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Formulation and characterization of novel nanostructured lipid carriers made from beeswax, propolis wax and pomegranate seed oil

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ABSTRACT

The objective of this study was to develop functional nanostructured lipid carriers (NLCs) using beeswax (BW), propolis wax (PW) and pomegranate seed oil (PSO). NLCs were prepared by a melt emulsification-ultra sonication technique. The influences of solid lipid composition, surfactant blend concentration (2, 4, and 6% of formulation) and PSO content (10, 30 and 50% of total lipid phase) were investigated. Statistical evaluations revealed that the formulation variables had significant effects on physical properties of NLC. The developed nanocarriers presented particle sizes ranging from 71 to 366 nm, leading to excellent physical stability. The optimum formulations with minimum particle size and high zeta potential value were PW and BW + glycerol behenate samples, containing 10% oil and 6% surfactant. DSC and XRD studies indicated that the addition of oil to the lipid phase could disturb the crystalline order and form lattice defects. TEM observations exhibited spherical morphology of the NLCs.

1. Introduction

Despite having noteworthy health benefits, lipophilic bioactive compounds pose a real challenge to food and pharmaceutical industries due to their poor water solubility and insufficient bio-accessibility which limits the development of formulations deliverable by oral and parenteral routes. Among different strategies to overcome the solubility issue and other problems, such as high melting point, instability, undesired organoleptic properties and interactions with food molecules associated with these components, lipid-based colloidal dispersions have attracted increasing attention (Tamjidi, Shahedi, Varshosaz, & Nasirpour, 2013).

Although several lipid colloidal carriers, including emulsions, liposomes and solid lipid nanoparticles (SLNs), have been studied extensively for the past few decades (Muller, Mäder, & Gohla, 2000), a great interest has focussed on nanostructured lipid carriers (NLCs). NLC, consisting of a binary mixture of a solid lipid and a distinct liquid lipid, is known as a delivery system with less-ordered crystalline structure, developed for overcoming the limitations of SLNs. The incorporation of oil into the core of a solid lipid offers several advantages, including higher efficiency and loading capacity, better physical and chemical stability, as well as controlled release after encapsulation of active components (Tamjidi et al., 2013). Yang, Corona, Schubert, Reeder, and Henson (2014) reported that considerably less surfactant was required to produce stable NLC compared to SLN which was attributable to both reduced particle shape change and increased mobility of surfactant molecules.

Lipids are the main ingredients of lipid nanoparticles that influence stability, drug loading capacity and sustained release behaviour of the formulations. Although lipid nanoparticle dispersions, based on a variety of lipid materials, including fatty acids, triglycerides or partial glycerides, have been broadly investigated, quite less attention has been paid to wax-based NLCs. Waxes are defined as simple esters of fatty acids with long chain alcohols (Jenning & Gohla, 2000). Due to differences in chemical composition, glycerides and wax display striking dissimilarities in physical properties and crystal order. Glycerides crystallize in three main subcell packings: hexagonal (α crystal), orthorhombic (β′ crystal) and triclinic (β crystal) and exhibit marked polymorphic transitions. β prime is the dominant polymorph in waxes and the polymorphic transition rate is very low due to the longer chains of fatty acids (Jenning & Gohla, 2000; Wong, Li, Bendayan, Rauth, & Wu, 2007). It has been reported that wax-based lipid nanoparticles could be physically more stable and show excellent particle size distribution (Jenning & Gohla, 2000). Beeswax is commercially the most important natural wax and contains a mixture of several compounds, mainly palmitate, palmitoleate, hydroxypalmitate and oleate

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esters of long chain (C30–C32) aliphatic alcohols. However, the composition of beeswax is very variable and depends on the genetic characteristics of the bees (Negri, Marcucci, Salatino, & Salatino, 2000a).

Propolis (bee glue) is a natural resinous substance produced by bees from plant-derived products (resins and gums). Propolis is well known for its antibacterial and antifungal properties. Bees use propolis mainly to repair the cracks of their hives and also as an antibiotic against hive colonization with diseases. In general, propolis is composed of 50% resin, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% other different substances, including organic debris (Tosi, Re, Ortega, & Cazzoli, 2007).

In order to provide more therapeutic benefits and improve delivery properties, different healthful oils such as corn oil (Liu & Wu, 2010), olive oil (Lacatusu et al., 2012), fish oil (Lacatusu et al., 2013) and grape seed oil (Lacatusu, Badea, Ovidiu, Bojin, & Meghea, 2012) have been successfully utilized as liquid lipid for NLC production.

Pomegranate seed oil (PSO) is an attractive nutraceutical ingredient possessing an enriched phytochemical composition with high punicic acid and antioxidant activity due to the presence of phenolic compounds, especially ortho-diphenols. Punicic acid has many outstanding functions related to human health, such as anti-carcinogenic, anti-diabetes, anti-hyperlipidemia, anti-obesity and anti-atherosclerotic properties (Cao, Wang, He, Zhang, & Wang, 2014).

Thus, we thought it was worthy of interest to formulate a novel nanostructured lipid carrier based on natural waxes (propolis wax and beeswax) as solid lipid and PSO as liquid oils for oral delivery of food bioactive components. The effects of PSO ratio in the lipid mixture, surfactant concentration, and solid lipid composition were investigated on the particle size and size distribution, surface charge and encapsulation efficiency of the resulting delivery systems. Eventually, thermal, morphological and antioxidant properties, as well as crystalline structure of the optimized NLC formulation, were evaluated.

2. Materials and methods

2.1. Materials

Propolis sample and beeswax (BW, melting point of 65–67 °C) were obtained from Espadana Mokamel Co. (Isfahan, Iran). Propolis wax (PW, melting point of 62–64 °C) was extracted using a Soxhlet apparatus and petroleum ether as solvent. PSO (with fatty acid composition of 78.1 ± 0.16% punicic acid, 7.6 ± 0.04% linoleic acid, 7.12 ± 0.04% oleic acid, 3 ± 0.04% palmitic acid and 2.52 ± 0.02% stearic acid) was provided by a local supplier. Compritol® 888 ATO US/NF (COMP, glyceryl behenate (GB), a mixture of ~15% monoglycerides, 50% diglycerides and 35% triglycerides of behenic acid, melting point of 71–74 °C), was kindly donated by Gattefosse (Saint-Priest, France). Lecithin (L-α-phosphatidylcholine) was purchased from Daejung Co. (Korea). All other chemicals were of analytical grade and supplied by the Merck Co. (Darmstadt, Germany).

