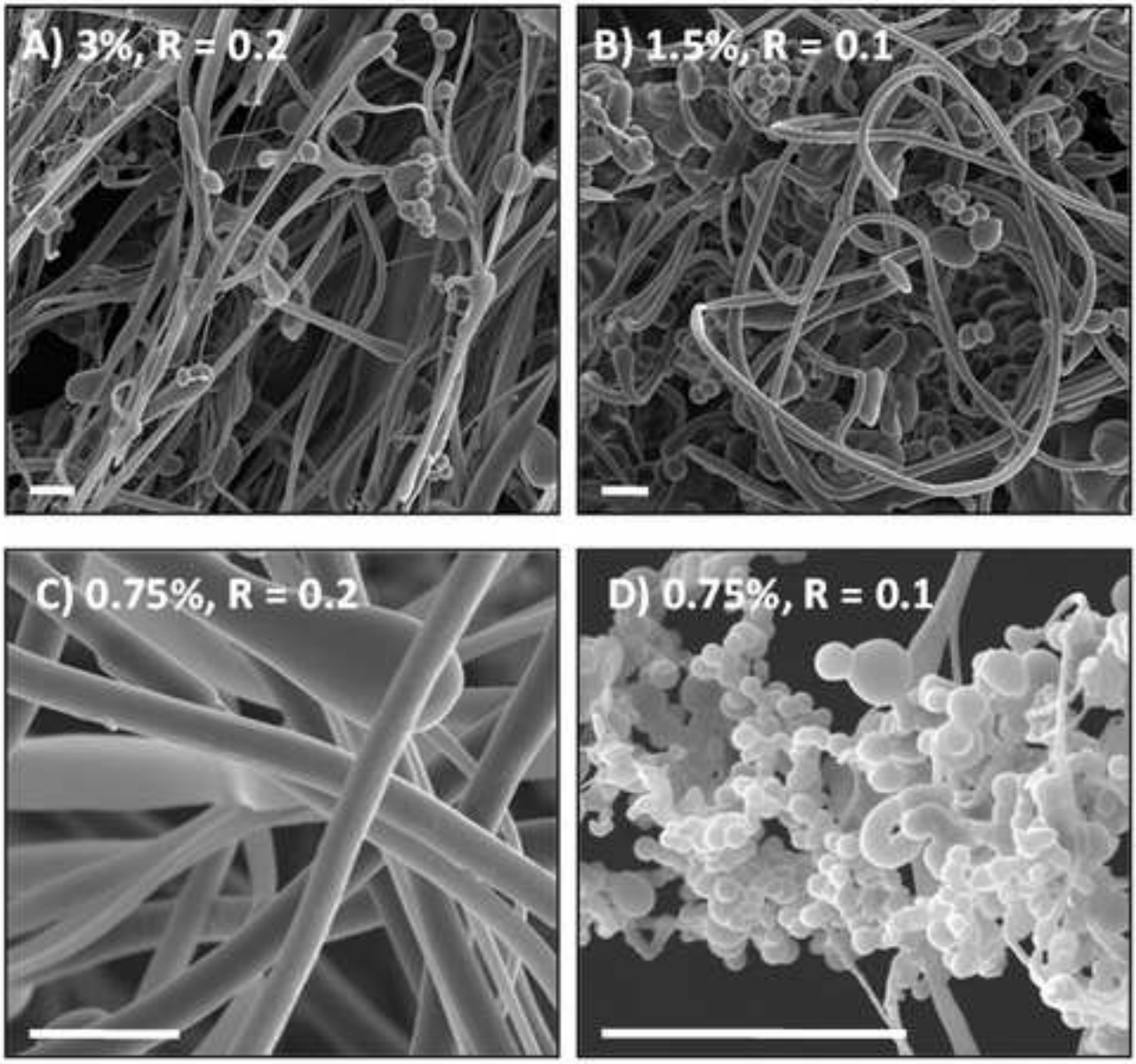

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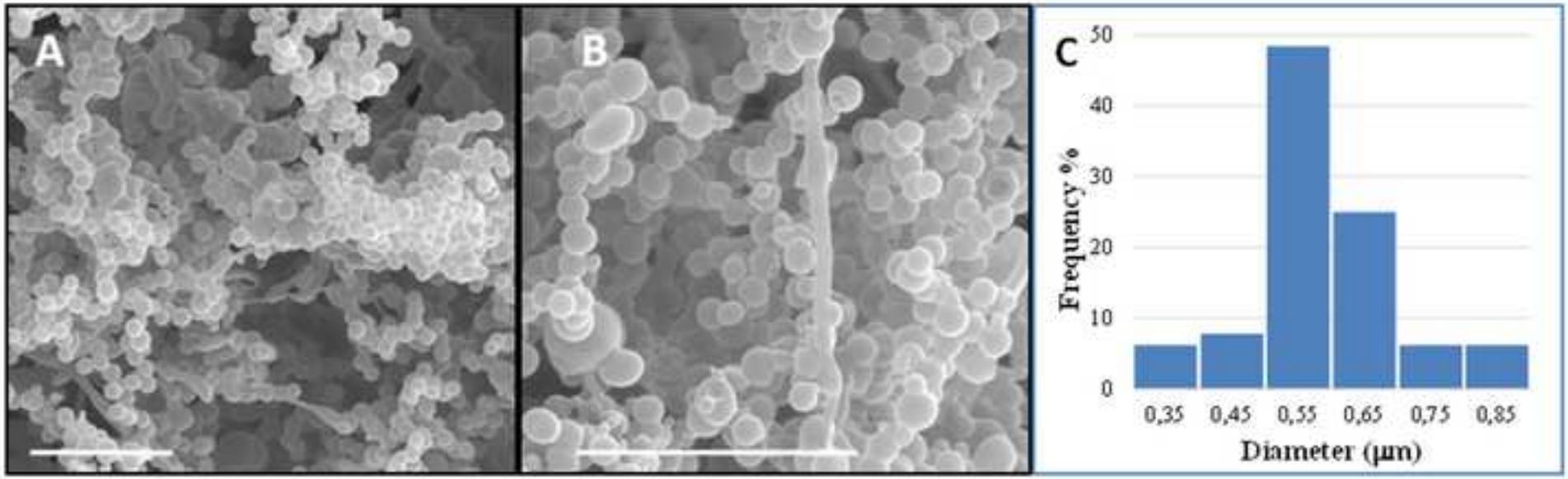

