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Poly (N-isopropylacrylamide) based hydrogels as novel precipitation and stabilization mediums for solid lipid nanoparticles (SLNs)

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Abstract

In this work, poly(*N*-isopropylacrylamide) (PNIPAM) based chemically cross-linked hydrogels are used as novel precipitation and stabilization mediums for solid lipid nanoparticles (SLNs) for the first time. The hydrogels and the hybrid thermoresponsive composite hydrogels with SLNs were characterized by SEM, DSC, DLS and rheometric analysis. The results showed that the SLNs obtained directly in the gel matrix by the newly devised method were well-dispersed and remained stable for one month. A remarkable advantage of this approach is that it yields the thermoresponsive nanocomposite hydrogels in a single step. This approach is a significant advancement in the preparation of hybrid thermoresponsive nanocomposite systems based on smart gels and SLNs for their use in biomedical applications.

Key words: Smart polymers, Thermoresponsive gels, Solid lipid nanoparticles (SLNs), Stability of SLNs, Thermoresponsive nanocomposites.

1. Introduction

The chemically cross-linked hydrogels based on water soluble thermoresponsive polymers such as poly (*N*-isopropylacrylamide) (PNIPAM) and its various copolymers have shown promising applications in biomedical field. PNIPAM based gels are biocompatable, non toxic and they have very high water retention capacity or swelling index [1-4]. Additionally, the properties of the gels can be tuned by changing the cross-linkers and by adjusting the cross-linking density. These gels have shown potential applications in drug delivery, biosensing and in environmental applications [3-6]. Due to the versatility of the PNIPAM based hydrogels they continue to capture additional fields of technological applications. Instead, solid lipid nanoparticles (SLNs) are colloidal carriers of the new generation of submicron-sized lipid emulsions where the liquid lipid (oil) has been substituted by a solid lipid [7-11]. SLNs offer unique properties such as small size, large surface area, high drug loading and the interaction of phases at the interfaces, and have shown the potential to improve performance of pharmaceuticals, neutraceuticals and other materials. There are various methods of the preparation of SLNs [12], among which, that developed by Gasco et al. [13] was the first one based on the use of microemulsions. Microemulsions were obtained by mixing low melting fatty acid, an emulsifier, co-emulsifiers and water with slight quantities of organic solvents and stirring at 65-70 °C until optical trasparency was obtained. The microemulsion was then dispersed in cold water (2-3 °C) under stirring to obtain the precipitation of SLNs [14]. At present, the development of different SLNs preparation methods based on cold microemulsion dilution, avoiding the use of highly toxic organic solvents is been given increased attention. In this work, a new method of preparation of SLNs called “cold dilution of microemulsion” is used [15]. It is as an evolution of the "solvent diffusion" method described in the literature [16]. This method involves the preparation of an O/W μE at room temperature whose hydrophilic and lipophilic phases are mutually saturated, using a solid lipid dissolved in a partially water soluble organic solvent as oil phase. SLN precipitate as lipid solid matrix following solvent removing upon water dilution of the μE. Such method combines the advantages of the emulsion solvent diffusion technique with the high stability and the super solvent properties of microemulsion systems. This technique does not require low or high temperatures, other than the method patented by Gasco et al. [13], which exploited a molten lipid substance added of a hot aqueous surfactant-cosurfactant dispersion.

Moreover, in this method there is no need of pH modification, ultrasonication, homogenization, or pressure variations. Also toxic organic solvents are not required, which is beneficial in the view of a future industrial production and therapeutic application.

The combination of thermoresponsive gels and SLNs leads to the formation of smart nanocomposite hydrogels. These smart nanocomposites have shown lot of potential applications in biomedical field [17,18]. However, the post-synthesis transfer and dispersion of SLNs in smart gel matrix to prepare the composites is difficult and it may lead to the aggregation of SLNs or even breaking of the SLNs structure. Hence, there is a need to invent and optimize new preparation methods for the SLNs, in which direct formation of SLNs in smart or responsive mediums is possible. In order to prepare SLNs based thermoresponsive hybrid composite drug delivery systems, in the present work we have demostrated that the PNIPAM based synthetic hydrogels or their suspensions can be used as efficient precipitation and stabilization mediums for SLNs. As pointed out earlier, PNIPAM based cross-linked hydrogels have been tested in various biomedical applications. Due to their varsatality and wide range of applications we chose this class of hydrogels as precipitation mediums for SLNs by the new method. This advancement provides a one-step preparation procedure of thermoresponsive hydrogel nanocomposites. The formation of the SLNs and their distribution in the gel matrix were studied. The SLNs prepared by the cold microemulsion dilution directly in gels have shown excellent dispersion of the SLNs and stability in the gel. This approach of the preparation of SLNs directly in smart gels or in their suspensions is a significant advancement in the field of preparation of smart composites for biomedical applications.

