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Itaconic anhydride based amphiphilic copolymers: Synthesis, characterization and stabilization of carboxyl functionalized, PEGylated nanoparticles

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Abstract

N-Vinyl-2-pyrrolidone (NVP) and itaconic anhydride (IA) copolymers were synthesized via radical polymerization. The synthesized copolymers were grafted with MPEG chains of different average molecular weights (350, 550, 750 Da). The grafted copolymers were used as surfactants in the synthesis of poly(ε-caprolactone) (PCL) nanoparticles in water by solvent evaporation technique. In order to further test the synthesized surfactants, the miniemulsion polymerization of vinyl acetate was performed. Two methods of obtaining miniemulsion were implied: a sonicator and a static mixer. The synthesized surfactants performed well in both type of experiments while in the case of static mixer nanoparticles with a lower polydispersity were obtained. Droplets with a mean diameter of 160 nm were obtained when using the sonicator while in the case of static mixer the mean diameter was 280 nm.

Keywords: MPEG grafted copolymers; PCL nanoparticles; Miniemulsion polymerization; Static mixer

1. Introduction

Pharmaceutical industry has started looking for new ways to attain drug delivery, rather than researching for new bioactive principles. It is the case of cancer therapy, very difficult because of the low bio-availability of the drug at the tumour site and the increased systemic toxicity of anti-cancerous drugs.

Many ways to achieve a better drug delivery have been employed, some of them purely physical – the port-a-cath® system, others based on new vehicles for the active principle.

One of the most promising ways to achieve cancer therapy is the employment of nanoparticles loaded with drugs [1]. The tumours are characterized by morphological and functional modifications of the host tissue, like an increased angiogenesis, leaky blood vessels and a low rate of lymphatic drainage. Because of these characteristics, the

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agglomeration of macromolecules within the tumour is facilitated by the so called enhanced permeation and retention effect (EPR) [2].

In order to achieve the drug targeting, certain techniques were employed. One of the most promising is to entrap the biological active compound into polymeric particles (nanoparticles [3], nanocapsules [4], micelles [5], drug–polymer conjugates [6] or liposomes [7]).

Nanoparticles are widely used as they can provide the transportation of the drug to the target tissue, allowing a longer circulation time than conventional drugs. The nanoparticles are very useful in the case of lipophilic drugs which can not be administered parentheral.

In order to obtain stable nanoparticles, generally a surfactant is employed, although there are authors who reported surfactant free nanoparticles [8]. The majority of nanoparticles for medical applications are stabilized by a polymeric surfactant, usually poly(vinyl alcohol) (PVA) [1,9] or a member of the PEO–PPO–PEO family (Pluronic®). Though Pluronic has been widely used, and some authors have proven that it actually enhance the activity of some anti-cancer drugs [10], some studies reported toxic side effects [11]. Therefore new surfactants are desired in the field of biomedical applications.

Many techniques for the preparation of polymeric nanoparticles were described. One of the first methods to obtain nanoparticles was the emulsion polymerization. Several authors described the mechanism of nanoparticle formation during the emulsion polymerization [12,13]. Another method to obtain nanoparticles is by using preformed polymers. Lee et al. [14] obtained nanoparticles of poly(lactide)–tocopheryl polyethylene glycol succinate (PLA–TPGS) by double emulsion technique in the presence of PVA. Spontaneous emulsification was described by Iyer [15] who obtained nanosized micelles of copoly(styrene-maleic acid)–zinc protoporphyrin (SAM–ZnPP). In this case, the hydrolized SAM acted as a surfactant. Similar results were obtained by Zeisser-Labouèbe et al. [16] using PLA and PLGA nanoparticles, loaded with hypericin. PLGA nanoparticles can also be obtained by dialysis as proved by Jeon et al. [8].

Miniemulsion polymerization is one of the most suitable techniques to obtain polymer nanoparticles from highly hydrophobic monomers. In miniemulsion polymerization, the nanodroplets contain all the components required for the polymerization and act as separate nanoreactors. Polymerization in these nanoparticles takes place in a parallel fashion, in 10^{12}–10^{18} nano-compartments per liter [17].