2.2. NLC preparation

Nanostructured lipid carriers were formulated and prepared by melt-emulsification, coupled with a ultrasonication technique (Zhu, Zhuang, Luan, Sun, & Cao, 2015). Total lipid phase concentration, consisting of solid fat and liquid oil, was kept constant at 10%. The effects of solid lipid composition [BW, PW and their binary mixture (1:1) with GB], PSO content (10, 30 or 50% of total lipid phase) and concentration of surfactant mixture (2, 4 and 6%) composed of Tween 80 and lecithin (1:0.25 w/w) on NLCs formation and their properties, were investigated. For NLC production, a certain amount of solid lipid phase, PSO and lecithin was melted at 80–85 °C, to form a homogeneous and clear oil phase. The aqueous phase [a solution of Tween 80 in phosphate buffer solution (SPB, 10 mM; pH = 7) containing 0.02% sodium azide], at the same temperature was then added to the molten lipid phase with gentle stirring with a magnetic stirrer (IKA RH Basic 2, Germany) at 300 rpm. The mixture was further dispersed with a high-speed mixer (Ultra-Turrax T25 basic, IKAStaufen, Germany) at 14,000 rpm and 85 °C for 10 min to produce the hot primary emulsion. It was then immediately treated by a probe-type sonicator (Adeeco, Iran, power: 250 W), for 8 min (on for 2 s at intervals of 2 s, 250 W) while maintaining the temperature around the melting point of the lipids. The attained emulsion was cooled in an ice bath for 30 min to recrystallize lipid and form NLC. Analysis of particle size (PS), polydispersity index (PDI), zeta potential and encapsulation efficiency was performed for all samples. Formulations showing the best properties were selected for stability study and further investigations. The freshly prepared NLC formulations were frozen at −80 °C for 24 h, and then lyophilized (ALPHA 2–4, Martin Christ Inc., Osterode, Germany) at −70 °C and 0.001 bar for 48 h for evaluation of thermal, crystalline structure and antioxidant properties.

The formulations of SLNs used in the differential scanning calorimetry (DSC) and X-ray diffraction (XRD) experiments were similar to the optimal composition of NLC except that the liquid oil (PSO) was replaced by respective solid lipids. SLNs were prepared by the same procedure as described previously.

2.3. Determination of PS, PDI and zeta potential

The analysis of PS (as Z-average) and PDI of NLC formulations was performed by photon correlation spectroscopy (PCS), using a Zetasizer (NanoSizer 3000, Malvern Instruments, Malvern, UK) at an angle of 90° in 0.01 m width cells at 25.0 ± 0.1 °C. The electrical charge (zeta potential) of lipid nanoparticles was determined in a capillary cell, using the same instrument which utilized the Helmholtz–Smoluchowski equation to convert the measured particle electrophoretic mobility into zeta potential. Prior to analysis, NLCs were diluted 1:100 with the same buffer solution to avoid multiple scattering effects.

2.4. Determination of entrapment efficiency for PSO

Entrapment efficiency (EE) was determined to assess the extent of PSO incorporation in the nanoparticles, using a centrifugation method. Briefly, a 0.5 ml of NLC dispersion was placed in a centrifugal filter tube with a cut-off of 10 kDa (Millipore, Bedford, MA, USA) and ultracentrifuged at 10,000 rpm for 10 min. The NLCs along with the encapsulated PSO remained in the outer chamber whereas the aqueous dispersion medium, containing the free unloaded oil, moved to the sample recovery chamber through the filter membrane. After separation, the amount of free oil was estimated by measuring the concentration of punicic acid (the most important bioactive compound in pomegranate seed oil) in the dispersion medium by gas chromatography. An agilent model 6890N gas chromatograph, equipped with flam ionization detector (FID) and HP-88 capillary column (100 m × 250 μm), was used. Fatty acid methyl esters were prepared by methylation with sodium methoxide (0.5 N). Injection (2 μl) was performed in the splitless mode. Nitrogen was the carrier gas with a flow rate of 1.1 ml/min. The column temperature was programmed to be increased from 150 °C (held for 1 min) to 190 °C at 5 °C min^{-1} held for 2 min, up to 240 °C at 5 °C min^{-1}, and held for 8 min. Injector and detector temperature were 150 °C and 250 °C, respectively.

The PSO entrapment efficiency was subsequently calculated from the following equations:

$$\text{Entrapment efficiency}(\%) = \frac{\text{Amount of PSO entrapped in NLCs}}{\text{Theoretical total amount of PSO added to NLCs}} \times 100$$

The amount of PSO entrapped was calculated by subtracting the amount of free PSO from the theoretical amount of PSO added to NLCs (Ali, El-Sayed, Sylvester, & Nazzal, 2010).

2.5. Long-term storage stability

2.5.1. General

To investigate the physical and chemical stability, the lipid dispersions were divided into aliquots of equal volume and stored in capped polyethylene microtubes at room (25 ± 1 °C) and refrigerator (4 ± 2 °C) temperatures for a period of 40 days. The analysis of PS, PDI, zeta potential, peroxide value and antioxidant activity by 2,2-diphenylpicrylhydrazyl ($DPPH^{\cdot}$) scavenging assay was performed at appropriate time intervals (0, 10, 20, 30, and 40 days) and the stability to creaming and phase separation checked every day.

2.5.2. $DPPH^{\cdot}$-scavenging assay

The free-radical-scavenging capacity of NLCs was evaluated, using the DPPH stable radical and following the methodology described by Kumar, Bhandari, Singh, and Bari (2009). Briefly, a 0.1 mM solution of $DPPH^{\cdot}$ in ethanol was prepared and 2 ml of this solution was added to 0.3 ml of NLC ethanolic solution (500 ppm) and allowed to react at room temperature. After 30 min, the absorbance values were measured at 517 nm against the blank (0.1 mM ethanolic DPPH solution). The radical-scavenging activity (inhibition percentage) was expressed as percentage of DPPH radical elimination calculated according to the following equation:

$$\text{inhibition percentage}\% = (A_{control} - A_{sample}/A_{control}) \times 100;$$

where $A_{control}$ is the absorbance value of blank and A_{sample} is the absorbance value of the sample at 517 nm.

2.5.3. Lipid oxidation assay

The lipid hydroperoxide concentration was measured to evaluate the oxidative stability of the NLC samples during storage. NLC dispersions (0.6 ml) were vigorously vortexed three times with 3 ml of isooctane:2-propanol (3:2 v/v), followed by centrifugation for 5 min at 1500 rpm. The upper layer was carefully removed and the solvent was evaporated under nitrogen gas. Then, the oil samples were weighed. Peroxide value (PV) was determined, using the International Dairy Foundation (IDF) standard method (74A:1991). Briefly, 3 ml of chloroform/methanol (7:3, v/v) were added to oil-containing test tubes and vortexed to dissolve. This mixture was then reacted with 15 µl of ammonium thiocyanate solution (30%, w/v) and 15 µl of ferrous iron solution and vortexed (Shantha & Decker, 1994).