2. Experimental

2.1 Materials

Gels

N-isopropylacrylamide (NIPAM 99.8%), acrylamide (AM 99.8%), polyehtylene glycol diacrylate (PEGDA 250, average Mn 250, 100 ppm MEHQ as inhibitor), potassium persulphate (KPS, 99%), *N,N*-methylenebisacrylamide (MBA, 99%), *N,N,N',N'*-tetramethylethylenediamine (TMEDA, 99.5%, purified by redistillation) were purchased from Sigma-Aldrich, Italy.

SLNs

Ethylacetate (EA 99.8%), trilaurin (99%)and taurocholic acid sodium salt (>97%) were purchased from Sigma Aldrich (Milan, Italy), Epikuron®200 (phosphadityl coline 92%) from Cargill (Minneapolis, US), Cremophor®RH 60 (PEG-60 Hydrogenated Castor Oil 100%) from BASF (Ludwigshafen, Germany). All the solvents used were of high purity and used as received.

2.2 Instruments and methods

Fourier-transform infrared (FTIR) spectra were recorded on a Perkin Elmer Spectrum 100 in the attenuated total reflectance (ATR) mode with a diamond crystal, using 32 scans per spectrum and a resolution of 4 cm^{-1} in the spectral range of 4000-650 cm^{-1}. DTGS was used as detector.

A differential scanning calorimeter (DSC Q200, TA Inc.) was used to collect DSC thermograms of the samples and to determine the transition temperature of gels. 10 mg of water saturated gel sample was put in an aluminium pan and was subjected to heating and cooling cycles under nitrogen atmosphere at a specified rate.

Oscillatory rheological measurements were carried out on a TA Instruments Discovery HR 1 equipped with 20 mm plate geometry and Peltier plate temperature control. Measurements were performed at an heating rate of 0,1 °C/min in the temperature range 20 – 40 °C. Samples were equilibrated for 30 min prior the experiments to allow relaxation of material and temperature equilibration (20°C). Three measurements were performed for each sample and the mean values are reported together with their standard deviations. The gelling temperature was determined graphycally as the inflection point on the curve of the viscosity as a function of temperature.

Scanning electron microscopy (SEM) analyses were carried out by using a ZEISS EVO50 XVP microscope with LaB6 source, equipped with detectors for secondary electrons collection. SEM micrographs were obtained after sputtering samples with ca. 15 nm of a gold layer to avoid any charging effect (Bal-tec SCD050 sputter coater).

SLN particle sizes, polydispersity index (PI) and Zeta potential were determined by using dynamic light scattering (90 Plus Particle Size Analyzer, Brookhaven, New York, USA). Size measurements were obtained at a 90° angle at 25 °C. SLNs suspensions were 1:20 diluted before analysis with MilliQ water for size determination or with 0.01 M KCl for Zeta potential determination, in order to achieve the suitable conductivity. Zeta potential was

calculated according to Smoluchowski equation: as SLNs reasonably have a charge layer much smaller than their particle size, it was assumed that the Smoluchowski equation is valid. All samples were prepared in triplicate and results are expressed as the mean ± SD of three runs on each.

2.3 Synthesis of hydrogels

The PNIPAM based cross-linked hydrogels were prepared by free radical polymerization method. The representative procedure is as below.

Weighed quantities of NIPAM and AM monomers in presence of PEGDA or MBA as cross-linkers were first dissolved in 16 ml of deionized (D.I.) water to form reaction solution. After slow stirring for few minutes, 480 µl of polymerization accelerator TMEDA was added into the reaction solution. Finally, the initiator KPS was added to the reaction mixture to initiate the polymerization. The polymerization reaction was continued at 70 ^{o}C for 4 h. At the end of the reaction, whitish swollen cross-linked mass of the hydrogel was obtained. The water left from reaction mixture with the gel mass was drained out completely by squeezing the gel as it may contain unreacted reagents. The gel mass was cut into small pieces and was transferred into a beaker with ample amount of distilled water and kept on stirring overnight at room temperature. The washed swollen gel mass was recovered by centrifugation and again kept on stirring in fresh water for at least 6 hours. These two prolonged water washing were done to purify the gels. The final pure swollen gel mass was recovered by centrifugation and transferred into a single neck round bottom flask. The gel mass was freeze-dried for 48 hours. After drying, a dry bright white gel was obtained as final product. Four gel samples were prepared by varing the monomer and initiator ratios and by chaning the cross-linkers. List of samples prepared is shown in the table below.