Miniemulsion generally implies a method that allows the generation of small stable droplets in a continuous phase by applying high shear stress [18]. Under high shear, the broadly distributed macrodroplets are broken into narrowly distributed, small nanodroplets. Usually homogeneous droplets in the size range between 30 and 500 nm with a narrow size distribution can be produced by the miniemulsion process [19].

The aim of the present study was to prepare an itaconic anhydride–*N*-vinyl-2-pyrrolidone copolymer and to determine whether it can be used as a surfactant for stabilization of nanoparticles, after grafting MPEG chains onto it.

2. Materials and methods

N-Vinyl-2-pyrrolidone was purchased from Sigma–Aldrich and was used as received. Itaconic anhydride was purchased from Fluka chemical company and used as received. The amount of itaconic acid, determined by ^{1}H NMR was less than 1%. Azobisisobutyronitrile (AIBN) was purified by recrystallisation twice from methanol. MPEG was purchased from Fluka chemical company and it was freeze-dried. All the solvents were purchased from Sigma–Aldrich and Acros and used as received.

2.1. Synthesis of NVP–IA copolymers

The copolymers of *N*-vinyl-2-pyrrolidone and itaconic anhydride were synthesized by classical radical copolymerization. The polymerization was carried out in ethyl acetate in the presence of AIBN (1% molar) as initiator at 70 °C for 8 h. A mixture of IA and NVP (molar ratio = 1:1) was dissolved in ethyl acetate and poured into a three necks round bottom flask fitted with a reflux condenser. The white precipitate was recovered by pouring the reaction mix in cold dichloromethane. The product was washed several times with chloroform in order to remove all traces of monomers. After the final washing, the product was dried under vacuum for 24 h. The product was characterized by ^{1}H, ^{13}C NMR, IR spectroscopy, size exclusion chromatography (SEC).

2.2. Synthesis of grafted copolymers

Grafted copolymers were synthesized by esterification of NVP–IA copolymers with MPEG. MPEG

of 350; 550; 750 g/mol were used. The reaction was carried out in a two necks round bottom flask, under inert atmosphere (argon). A mixture of 1 g NVP–IA copolymer and 3.3 g MPEG750 was stirred for 2 h at 110 °C then the temperature was increased to 130 °C for another 2 h. The final product was washed with THF several times, in order to remove the non grafted PEG. All of the grafted copolymers were water soluble. The products were characterized by IR spectroscopy and SEC.

2.3. CMC determination

The critical micelle concentration (CMC) was determined by measuring the surface energy by Wilhelmy plate method. A Kruss K 1000 processor tensiometer was employed for the purpose. The system was fitted with two dosing units in order to attain automatic operation. One dosing unit was used to add solvent (water) and the second to substract the amount of liquid previously added by the first one thus lowering the concentration while maintaining a constant volume.

Solutions of up to 10 g/L were obtained in order to measure the CMC.

2.4. Nanoparticles preparation

Polycaprolactone nanoparticles were prepared by solvent evaporation technique. A solution of PCL in AcOEt was prepared (4 g/L). The copolymer (0.4 g) was dissolved in the same volume of water resulting a solution of 0.4 g/L. The two solutions were mixed, under moderate magnetic stirring and then sonicated at 450 W for 2 min using an ultrasound generator (Bioblock Scientific). The resulting emulsion was kept under reduced pressure in order to remove the organic solvent.

2.5. Miniemulsion polymerization

Miniemulsion polymerization of vinyl acetate was performed. In order to verify the stabilizing capacity of synthesized copolymers, two methods of obtaining emulsion were implied. The first method, rather classical, involved an ultrasound generator (Bioblock Scientific France – 750 W) operated at 450 W.

The second device used to generate miniemulsions was a simple static mixer (8 mm diameter, 165 mm length) obtained from Bioblock. The emulsion was circulated through the mixer with a centrifugal pump with a flow rate of up to 957 mL/min (Fig. 1).