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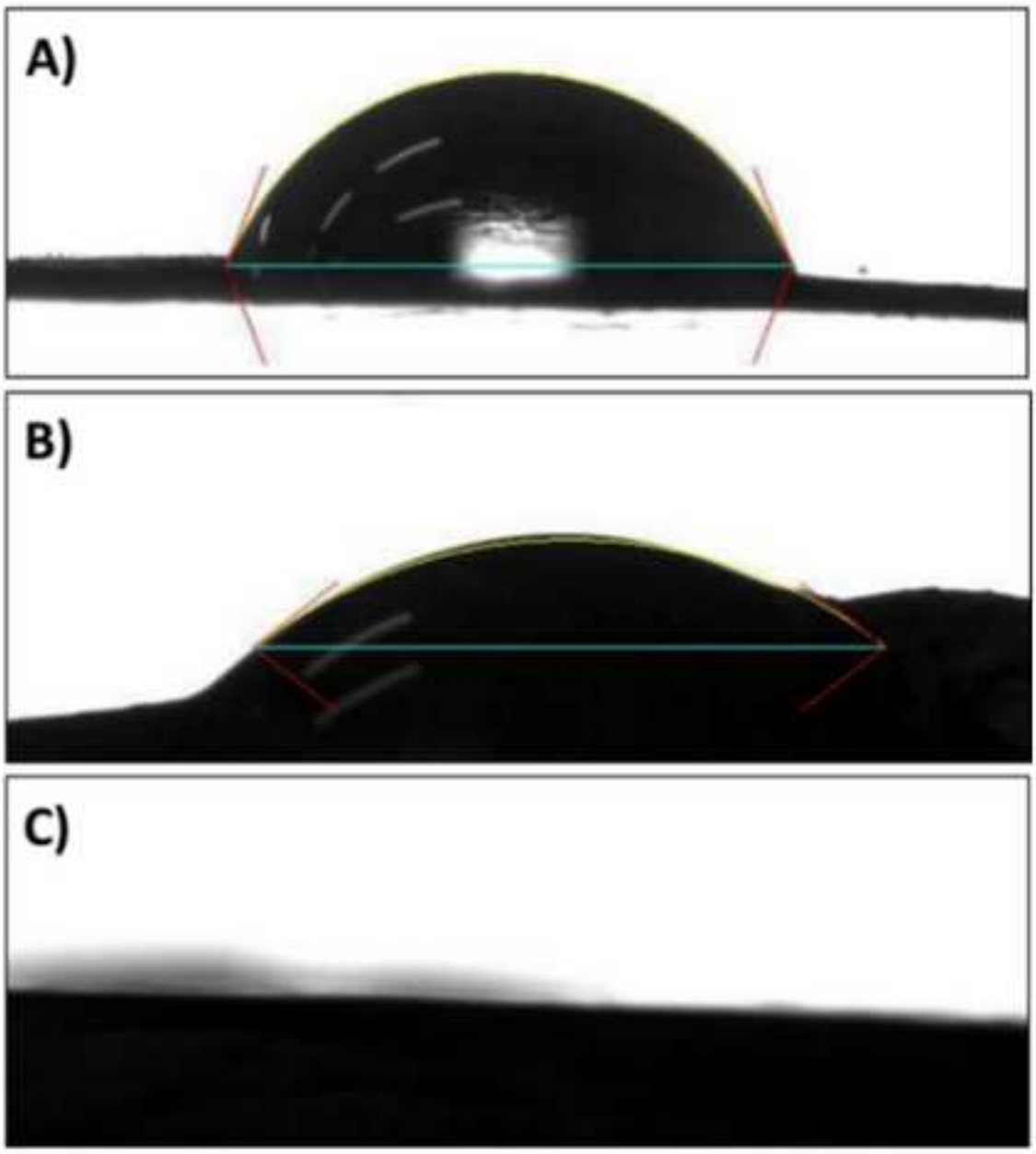