2.4 Preparation of the microemulsion (µE)

EA and water were mutually pre-saturated before use. The O/W µE was obtained by mixing appropriate amounts of water-saturated ethylacetate (s-EA), trilaurin, Epikuron®200, Cremophor®RH 60, taurocholic acid sodium salt in a glass tube and adding EA-saturated water (s-water) drop by drop to the lipid phase by vortexing at room temperature up to transparency.

2.5 Precipitation of SLNs in gels

SLNs were prepared using the innovative technique called "cold dilution of microemulsion". This technique involves the preparation of an O/W µE using a partially water-soluble organic solvent as disperse oil phase. Following the dilution of µE with water, the dissolution of the organic solvent in water occurs, with the consequent SLN precipitation. The use of a stabilizer, typically from 0.2 to 2% w/w, is also necessary to reduce the lipid/water interfacial tension and thus to prevent aggregation phenomena. Polymeric stabilizers (poloxamers, polyvinyl alcohol or polyvinylpyrrolidone) are generally employed for this purpose. In the present work, all above described gels (PP 20:1, PP 10:1, NAM, NAP) were used separately to support SLNs precipitation. More precisely, 1% w/w of each gel was dispersed in water according the procedure reported above to obtain four aqueous gels. Then 5 mls of each gel were added dropwise by vortexing to 1 ml µE to dilute it and to dissolve the organic solvent in water with the consequent precipitation of SLNs.

3. Results and discussion

Synthesis of hydrogels

The hydrogels in this study were prepared by free radical polymerization where TMEDA was used as the polymerization accelerator [19,20]. As listed in table 1, four samples prepared with varying mole ratios of the monomers PNIPAM and AM or by changing the cross-linkers MBA and PEGDA were obtained. The ATR-FTIR spectra of the gels (figure 1) confirm the formation of the cross-linked polymer and presence of the cross-linkers in the gel structure. All the spectra of gels (A to D) show typical absorption peaks for PNIPAM at 1387, 1479 and at 1565, 1667 cm^{-1} which are attributed to C–H bending typical of the isopropyl group,-CH_3 bending, C-N (amide) stretching and C=O (amide) stretching, respectively [21-25]. The spectra A, B and D clearly show the peak at 1723 cm^{-1} due to the carbonyl group of the PEGDA cross-linker, while the peaks due to the MBA cross-linker were overlapped by the broad PNIPAM peaks.

Preparation of SLNs

Following a preliminary formulation study to screen several excipients and organic solvents suitable to form a µE, lecithin and trilaurin were chosen as biocompatible lipid matrices and s-EA as organic internal phase. The resulting µE composition is reported in Table 2. In "cold dilution of microemulsion" method for the preparation of SLNs, cold water is used as a non solvent to precipitate SLNs. Hence the formulation of microemulsion contains surfactants to stabilize the SLNs formed immediately upon precipitation of the lipid in water. Recently, the use of dilute solutions (1-3%) of polymeric stabilizers, such as

Pluronics® or polyvinyl alcohol, in the dispersive medium of SLNs was tested [26,27]. But as the concentration of Pluronics® was low, their major role was to act as surfactants/stabilizers (due to the amphiphilic nature of the polymer). In such low concentrations, Pluronics® do not show the typical gelation properties normally shown by a 20% w/v solution of various Pluronics® in water [28]. Instead, in the present approach we used pre-formed synthetic cross-linked gels saturated (swollen) with water as precipitation and stabilization mediums for the SLNs. The SLNs were directly prepared in the gels and the precipitation was checked in triplicate to test the reproducibility of the method. The remarkable advantage here is that the lipid nanocarriers (SLNs) are directly formed by precipitation in the gel matrix. As the gels are thermoresponsive, the process directly yields a SLNs based thermoresponsive composite. The thermosensitive composite system provides two advantages: first the poorly water soluble hydrophobic drugs can be encorporated inside SLNs [29] and second, a thermoresponsive release of those drugs from the composite drug delivery system can be obtained [30].