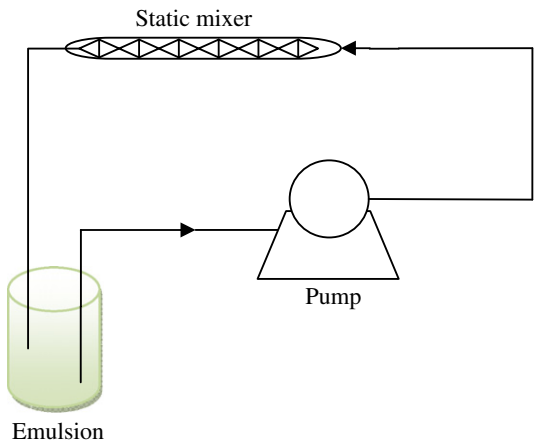


Fig. 1. Schematic representation of the static mixer. According to Ouzineb [20].

Two different formulations were tested for each of the method, which were chosen taking into account the influence of both mixing method and surfactant concentration over the size of nanodroplets. Pretests were conducted using the similar formulations, in the presence of Pluronic F-68 as surfactant in order to verify that the system is stable.

2.6. Particle size determination

Particle size, polydispersity index and zeta potential were determined using a Malvern Nanosizer ZS instrument. Size measurements were performed following a 1/100 (v/v) dilution of the nanoparticles suspension in deionised water.

3. Results and discussion

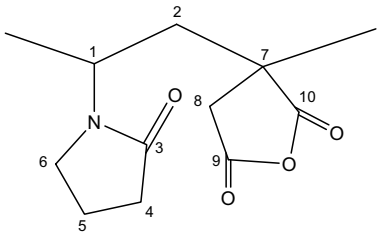
3.1. Copolymer synthesis and characterization

The copolymerization of NVP and IA went by a free radical mechanism. Many authors [21,22] proved that the copolymerization of an electron donor (NVP) with an electron acceptor (IA) monomer goes by formation of a charge transfer complex (CTP).

The ^{13}C NMR data for the copolymer are presented in Table 1.

The IR spectra shows the presence of the two anhydride bands (1863 cm^{-1} and 1778 cm^{-1}) – the C=O stretching vibrations; the amide band (1680 cm^{-1}); CH_2 polymer band (2960 cm^{-1}).

Table 1
The results of ^{13}C NMR analysis



Atom	Chemical shift
1	42.4
2	30.8
3	177.3
4	32
5	16.4
6	45
7	171–175
8	36.5
9,10	47.5

As observed from the RMN analysis, modifying the ratio between the comonomers does not significantly changed the copolymer composition.

While some authors reported copolymers of itaconic anhydride with different comonomers, with anhydride sequences longer than one unit [23], for this particular system the copolymer was alternant. The advantages over the NVP–maleic anhydride copolymer are given by the nature of anhydride. The itaconic acid can be oxidized in the liver to lactate that can afterward enter the tricarboxylic acid cycle [24].

3.2. Grafted copolymers

The IR spectra of the grafted copolymers show the formation of an ester band (1730 cm^{-1}) and the decreasing of the anhydride bands (Fig. 2). The anhydride bands do not disappear completely as that was not the aim of this study. By adding an excess of MPEG it is possible to completely open the anhydride rings, but keeping a small fraction of anhydride rings allows to further modulate the structure and the amphiphilic properties of the final compound. In order to increase the hydrophobic content of the copolymer, 1-dodecanol can be grafted by opening the rest of the anhydride rings.

The grafting reaction can be followed by IR spectroscopy through the integration of characteristic bands. The percentage of remaining anhydride units can be determined by the formula:

$$\% \text{ Anhydride} = 1 - \frac{(A_1 + A_2)t_1}{(A_1 + A_2)t_0}$$

wherein the A_1 and A_2 represent the surface of 1780 cm^{-1} and 1860 cm^{-1} bands, respectively.