2.6. XRD analysis

The XRD patterns of selected nanocarrier formulations were obtained by collecting intensity data measured by a Philips MPD-X'PERT diffractometer (Amelo, The Netherlands), using Cu Kα radiation (λ = 1.540598 Å; voltage 40 kV; current 40 mA) with a step width of 0.05°/s over the range of 5–40°2θ.

2.7. DSC analysis

The thermal behaviour of nanocarriers was studied by differential scanning calorimetry (DSC 200 F3, Netzsch, Germany). The samples (≈7 mg) were weighed into standard aluminium pans and heated from 20 °C to 100 °C with a scan rate of 5 °C/min. An empty aluminium pan was used as reference.

2.8. TEM analysis

The morphological characteristics of selected NLC formulations were studied by TEM. NLC dispersions were diluted with deionized water at a ratio of 1:10 (v/v) and a drop of the sample was placed on a carbon-coated copper grid and dried at room temperature. The sample was then observed by a Zeiss EM 900 (Carl Zeiss Microscopy, Oberkochen, Germany) for transition electron microscopy.

2.9. Statistical analysis

All measurements were carried out in triplicate. Data collected in this study were analyzed using a SAS statistical software package (version 9.4) by one-way analysis of variance (ANOVA), followed by comparisons with a least significant difference (LSD) procedure and the results of the statistical analysis were considered significant if their corresponding p-values were less than .05. Principal component analysis (PCA) was employed to determine the relationship between the response functions and the formulation variables.

3. Results and discussions

3.1. Physical characteristics of wax-based NLCs

3.1.1. General

It is well documented that the nature of the lipid matrix and the surfactant blend are significant parameters which determine the size and aqueous stability of NLC suspensions. In this study, experiments have been conducted to examine the effect of formulation variables on the formation and characteristics of wax-based NLC.

3.1.2. Particle size

The results of particle size analysis carried out on the NLC suspensions are presented in Fig. 1(a–c). All freshly produced wax-NLC formulations yielded particles with desirable size which ranged from 71.4 (for BW + GB, 30% oil and 6% emulsifier) to 366.3 nm (for BW, 10% oil and 2% emulsifier). Although, all samples were stable after preparation and maintained their stability for at least 2 months, only NLCs produced with 6 % emulsifier exhibited a size less than 100 nm and remained unchanged for up to 4 months. It could be clearly observed that the average size of all NLC samples decreased significantly ($P < .05$) with increase of surfactant amount from 2 to 6% (Fig. 1a). Other researchers have also reported reduction of particle size at higher surfactant concentrations in NLC production (Pezeshki, Ghanbarzadeh, Mohammadi, Fathollahi, & Hamishehkar, 2014; Tamjidi, Shahedi, Varshosaz, & Nasirpour, 2014). From the results of size measurement, it can be concluded that a 6% surfactant blend is an appropriate concentration for production of smaller particle sizes with high stability.

The surfactant nature (and concentration) plays a prominent role in particle size and emulsion stability. By adsorbing onto the surface of a system or interface, surfactants reduce the surface free energy and consequently decrease interfacial tension between lipid matrix and the aqueous phase (Corrigan & Healy, 2006) and cause easier disruption of particles during homogenization and formation of smaller ones. Generally, low surfactant concentrations cause inadequate repulsion between particles and consequently aggregation and instability during storage. On the other hand, the increased surfactant concentration results in size growth, probably due to the depletion flocculation of micelles which have been formed by excessive surfactant molecules in continuous phase (Pezeshki et al., 2014). Therefore, a particular surfactant concentration is necessary to yield a stable system.

PS was also affected by the amount of PSO in the formulations. The interaction effect of solid lipid type and oil concentration is presented in Fig. 1b. The PS of BW-NLCs decreased significantly with an increase in oil concentration, which is in agreement with several previous reports (Ali et al., 2010; Hejri, Khosravi, Gharanjig, & Hejazi, 2013; Zhu et al., 2015). This behaviour could be associated with a progressive reduction of core viscosity by increase of the oil content which promotes fluidity and formation of smaller particles. However, further increase of the oil content may disrupt the wall of lipid carriers and cause aggregation and size growth (Hu et al., 2005). By contrast, PW-NLCs showed the opposite trend and PS increased with higher concentration of PSO. Increase in size of nanoparticles with increasing liquid oil has also been reported by other research groups. Enhanced interfacial tension (Tamjidi et al., 2014) or swelling of the core of the nanoparticles (Dai