Rheology

An oscillatory shear rheometer was used to check the viscosities at different temperatures of the prepared gels. The viscosities for all gel samples were measured with a heating rate of 1 °C/min in the 20−40 °C temperature range. Figure 2 shows the thermosensitive trend behavior of all four gels suspended in D. I. water at 1% (w/v). The plots of viscosity shows a slight lowering of viscosity values as a function of the temperature, followed by an inflection point from which the viscosity begins to increase. This inflection point may be considered as the transition temperature [31]. This increase of viscosity was from 1.9 to 4.8 mPa.s for PP20:1, 0.82 to 0.88 mPa.s for PP10:1, 0.1 to 0.34 mPa.s for NAM and 0.086 to 0.270 mPa.s for NAP gel samples respectively. The viscosity measurements were also carried out on gel samples with SLNs suspended in them. An inflection point in the plot of viscosity versus temperatures was also observed in the presence of SLNs (figure 3). This confirms that even in the presence of SLNs the gels show a thermoresponsive behavior. A slight lowering of the transition temperature values in the presence of SLNs was observed for all gel samples. The increase in viscosity values for gels in the presence of SLNs were from 2.5 to 3.7 mPa.s for PP20:1, 3.3 to 3.6 mPa.s for PP10:1, 2.9 to 5.0 mPa.s for NAM and 3.1 to 6.0 mPa.s for NAP samples respectively. It is important to mention that, as expected, the viscosity values of the gels were higher in the presence of SLNs. The transition temperature values were also determined by the DSC analysis and they are

reported in table 3 together with the values obtained from viscosity transitions. It was observed that the presence of SLNs in the gels does not affect the thermoresponsive transition properties of the gels, hence it makes them good stabilization mediums for SLNs. The efficiency of the gels to stabilize SLNs was further tested by SEM and DLS analyses.

SEM

The SEM images of SLNs precipitated directly in the gels are shown in figures 4A to 4C. The formation of spherical or quasispherical and well dispersed SLNs with narrow particle size distribution in the range of 350 to 400 nm was observed. The size of SLNs in each gel was further confirmed by DLS measurements. As mentioned before, the gel structure was found to prevent the aggregation of the particles. This was observed for all four gel samples used in the study. This confirms that the gels act as pecipitation medium and also play an important role to stabilize SLNs for a prolonged time.

Stability of SLNs in gels

After the formation of SLNs directly in the gel matrix, their stability in the gels for a prolonged period up to app. one month was checked. This was done by monitoring the important parameters such as size (hydrodynamic diameter, HD), Zeta potential (Zp) and pH of the precipitated SLNs. Mean HD (± standard error, S.E.), polydispersity index (PDI) and Zp of the four different SLNs suspensions were determined by DLS technique. The precipitated SLNs inside the gels showed remarkable stability and no any major variations of all the parameters monitored were observed (figure 5). The SLNs showed negative values of Zp [32,33]. Although very slight variations in the values of Zp with time were noted that may be due to the changes in the surface interactions of SLNs with gels. This may be due to the increased interactions of the lipid particle surface with the gel matrix and presence of water (which may vary with time) at the interface between them. The stability of the SLNs was high due to the fact that the swollen cross-linked gel matrix not ony provides the precipitation medium (water as non solvent for lipid) for the SLNs but it also helps to prevent the SLNs from aggregation by virtue of the cross-linked polymer (gel) structure. Once the SLNs are formed, they remain sort of incorporated and well dispersed in the gel. The hydrophobic lipid surface remain in contact with the highly hydrophobic gel making it a stable combination and preventing any sort of hydophobic-hydrophobic interactions which may lead to the aggregation of the particles. So we make a speculation based on the results obtained in this work that potentially hydrogels prepared from various sources can be used for the preparation of SLNs.

4. Conclusions

In this work, we have demonstrated that the synthetic and chemically cross-linked gels based on thermoresponsive water soluble polymer PNIPAM can be used as new precipitation and stabilization mediums for SLNs. The results showed the formation of stable and well dispersed SLNs inside the thermoresponsive hydrogels. Additionally, the stability study of the SLNs performed by monitoring the key parameters showed that the SLNs were stable for one month that is for considerable long period. This strategy can be easily reproduced to prepare SLNs in various stimuli-responsive gel systems to prepare directly stimuli-responsive nanocomposite hydrogels in a single step. This approach can be further extended to prepare thermoresponsive nanocomposites containing drug loaded SLNs in a single step.

Conflicts of interest

The authors confirm that there are no conflicts to declare.

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Figure and table captions

Fig. 1. ATR-IR spectra of A) PP20:1 B) PP10:1 C) NAM and D) NAP gels.

Fig. 2. Change in viscosity of gels with temperature.

Fig. 3. Change in viscosity of gels plus SLNs with temperature.