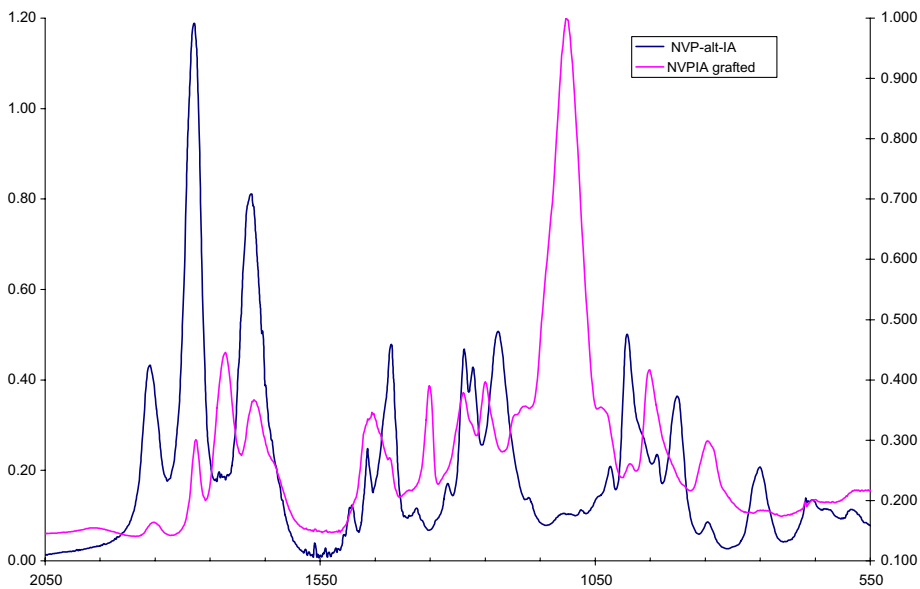


Fig. 2. The IR spectra of NVP-*alt*-IA copolymer and PEG grafted NVP-*alt*-IA copolymer. It is clearly visible that some of the anhydride units remain intact after the grafting reaction.

3.3. CMC determination

The CMC was determined by Wilhelmy plate method, using an automated system. The surface tension is determined with the equation:

$$\sigma = \frac{F}{L\cos\theta}$$

where σ is the surface tension, F – the force acting on the balance, L – wetted length and θ – the contact angle. The Wilhelmy method has some advantages: it does not require that the densities of the liquids are known; it does not require corrections like the ring method and does not alter the equilibrium state, as the plate does not move once the surface has been reached. In conclusion, the Wilhelmy method fits better for measuring the CMC of macromolecules. A possible explanation of the rather high values obtained could be the particular behavior of polymers in water, when equilibrium is reached after a very long time.

The measured values of CMC are presented in Table 2.

The M_n values were measured by SEC. As expected, the average molecular weight of the grafted copolymers increased with the molecular weight of grafted MPEG and with the PEG/IA ratio.

The aspect of a surface tension vs. concentration curve is presented in Fig. 3.

3.4. Nanoparticles preparation

PCL nanoparticles were stabilized with synthesized copolymers as well as with a commercially available surfactant, Pluronic-F68 for comparison. In all the cases a PCL of an average molecular weight of 14,000 was used. The results are presented in Table 3.

The copolymer:Pluronic ratio was 5:1 in all cases where a mixture was used.. The concentration of Pluronic alone was well under the CMC value.

Table 2
Average molecular weights and CMC of synthesized copolymers

Sample	PEG	PEG/IA	M_n (g/mol)	IP	CMC (g/L)
LC14	350	1:1	22,300	3.18	4.08
LC15	350	3:4	22,000	1.65	4.70
LC16	550	1:1	25,630	3.15	4.07
LC17	550	3:4	32,110	1.77	5.28
LC18	750	1:1	22,810	4.42	5.01
LC19	750	3:4	25,600	3.86	4.86
Pluronic		F-68	8000	–	2.66