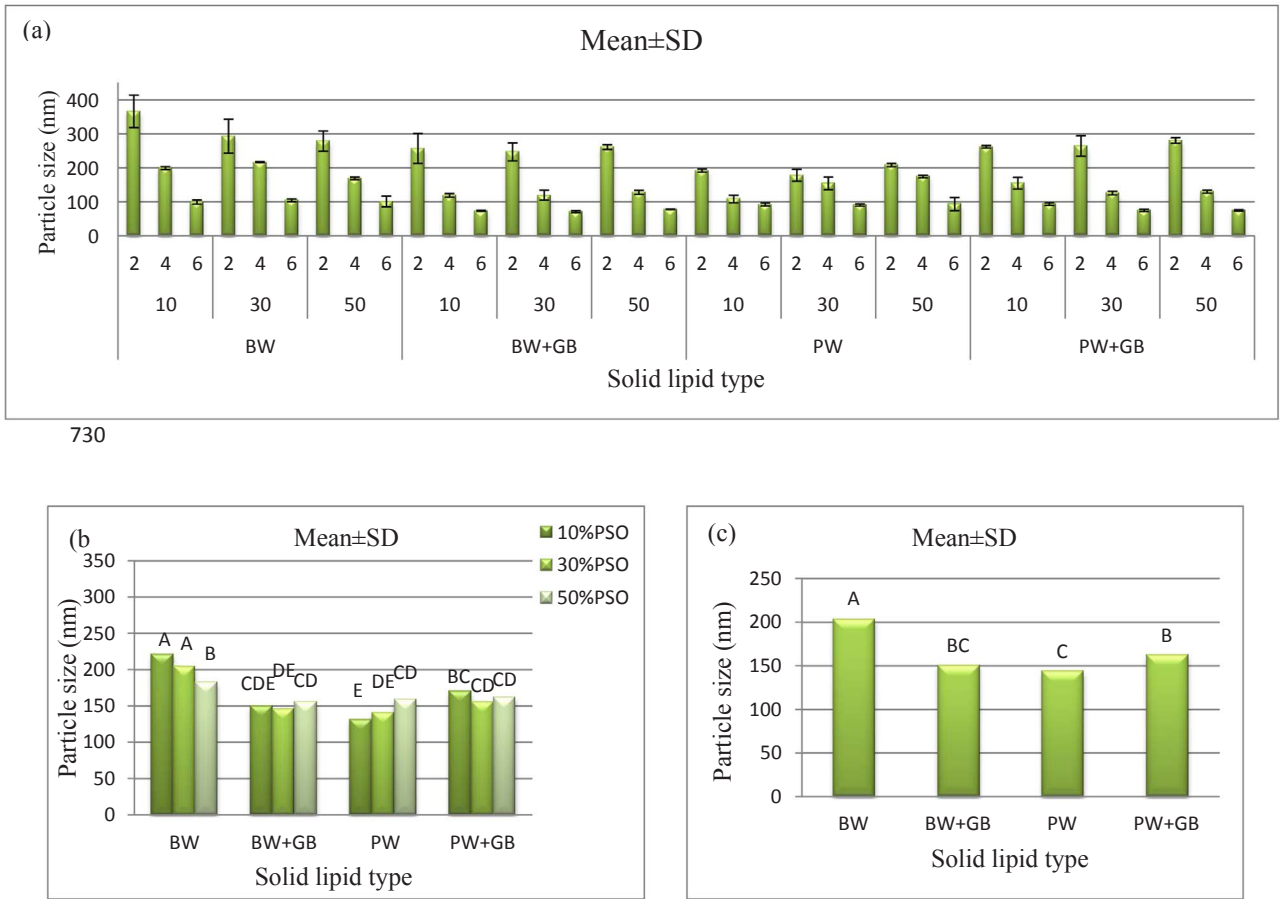

Fig.1. Mean particle diameter of NLC dispersions. (a) NLCs made from different solid lipid types at different surfactant (2, 4, and 6%) and oil concentrations (10, 30, and 50%), (b) the effect of solid lipid type, (c) the interaction effect of solid lipid type and oil content. Different letters in the same row indicate a statistically significant difference ($p < .05$).

et al., 2010), as a result of greater oil content, is a possible explanation. The particle size of mixed solid lipid NLCs did not change significantly ($P > .05$) with varying liquid oil content (Fig. 1b) which reflects a good compatibility among the solid lipids and oil.

In regard to the overall impact of solid lipid type (Fig. 1c), the samples comprised of BW showed the biggest NLC particles (203 nm), followed by PW + GB and BW + GB NLCs, although they were not significantly different ($p > .05$). On the other hand, PW made the smallest NLC particles (143 nm). Bees wax and propolis wax are complex mixtures of organic compounds belonging to several chemical classes. The composition of these waxes is very variable and depends on the genetic characteristics of the bees, as well as the site of collection. Generally, monoesters of carboxylic acids and long chain alcohols are the predominant class of constituents of both waxes, followed by hydrocarbons. However, a wide variation in fatty alcohol, hydrocarbon and carboxylic acid contents, chain lengths and patterns of different samples has been identified (Negri et al., 2000a). The analysis of the PW sample presented exclusively palmitic acid and lignoceric acid, hydrocarbons with 27 and 28 carbon atoms and alcohols in the range with 15–24. In bees wax, however, a longer series of carboxylic acid homologues, ranging from 16 to 28, alcohols and hydrocarbons in the range of 24–34 and 23–35, respectively has been identified (Negri et al., 2000a).

Therefore, the observed differences in PS of BW and PW could be attributed to their different chemical compositions and corresponding melting points, as small differences in the lipid composition might have considerable impact on size and quality of nano lipid particle dispersions (Mehnert & Mäder, 2012). The influence of lipid composition on particle size was also confirmed by Ahlin, Kristl, Smid-Kobar, and Kobar (1998) who produced SLN via high-shear homogenization and Zheng, Falkeborg, Zheng, Yang, and Xu (2013) who reported that a high proportion of long chain triacylglycerols contributed to larger NLC particles. Our results were also in consonance with the findings of Siekmann and Weatesen (1996) that lipids with higher melting point increased the mean particle size of nano lipid dispersions produced via a hot homogenization method, due to the higher viscosity of the dispersed phase. The smaller particle size observed with GB + BW NLCs, compared to BW alone, could be attributed to the presence of partial glycerides in GB (15% monoglycerides and 50% diglycerides) that promote emulsification and facilitate droplet disruption, as opposed to the waxy nature of BW. These partial glycerides create lower interfacial tension with a consequent decrease in particle size (Tamjidi et al., 2014). Ali et al. (2010) also observed that the presence of partial glycerides in the solid lipids promotes size reduction. Surprisingly, the addition of GB caused little growth in the size of PW-containing samples. The larger particle size of PW + GB NLC could be attributed to the higher melting point of GB (74 °C) in comparison with PW (64 °C) and formation of a more viscous dispersed phase. This could also be attributed to the chemically different structure of the ternary mixture of lipid matrix and thus poor compatibility.

3.1.3. PDI and zeta potential

PDI, as a measure of PS distribution, ranges from 0 to 1. While low PDI values (0.1–0.25) show a fairly narrow size distribution and favour long-term stability of nanodispersions, values greater than 0.5 indicate a very broad distribution (Tamjidi et al., 2014). In the present study, the PDI value of formulations ranged from 0.14 to 0.36. BW + GB formulation with 10% oil and 6% surfactant showed the lowest value of PDI and particle size while BW + GB, containing 10% oil and 2% surfactant, had the greatest one (Fig. 2a). The preparation of NLC was