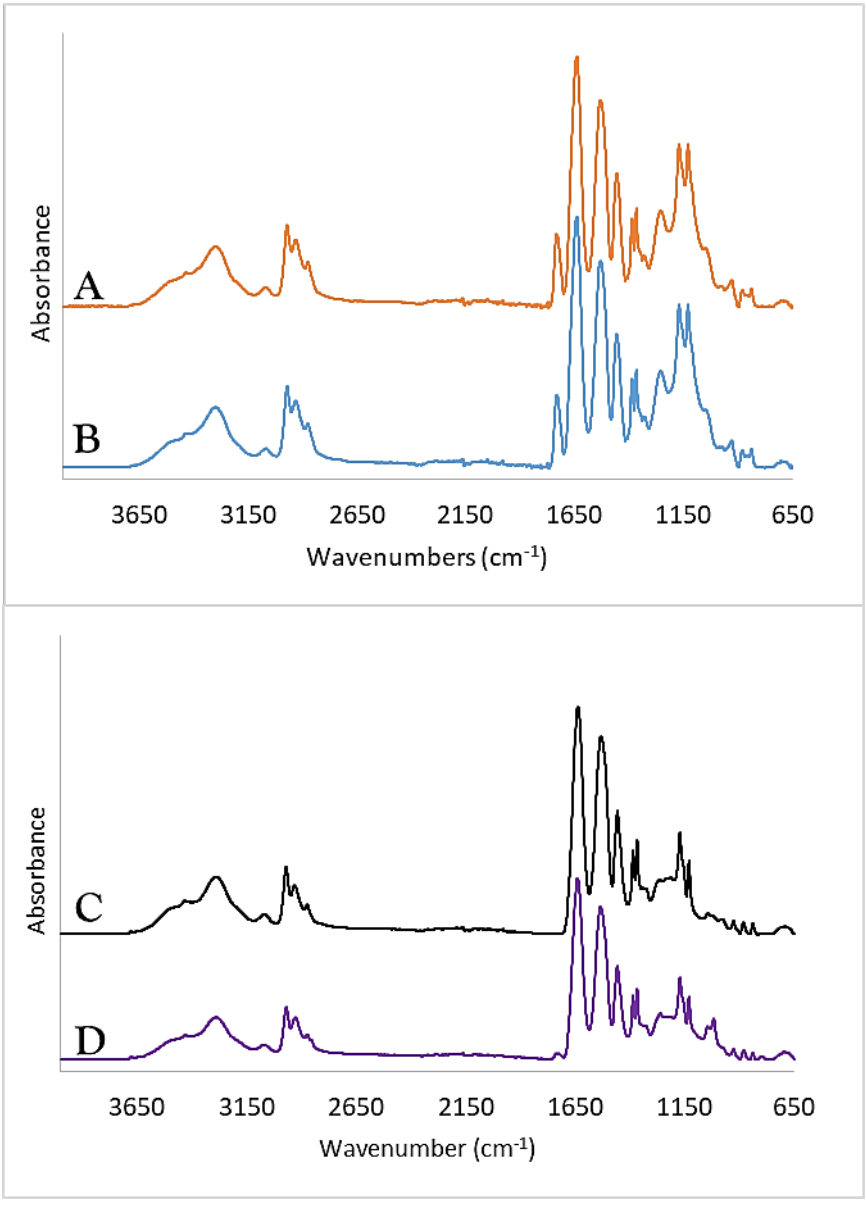
Fig. 4. SEM images of SLNs precipitated direclty in gel suspensions A) PP20:1 SLNs B) PP10:1 SLNs C) NAM SLNsand D) NAP SLNs. Inset red circles show the SLNs.

Fig. 5. Stability study of SLNs by monitoring size, Zeta potential and pH of SLNs.

Table 1. List of gels prepared.