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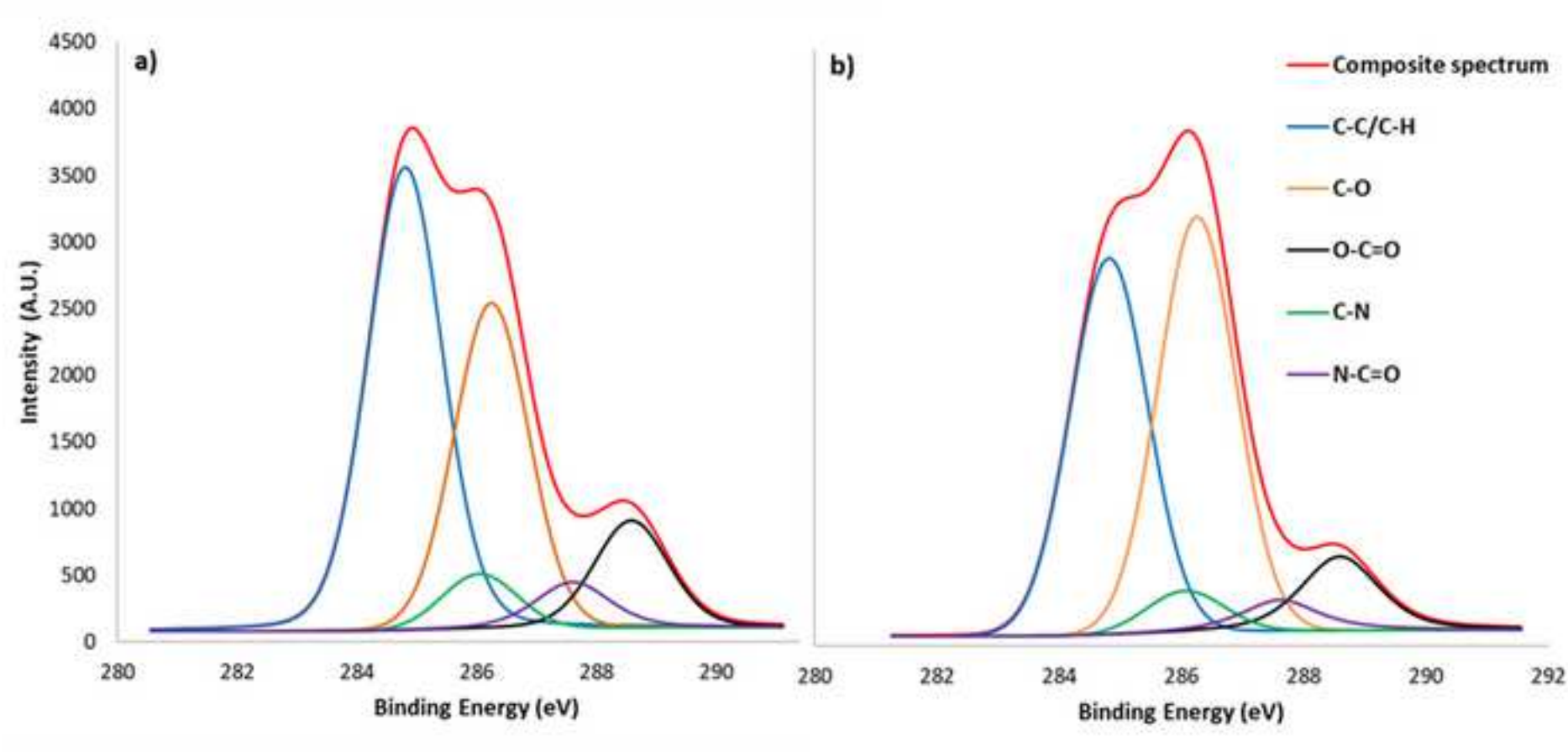