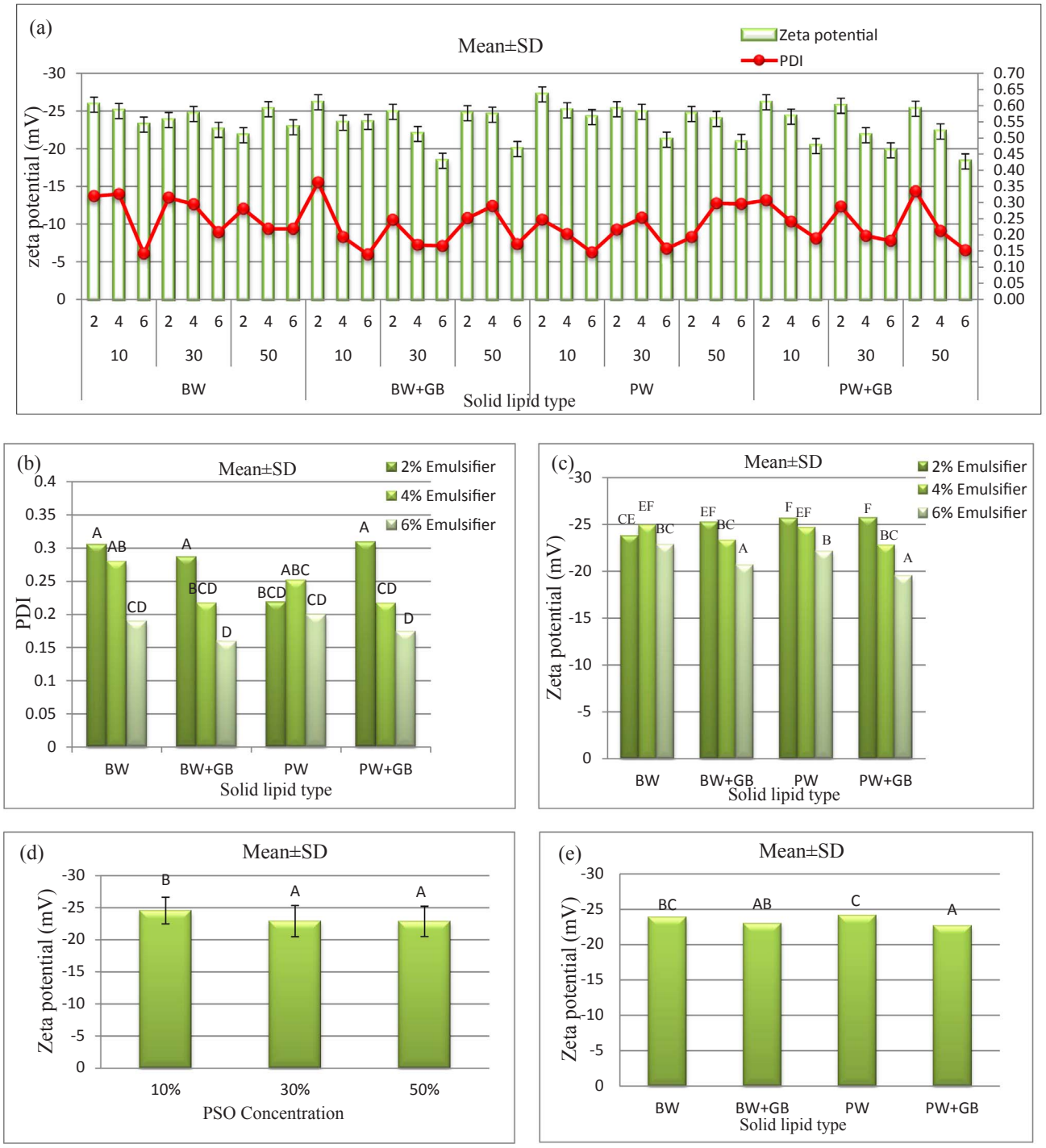

Fig.2. Zeta potential and polydispersity index of NLC dispersions (a) NLCs made from different solid lipid types at different surfactant (2, 4, and 6%) and oil concentrations (10, 30, and 50%), (b) the interaction effect of solid lipid type and emulsifier concentration on PDI, (c) the interaction effect of solid lipid type and emulsifier concentration on zeta potential, (d) the overall effect of oil concentration on zeta potential, (e) the overall effect of solid lipid type on zeta potential: different letters in the same row indicate statistically significant difference ($p < .05$).

performed using an ultrasonication method. This instrument produces a strong vibration that can break lipid nanoparticles into smaller ones. Compared to continuous techniques such as high pressure homogenization, probe sonication usually yields higher PDI values (Tamjidi et al., 2014). Altogether, the observed low PDI values (< 0.35) for the nano carriers indicate that particles were in a state of desirable monodispersity, suggesting good storage stability (Ali et al., 2010). PDI decreased significantly with an increase in surfactant concentration (Fig. 2b) which confirms that the emulsifier mixture was efficient in reducing the surface tension to lower mean diameter and PDI. Similar decrease in PDI was also reported in previous works (Tamjidi et al., 2014; Zhu et al., 2015). None of the remaining variables and interactions (data are not shown) had any major effect on the PDI of NLCs ($p > .05$).

A deeper consideration of physical stability of colloidal dispersions requires the knowledge of zeta potential values. Zeta potential reflects the strength of repulsion between charged particles. Typically, a minimum zeta potential of ± 30 mV is required for achieving a good stability for an electrostatically stabilized nano suspension, but for a nanosuspension stabilized with a combination of electrostatic and steric forces, a zeta potential of ± 20 mV is sufficient, because the coating of hydrophilic surfactant can further improve the stability of NLC by hydration in the surface layer (Tamjidi et al., 2014). All wax-NLC formulations showed a negative zeta potential ranging from −18.3 to −27.2, indicating long-term physical stability which was further confirmed by the absence of visible particle aggregation. The lowest zeta potential related to GB + PW formulation with 50% oil and 6% surfactant and the highest one pertaining to PW formulation contained 2% surfactant, and 10% PSO (Fig. 2a). This charge is mainly due to the presence of amphoteric lipophilic surfactant (i.e. lecithin) which is negatively charged at the applied neutral pH.

Fig. 2(c, d and e) shows the variation in zeta potential of wax-NLCs as a function of surfactant and oil concentration as well as solid lipid type. It can be clearly seen that various concentrations of surfactant had significant effects on the zeta potential and in all solid lipid types more negative zeta potential was observed with lower amounts of surfactant (Fig. 2c). The decrease of zeta potential of nanoparticles by increasing surfactant concentration emanates from differences in mobility and redistribution of used surfactants. High concentrations of the emulsifier reduce the surface tension and facilitate the particle partition during homogenization, leading to decrease in particle size and consequently rapid increase in surface area. The time scales of the redistribution process of emulsifier molecules to cover the new surfaces differ. While, low molecular weight surfactants adsorb rapidly at the interface, this process will take a longer time for high molecular weight surfactants such as lecithin. Therefore, lecithin, as internal lipophilic surfactant, is covered by external hydrophilic non-ionic surfactant (i.e. Tween 80) and thereby the zeta potential decreases (Mehnert & Mäder, 2012).