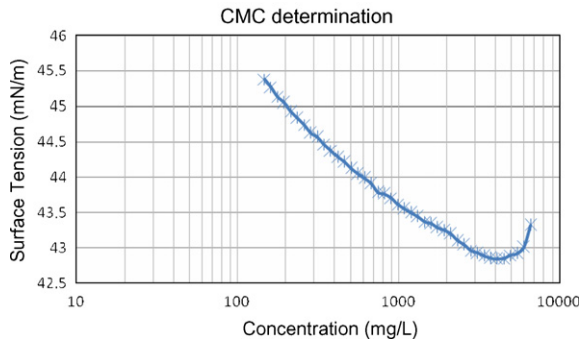


Fig. 3. Surface tension vs. concentration plots for NVPIAMPEG550 copolymer. The CMC is to be found at the intersection of descending segment with the ascending one. All the other CMC curves had the same shape.

Table 3
Average particles diameter, measured by DLS

Surfactant	Average size (nm)
Pluronic F68	153
NVPIAMPEG350/Pluronic F68	160
NVPIAMPEG350/Pluronic F68	160
NVPIAMPEG550/Pluronic F68	159
NVPIAMPEG550/Pluronic F68	158
NVPIAMPEG750	158
NVPIAMPEG750	157

The zeta potential value was -70 ± 10 mV for all the samples.

The average particles diameter was not affected by the type of surfactant used. In all the cases a Pluronic/copolymer mixture was used, as the copolymer alone did not manage to keep the PCL nanoparticles from precipitating. In the experiments where only NVP–IA copolymer, grafted with MPEG350 or MPEG550 was used as a stabilizer, the nanoparticles precipitated after a few days, but the nanoparticles were easily resuspended by a gently shake, so the flocculation did not occurred. Increasing the concentration of NVP–IA–MPEG copolymer led to an increase of the stabilization efficiency, with nanoparticles that were stable for more than 3 months.

The nanoparticles were stable in time, at room temperature for more than 12 weeks. The mean diameter was measured every week, in order to determine the stability of the suspension. The measurements showed that the nanoparticles are stable at room temperature, after several weeks only 5% variation of mean diameter was detected (Fig. 4).

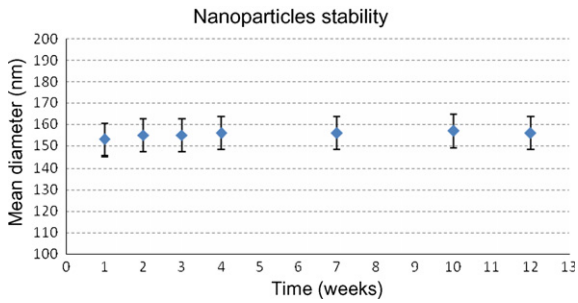


Fig. 4. The mean size of nanoparticles versus time (nanoparticles stabilized with a Pluronic/NVP–IA–PEG copolymer mixture).

From the presented data, one can see that although the size of nanoparticles is not as small as in the case of Pluronic F-68, the synthesized copolymers performed well in term of stabilizing PCL nanoparticles. The interest in using grafted copolymers is given by the fact that the nanoparticles stabilized with grafted copolymers have a lot more PEO units on the surface than Pluronic stabilized nanoparticles. In the case of Pluronic stabilized nanoparticles, the PPO block covers the hydrophobic surface while the PEO chains move freely in the solution, conferring steric stability. In the case of grafted copolymer, the minimum distance between two neighbour PEO chains is 7 Å, less than the length of PPO block of Pluronic F-68. One can see that more PEO chains can be expressed on the surface of NVP–IA–MPEG stabilized nanoparticles.

The presence of PEO chains on the surface of nanoparticles is very important as PEO acts as a very efficient anti-opsonization agent. Apart from the unspecific protection against opsonization of PEO, the synthesized copolymers have a large number of carboxyl groups on the surface, which generates a negative zeta potential, therefore increasing even further the resistance of nanoparticles against opsonisation (Fig. 5).

In terms of biocompatibility the synthesized copolymers are expected to perform much better than the commercial Pluronic F-68, due to increased number of PEO chains and negatively charged surface of nanoparticles.

3.5. Miniemulsion polymerization

The conditions for test formulations are presented in Table 4.