Table 2. microemulsion composition

Table 3. Transition temperatures of gels



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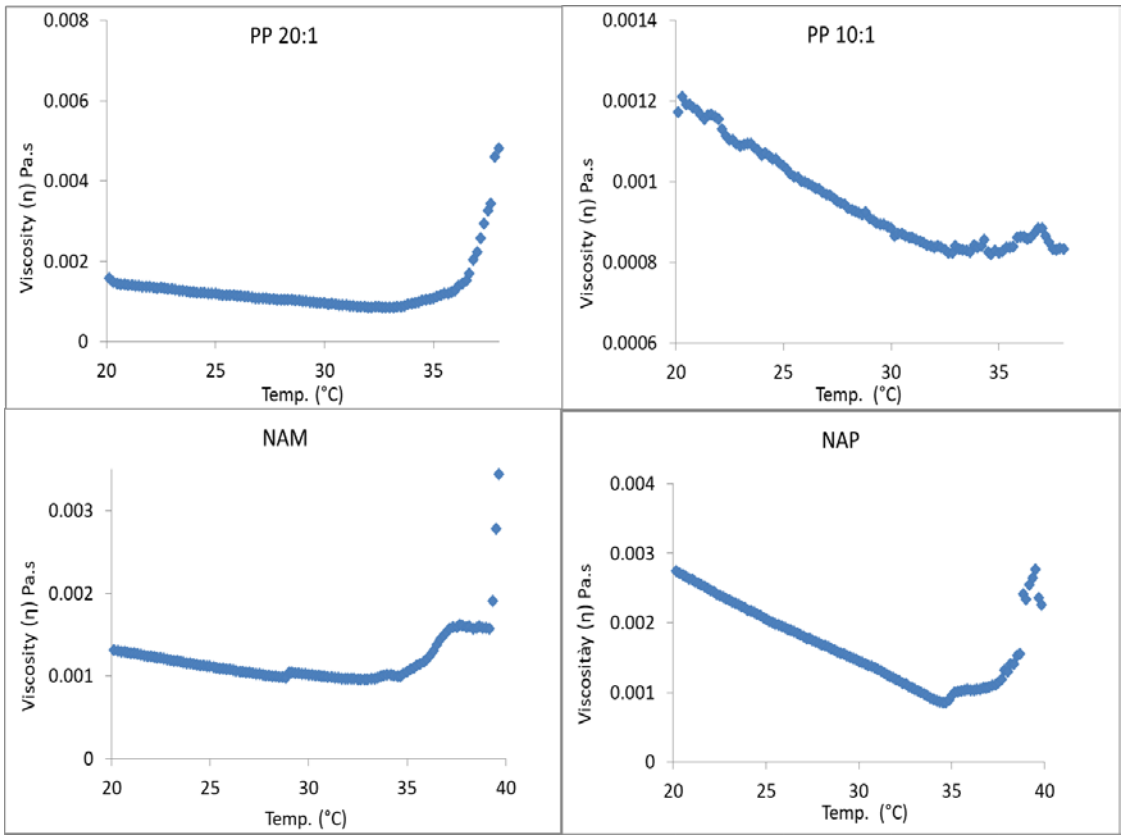

Fig. 2. Change in viscosity of gels with temperature.

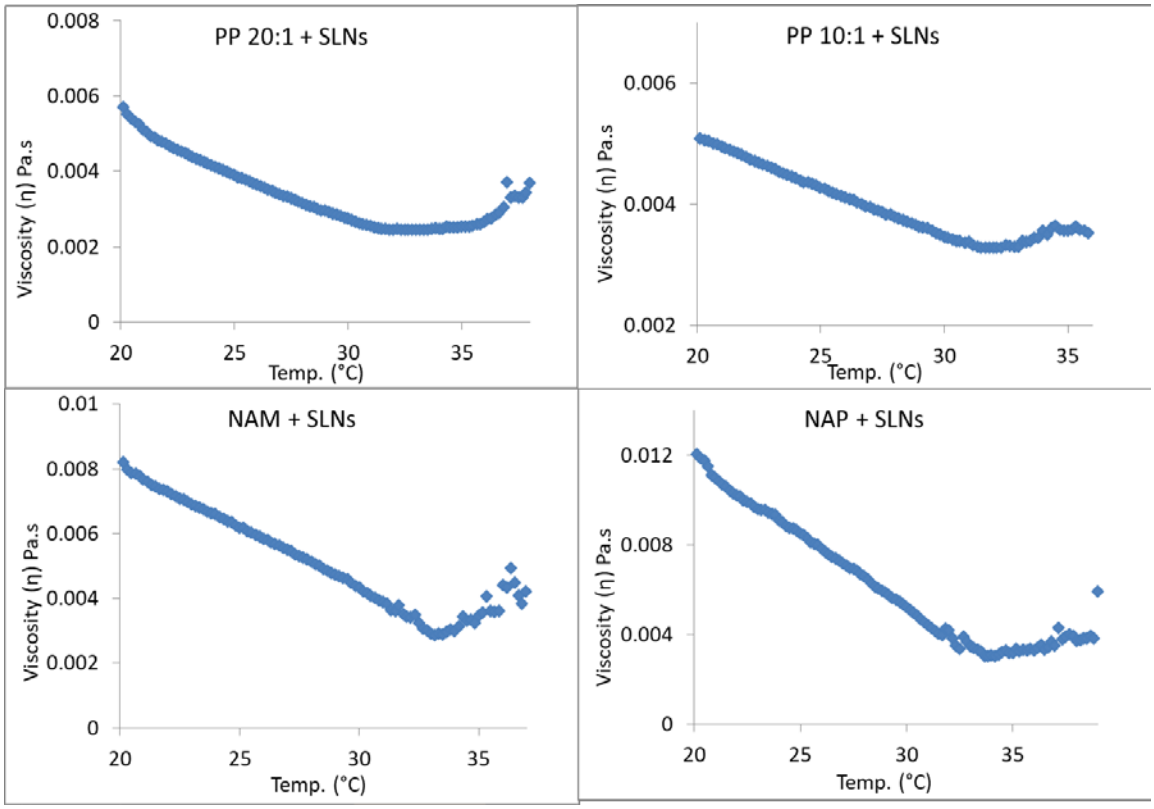

Fig. 3. Change in viscosity of gels plus SLNs with temperature.