According to Fig. 2d, the absolute value of zeta potential of lipid carriers decreased with increase in the amount of oil ($p < .05$). This observation is in good agreement with previous studies where zeta potential was slightly increased by decreasing the oil concentration (Niculae, Lacatusu, Badea, Meghea, & Stan, 2014; Wang et al., 2014). One possible explanation could be the disruption of the surfactant shell at higher concentration of oil which leads to rearrangement of surface charge and results in a change of zeta potential values (Wang et al., 2014). The investigation of overall effect of solid lipid type showed that zeta potential was higher when BW and PW were used alone (Fig. 2e) and could be associated with the presence of free hydroxyl groups and fatty acid present in the composition of waxes (Niculae et al., 2014).

3.2. Encapsulation efficiency

NLCs with high content of oil have been reported to have lower EE, as excess liquid lipid would be expelled during crystallization (Ali et al., 2010; Zhu et al., 2015). Therefore, to confirm the entrapment of PSO within the solid matrix of the NLCs, oil entrapment efficiencies (EE) of fresh samples were determined by measuring the concentration of free PSO in the aqueous phase of the nanoparticle. The data generated from the GC analysis, revealed 96–99% PSO entrapment efficiency of all samples. A mixture of lipids with heterogeneous composition forms an imperfect solid matrix with variable distances. At low concentrations, oil molecules are easily dispersed into the lipid matrix. Higher content of oil leads to liquid molecules being entrapped in the form of tiny oily nano-compartments completely surrounded by the solid lipid matrix (oil-in-lipid-in-water structure) (Shah, Eldridge, Palombo, & Harding, 2015).

The excess oil, which exceeds the holding capacity of the solid lipids, would form an oily layer on the surface of nanoparticles. Ali et al. (2010) reported that bimodal oil distribution on the surface and inside the nanoparticles had no influence on the overall entrapment efficiency, as reflected by the high EE values (90–110%). These data led to the conclusion that prepared NLCs have appropriate capacity to accommodate the oil.

3.3. Further analysis of optimum formulations

3.3.1. General

Principal component analysis was conducted to classify samples according to their physical characteristics and determine optimum formulations, possessing smaller particle size and size distribution with high zeta potential value. Developing an NLC with a minimal particle size was a goal of this study, due to the unique physical and biological properties of nanoparticles of these sizes (How, Abdullah, & Abbasalipourkabir, 2010). Small-sized droplets have better chances to adhere to tissue surfaces because the large surface/volume ratio ensures close contact with tissues, easy penetration of barriers, and more-controlled delivery. Furthermore, the small size of nanoparticles makes the dispersion kinetically stable against sedimentation, since slower Brownian motion is correlated with larger hydrodynamic diameter (Liu & Wu, 2010).

The resulting two principal component projections are shown in Fig. 3. The scores plotted along the first two principal components accounted for 93% of total variance. Accordingly, PW and BW + GB samples containing 10% PSO, stabilized with 6% surfactant blend, were identified as optimum formulations and used for further analysis. The PS, PDI and surface charge of these formulations were 73 nm, 0.14 and −23 for BW + GB and 92 nm, 0.15 and −24 for PW, respectively.

3.3.2. Storage stability

3.3.2.1. Physical stability. Long-term stability of selected NLC systems to coarsening was monitored for up to 6 weeks at room and refrigerator temperatures by measuring changes in their PS, PDI and surface charge (Fig. 4). It seems that the NLCs were fit for storage at 25 °C and also at 4 °C as no signs of instability in form of phase separation or creaming and no significant change of PS ($P > .05$) were detected during the storage period. The high stability of developed nanoparticles against particle aggregation could be related to their high zeta potential value and low kinetic energy of carrier system at low temperatures. A similar trend was observed in other NLC preparations when they were stored at 4 or 25 °C. Wang et al. (2014) reported the high physical stability of

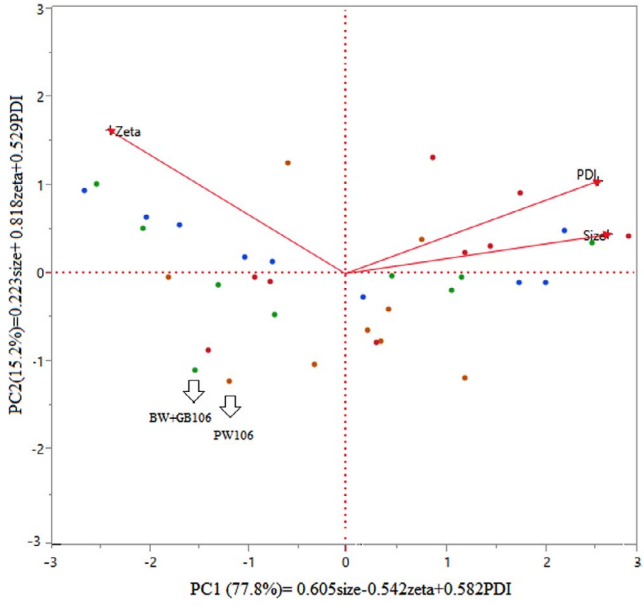

Fig. 3. Principal component analysis of developed nano carriers.