The evolution of mean diameter of nanodroplets during miniemulsion generation was followed by SLS techniques. In the case of static mixer, the minimum diameter was about 280 nm, while sonication led to a mean diameter of 160 nm.

The static mixer was coupled with a centrifugal pump in order to obtain miniemulsions. For both methods the same volume of solution was used, in order to eliminate the differences that may have been induced by mass variation. The volume was about 100 mL, as no bigger volumes can be easily emulsified using a lab scale sonifier.

As can be seen in Figs. 6 and 7, the mean particle diameter decreases with the mixing time for both devices. As noted by other workers [25,26], the final

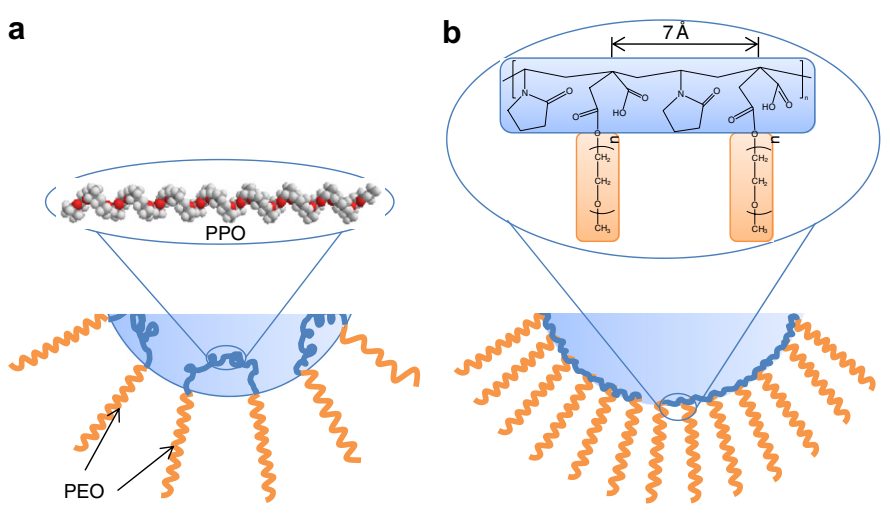


Fig. 5. Comparison between Pluronic F-68 (a) and NVP–IA–MPEG stabilized nanoparticles (b).

Table 4
Conditions for test formulations

Sample	Power output (W)	Flow rate (mL/min)	Time (min)	Vinyl Acetate (% total weight)	Miglyol (% total weight)	Copolymer (% total weight)	Pluronic F-68 (% total weight)
US1	450	–	6	13	11	1.5	1
US2	450	–	6	13	11	3.5	–
SM1	–	957	90	14.4	9.24	2.19	3.81
SM2	–	957	90	21.77	7.52	1.15	1.15

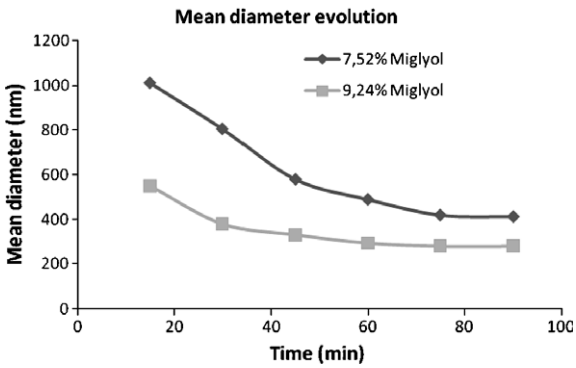


Fig. 6. The evolution of mean diameter vs. mixing time in the case of static mixer.