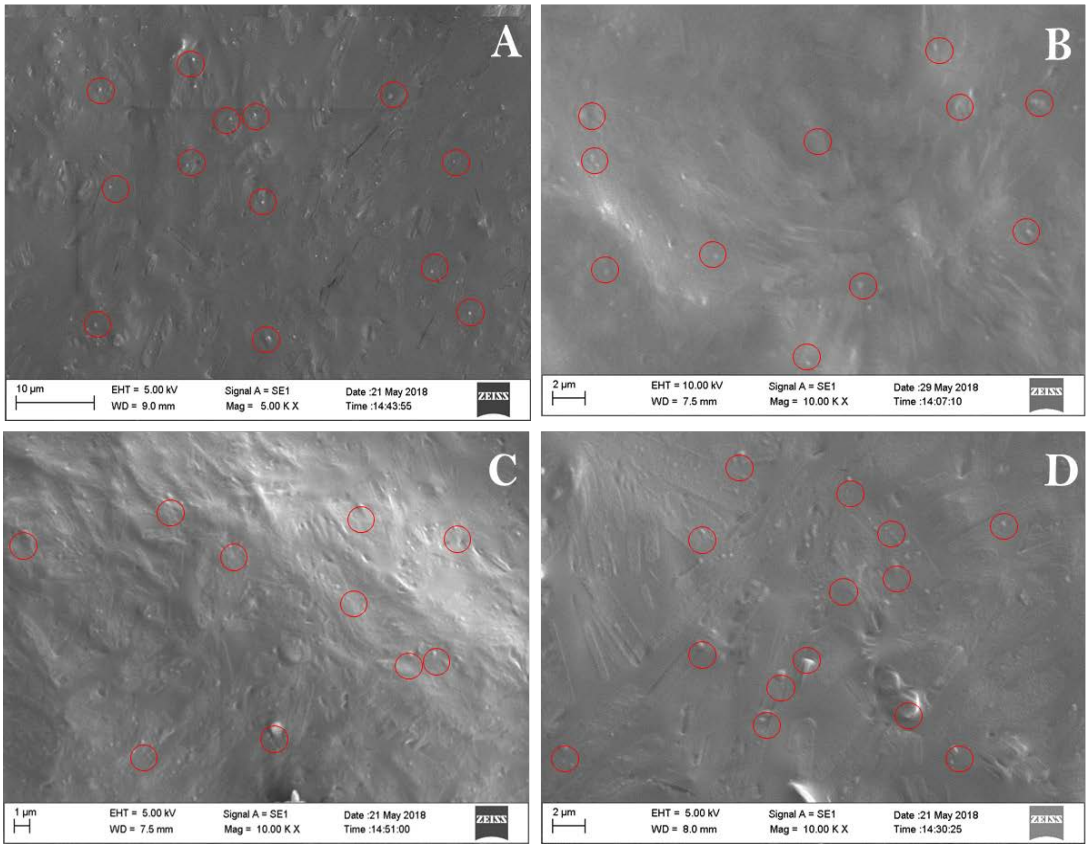

Fig. 4. SEM images of SLNs precipitated direclty in gel suspensions **A)** PP20:1 SLNs (scale bar shown 10 µm) **B)** PP10:1 SLNs (scale bar shown 2 µm) **C)** NAM SLNs (scale bar shown 1 µm) and **D)** NAP SLNs (scale bar shown 2 µm). Inset red circles show the SLNs.