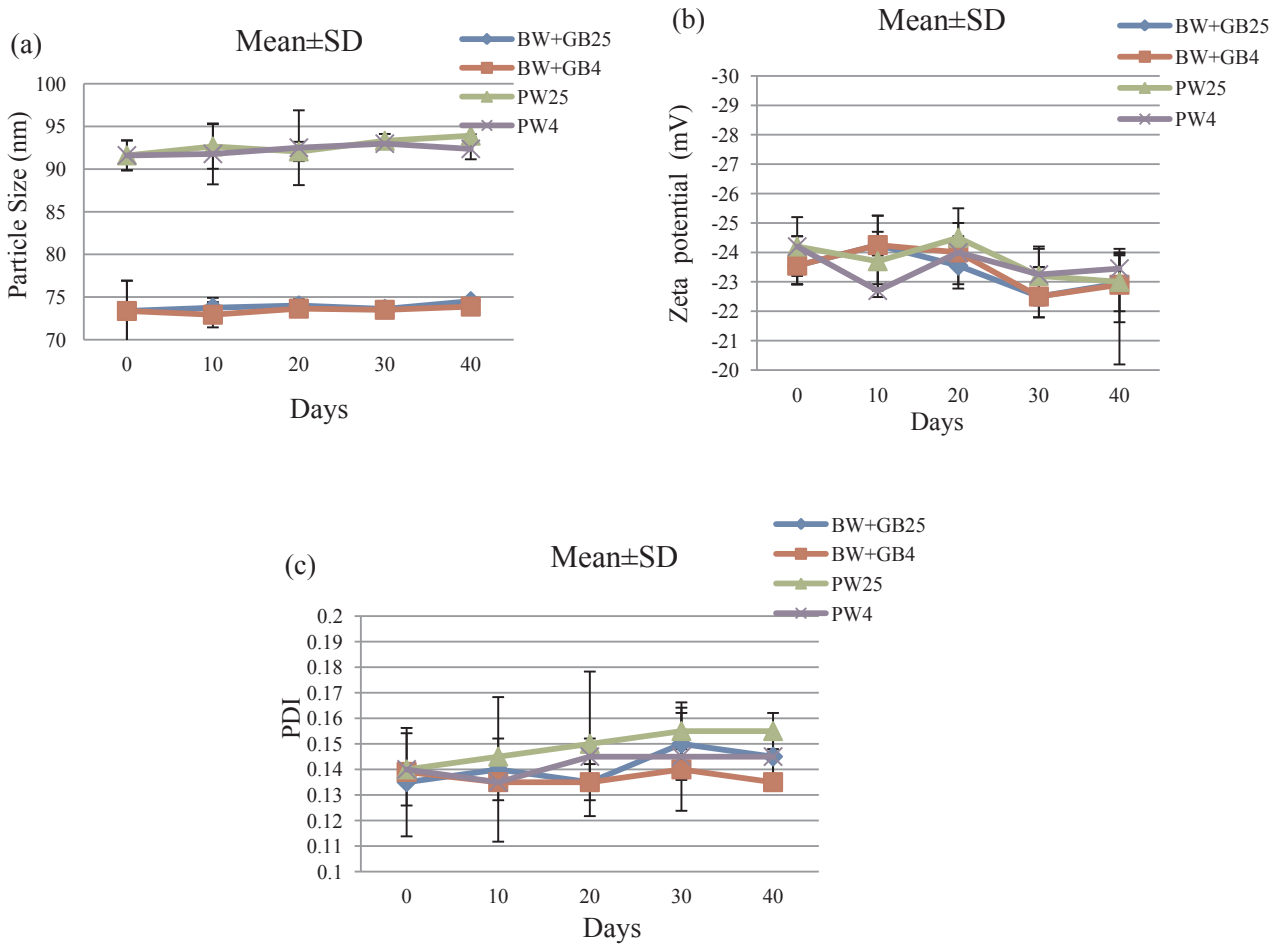

Fig.4. Effects of storage on particle size (a) zeta potential (b) and PDI (c) of selected NLC dispersions at different temperatures (4 and 25 °C). NLCs have been prepared by using 6% surfactant mixture of Tween80/Lecithin (1:0.25). The concentration of PSO was 10% of total lipid phase. BW + GB and PW represent NLC formulated with binary mixture of beeswax and glyceryl behenate (1:1) and propolis wax, respectively.

microalgae oil-loaded NLCs during storage as reflected by the particle size and size distribution. Good long-term stability of lipid nano particles at 4 and 25 °C, with no significant modification in the particle size, was also found by other researchers (Li, Zahi, Yuan, Tian, & Liang, 2016; Zhu et al., 2015). However, higher storage temperatures may lead to aggregation and thus rapid increase of particle size by accelerating the collision of small particles (Wang et al., 2014; Zhu et al., 2015). There was also no obvious change in the PDI and zeta potential of formulations during the storage period ($P > .05$) which is in agreement with results reported for other NLC preparations (Fathi, Varshosaz, Mohebbi, & Shahidi, 2013; Tamjidi et al., 2014).

3.3.2.2. Chemical stability. Due to the application of PSO consisting of highly polyunsaturated fatty acids, the quality of NLCs were also evaluated by assessing the peroxide value (PV) and their antioxidant activity during storage. The PVs of the fresh NLCs were 3.51 ± 0.66 (PW NLC) and 5.07 ± 0.21 meq/kg (BW + GB NLC) which were elevated to 7.1 and 9.7 (BW + GB NLC) and 6.6 and 6.8 meq/kg (PW NLC) at 4 °C and 25 °C, respectively during storage. However, the final PV value was still lower than 10 meq/kg, even after 40 days of storage (Table 1). This behaviour could be related to a chemically protective effect of solid core of the nanoparticles that prevents the oxygen from reaching the oil (Liu & Wu, 2010). The results are in agreement with findings of the study reporting the high oxidative stability of fish oil enriched with ω-3 fatty acids after processing at high preparation temperature (85 °C) and incorporation into Carnauba wax NLC formulations.

Salminen, Gommel, Leuenberger, and Weiss (2016) also found low rate of lipid oxidation in NLC containing ω-3 fish oil stored at 25 °C during 51 days of storage. The high antioxidant potential of surfactant solution and presence of high melting phospholipid were reported to be responsible for improving oxidative stability.

Compered to the BW + GB sample, lower PV was observed for PW NLC ($p < .05$) which was further confirmed by the results of antioxidant activity measurement. At the end of the storage time, a lower PV level ($p < .05$) was obtained at 4 °C than at 25 °C.

DPPH free radical-scavenging is an accepted mechanism by which hydrogen-donating antioxidants act to inhibit lipid oxidation by reducing the stable radical DPPH˙ to the yellow-coloured non-radical form, diphenyl-picrylhydrazine. The antioxidant activity of selected NLCs (at the concentration of 500 μg/ml) throughout the storage is presented in Table 1. PW NLC exhibited a better percentage of antioxidant activity which might be the reason for the lower peroxide value compared to BW + GB NLC. Propolis wax is known to manifest antioxidant activity due to the presence of pentacyclic triterpenoids such as lupeol and α and β amyrin in its composition which are considered among the components with biological and antioxidant activity (Sunil et al., 2014). Triterpenoids are plant defensive compounds, commonly found in plant waxes and in other plant secretions, such as resins. They are collected by bees along with other classes of compounds, e.g. phenolics, that are well known as propolis constituents. Triterpenoids are extracted with the common wax constituents due to their highly lipophilic nature (Negri, Marcucci, Salatino, & Salatino, 2000b). Regarding the

Table 1
Peroxide value and antioxidant activity of selected NLCs during storage at different temperatures (4 and 25 °C).