Highlights

- Synthesis of novel pegylated polycaprolactone/polyaspartamide copolymers.

- Processability of obtained materials by several techniques.

- Setting up of experimental conditions to get various types of systems potentially useful in the biomedical field, such as fibers and particles.

- Possibility to modulate shape, dimensions and surface characteristics of obtained systems.

Table(s)

Table 1. Experimental conditions, molar ratios, weight-average molecular weight ($\bar{M}_w$) and polydispersity index ($\bar{M}_w/\bar{M}_n$) values of obtained PHEA-g-SUCC-PCL graft copolymers.

Graft copolymers	Experimental conditions	Molar ratios			Molecular weight		Yield
	t_{tREACT}/T_{REACT} (hrs/°C)	R_3	R_4	R_5	($\bar{M}_w$) (kDa)	$\bar{M}_w/\bar{M}_n$	wt/wt % based on all starting materials
PHEA-g-SUCC-PCL$_{(A)}$	24/40	3	0.3	0.06	114.0	1.6	30.6
PHEA-g-SUCC-PCL$_{(B)}$	44/40	3	0.3	0.06	134.0	1.5	39.2
PHEA-g-SUCC-PCL$_{(C)}$	68/40	3	0.3	0.06	158.5	1.2	41.4
PHEA-g-SUCC-PCL$_{(D)}$	24/40	3	0.3	0.12	108.5	1.4	22.9
PHEA-g-SUCC-PCL$_{(E)}$	44/40	3	0.3	0.12	153.0	1.3	28.0
PHEA-g-SUCC-PCL$_{(F)}$	68/40	3	0.3	0.12	187.0	1.2	37.5

R_3 = CDI / PCL-SUCC molar ratio
R_4 = DEA/ hydroxyl-carrying PHEA repeating unit molar ratio
R_5 = PCL-SUCC/ hydroxyl-carrying PHEA repeating unit molar ratio

Table 2. XPS Surface chemical composition of obtained particles[a].

Sample	C 1s	O 1s	N 1s
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[b]Obtained by nanoprecipitation.
[c]Obtained by microfluidics.

***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Credit author statement

Emanuela Fabiola Craparo: Conceptualization, Methodology, Formal analysis, Investigation, Data curation. Salvatore **Emanuele Drago:** Investigation, Formal analysis. **Gaetano Giammona**: Conceptualization, Supervision. **Gennara Cavallaro**: Conceptualization, Supervision, Data curation.