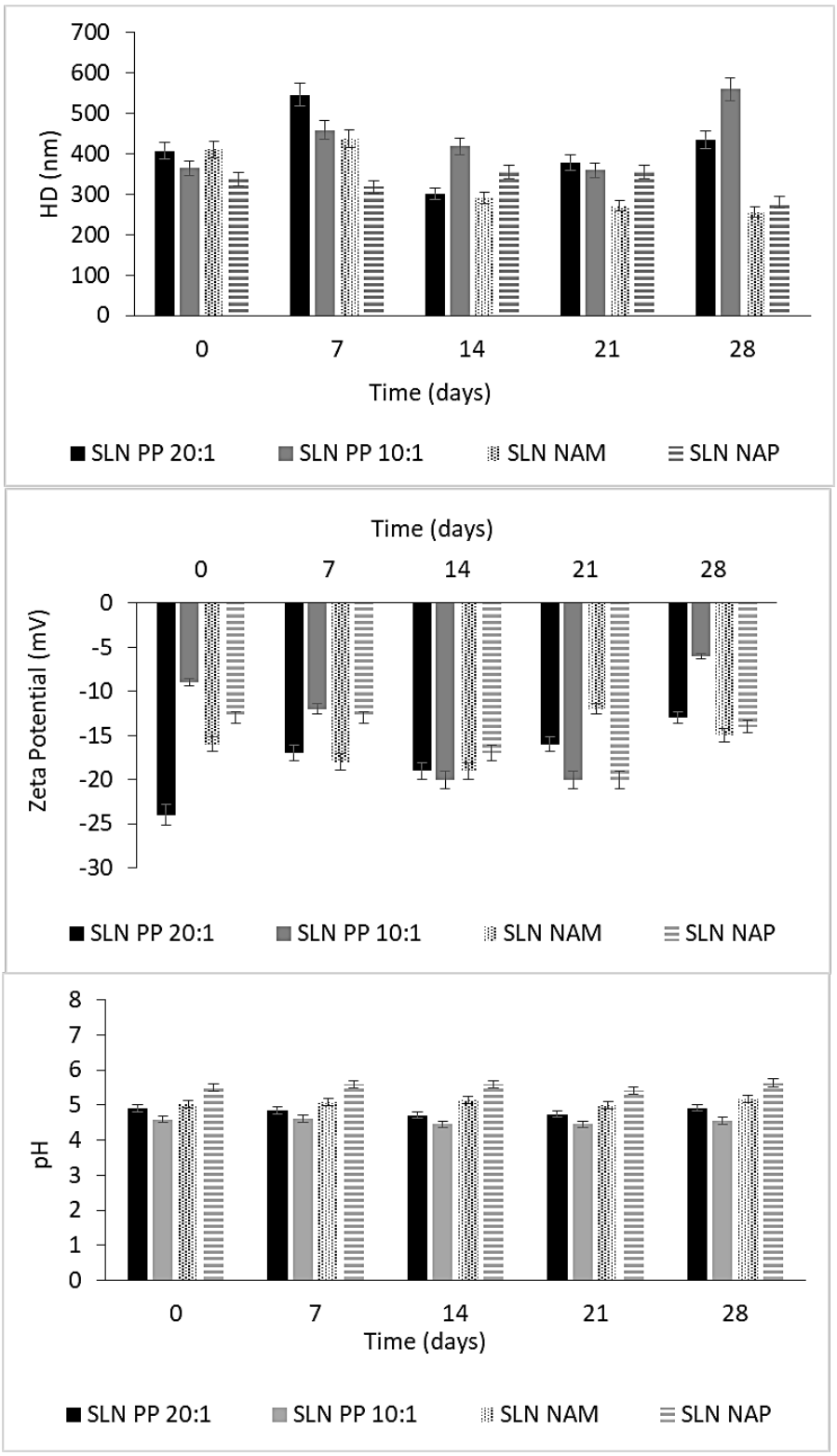

Fig. 5. Stability study of SLNs by monitoring size, Zeta potential and pH of SLNs. Each bar represents the mean ± SD obtained by three runs on three different samples.

Table 1. List of gels prepared

Gel	NIPAM g	PEGDA g	AM g	MBA g	TEMED µl	KPS g	Water ml
PP20:1	1.6	0.180	-	-	480	0.640	16
PP10:1	1.6	0.400	-	-	480	0.640	16
NAM	1	-	0.0125	0.0135	300	0.200	10
NAP	1	0.0221	0.0125	-	300	0.200	10

Table 2. Microemulsion composition

Ingredients	**mg**
s-EA	200
Trilaurin	60
Epikuron®200	170
Cremophor®RH60	50
Taurocholic acid sodium salt	10
s-Water	700

Table 3. Transition temperatures of gels

Gel	**Transition temp. of gels (°C) by DSC (±0.10°C)**	**Transition temp. of gels (°C) by Rheometer (±0.14°C)**	**Transition temp. of gels + SLNs (°C) by Rheometer (±0.14°C)**
PP20:1	36.3	35.5	34.8
PP10:1	36.5	34.4	33.9
NAM	36.1	34.8	33.7
NAP	34.5	35.4	35.1