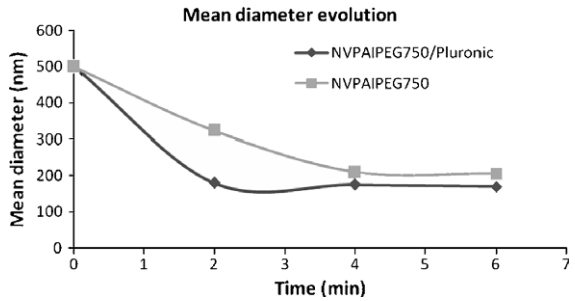


Fig. 7. Evolution of mean particle diameter in case of the sonicator. NVPIAMPEG750 copolymer and a mixture of it with Pluronic were used. Changing the surfactant system led to a change in the kinetic of miniemulsion generation, but the final droplet size was very similar in both cases.

size is strongly influenced by the hydrophobic component concentration (Miglyol). Values of around 9% were found to perform best for the studied systems.

The final size of the nanoparticles is highly influenced by the amount of energy received during the miniemulsion generation phase. One can see that sonication (at 450 W) generates much smaller particles. In terms of energy efficiency, the amount of energy used to obtain smaller particles, found by Ouzineb et al. [20], was about 1–4% of total energy in the case of static mixer, while the rest represents energy lost in coalescence and heating processes. For the sonicator, same authors have calculated an efficiency of 0.002–0.007%.

From the presented data it is clear that although the efficiency of static mixer is not very high, it is still better than other means of generating miniemulsions. One of the greatest advantages of this mixing method is that relatively large volumes of solution can be homogenized with an inexpensive piece of equipment. The increased efficiency is due to the time required for the solution to pass through the mixer, about 0.3 s. Another advantage of the static miser is the low heat generation. Temperature sensitive systems can be emulsified using this technique, which opens a lot of possibilities for the industry.

In terms of stability of both polymerized and not polymerized latexes, the synthesized copolymers performed well, no phase separation being observed after two weeks. The diameters of the polymer particles were in agreement with the size of particles before polymerization. In the case of static mixer generated miniemulsions, the average size of nanoparticles was about 360 nm while for the sonicator was 180 nm.

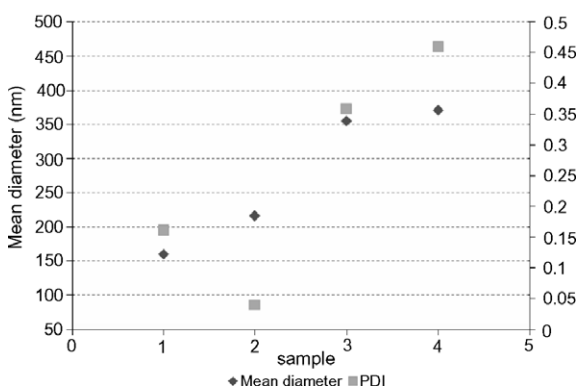


Fig. 8. The mean diameter and PDI of polymerized nanoparticles. Samples are noted as follows: 1 – US1; 2 – US2; 3 – SM1; 4 – SM2.

In the case of US2, where the stabilization was obtained implying only the grafted NVP–IA–MPEG copolymer, the mean diameter was 35% bigger than for US1 (mixed tensioactive system: Pluronic F-68 and NVP–IA–MPEG grafted copolymer) while PDI was the lowest of all experiments (0.03) (Fig. 8). The emulsions were stable for more than 6 weeks.

4. Conclusions

NVP–IA copolymers were prepared by classic radical copolymerization. Since the polymerization takes place by a charge transfer complex, the copolymer obtained is an alternant one, as shown also by NMR studies.

NVP–IA copolymers were grafted with PEG in order to obtain water soluble amphiphilic copolymers. The grafted copolymers were characterized by IR and the CMC was determined by Wilhelmy plate method.

Nanoparticles of PCL were stabilized by using the synthesized surfactant. In order to decrease the amount of grafted copolymer used for the stabilization studies, a small amount of Pluronic was added to the emulsion. However, the amount of Pluronic was much lower than its CMC, so that the main stabilizing effect was induced by the synthesized copolymer.

Miniemulsion polymerization of vinyl acetate was performed in the presence of a mixture grafted copolymer/Pluronic as well as in the presence of the synthesized copolymer alone. When using the copolymer alone, the lowest PDI was obtained but with a slightly increased mean diameter. The results were quite promising, for both sonication and static mixer systems.

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