Formulation*	Temperature	Peroxide value (meq/kg oil)				
		Day 0	Day 10	Day 20	Day30	Day 40
BW + GB	25	$5.07 \pm 0.11^{C}_{a}$	$5.4 \pm 0.14^{BC}_{a}$	$6.06 \pm 0.03^{BC}_{a}$	$7.35 \pm 0.22^{C}_{a}$	$9.74 \pm 1.69_{a}$
BW + GB	4	$5.07 \pm 0.11^{D}_{a}$	$5.18 \pm 0.03^{CD}_{a}$	$5.7 \pm 0.18^{C}_{a}$	$6.33 \pm 0.32^{B}_{b}$	$7.19 \pm 0.3_{b}$
PW	25	$3.51 \pm 0.66^{C}_{b}$	$4.65 \pm 0.21^{BC}_{b}$	$5.79 \pm 0.74^{AB}_{a}$	$6.5 \pm 0.4^{A}_{b}$	$6.87 \pm 0.08_{b}$
PW	4	$3.51 \pm 0.66^{D}_{b}$	$4.59 \pm 0.1^{C}_{b}$	$5.03 \pm 0.07^{BC}_{a}$	$5.47 \pm 0.13^{B}_{c}$	$6.6 \pm 0.07_{b}$
		Antioxidant activity (%)				
BW + GB	25	$24.4 \pm 0.53_{b}$	$24.1 \pm 0.11_{bc}$	$23.3 \pm 3.2_{b}$	$22.6 \pm 2.13_{c}$	$22.6 \pm 2.13_{b}$
BW + GB	4	$24.4 \pm 0.23^{A}_{b}$	$22.9 \pm 0.52^{B}_{c}$	$24.8 \pm 0.53^{A}_{ab}$	$24.1 \pm 0.1^{A}_{bc}$	$24.2 \pm 0.1^{A}_{ab}$
PW	25	$30.1 \pm 1.06_{a}$	$27.8 \pm 2.13_{ab}$	$27.7 \pm 0.69_{ab}$	$26.5 \pm 1.3_{ab}$	$28.0 \pm 0.1_{a}$
PW	4	$30.1 \pm 1.06_{a}$	$28.2 \pm 1.59_{a}$	$29.1 \pm 1.06_{a}$	$28.9 \pm 1.1_{a}$	$28.3 \pm 0.02_{a}$

* NLCs have been prepared by using 6% surfactant mixture of Tween80/Lecithin (1:0.25). The concentration of PSO was 10% of total lipid phase. BW + GB and PW represent NLC formulated with binary mixture of bees wax and glyceryl behenate (1:1) and propolis wax as solid lipid, respectively. Different a, b, c… letters in the same column and different A, B, C … letters in the same row indicate a statistically significant difference ($p < .05$).

storage condition, no significant variation in antioxidant activity of each formulation was observed at different temperatures ($P > .05$). Furthermore, antioxidant activity of samples revealed no statistically significant change after 40 days of storage.

3.3.3. XRD, DSC and TEM analysis

DSC was employed to check any variation of crystalline properties of lipid carriers after oil incorporation. In order to do that, SLNs were prepared in exactly the same manner only by replacing PSO with respective solid lipid. Fig. 5a shows the melting behaviour of lyophilized formulations of BW + GB SLN, BW + GB NLC, PW SLN and PW NLC. Samples prepared with PW revealed a single broad melting peak at 62 °C with a total enthalpy of 105.58 J/g (PW SLN) and at 61.56 °C with a total enthalpy of 94.09 J/g (PW NLC). These broad endotherms were attributed to the complex and heterogeneous composition of PW which affected its thermal profile. Lipid matrices which form highly crystalline structures with a perfect lattice show very sharp and narrow endothermic peaks, which lead to drug expulsion, while more complex lipids or lipid mixtures containing fatty acids of different chain lengths form less perfect crystals with many imperfections offering space to accommodate drugs (Attama, Schicke, & Müller-Goymann, 2007).

On the other hand, the 1:1 mixture of BW and GB presented a large broad split peak at 61.1 °C and two small peaks at 67.23 °C and 68.78 °C for the SLN sample (total enthalpy 132.13 J/g) and at 59.22 °C, 66.79 and 68.4 °C for the NLC sample (total enthalpy 95 J/g), indicating overlapping of melting endotherms of BW with that of GB. The GB shows a single endothermic peak at 74.5 °C (Tamjidi et al., 2014). Absence of melting peak at this temperature confirms that structural modification occurs on mixing GB with BW.

The presence of several endothermic peaks indicates a certain heterogeneity and disorder in the matrices and further proves the existence of the lipid matrices in low crystalline states which will favour drug loading when used in pharmaceutical and food formulation (Attama, Schicke, Müller-Goymann, 2007).

As was expected, the NLC formulations showed endothermic peaks with lower melting points and enthalpies compared to SLNs. The degree of crystallinity was calculated by comparing the enthalpy of the NLCs with their SLNs (Liu & Wu, 2010) and found to be 71 and 90% for BW + GB and PW, respectively. The results showed the lower order crystalline structure of NLCs, and suggested that the oil is dispersed in the fat matrix. Reduction of melting point in a concentration dependent manner, by adding the liquid oil to the carrier, was also observed by other researchers (Jenning, Thunemann, & Gohla, 2000). A less ordered crystal lipid matrix of NLC is favourable for entrapping more encapsulant molecules in comparison to the relatively ordered crystal of SLN.

The crystalline states of NLC and SLN samples were also investigated by XRD between angles $2\theta = 5–40°$ (Fig. 5b). The diffractograms of wax NLC samples revealed a high intensity reflection at 21.12° and a medium intensity reflection at 23.64, indicating short spacings of fatty acid chains at 0.42 and 0.376 nm, respectively, which are typical for the orthorhombic metastable polymorph (β′) (Jenning et al., 2000; Weiss et al., 2008). A small third peak was also found in diffractograms of BW samples (19.24°) and PW NLC (19.13°) and PW SLN (19.24°) which correspond to short spacing at 0.46 nm, representing the partial formation of triclinic-orthorhombic (βi) polymorph (Jenning et al., 2000).

The lipid composition determines the type of crystal that is generated upon cooling, thereby influencing the stability and the release characteristics of solid lipid nanoparticles (Weiss et al., 2008). Jenning and Gohla (2000) reported that waxes like beeswax, in contrast to glyceride solid nanoparticles, show typical reflections of orthorhombic subcell at 0.38 nm and 0.42 nm which will remain unchanged, even under stress conditions (shaking, exposure to electrolytes or water evaporation) and after long term storage. The surfactant also plays an important role in the crystallization process. Mixtures of nonionic surfactants and ionic surfactants are commonly used to control lipid crystallization (Weiss et al., 2008).

The SLN formulations also revealed reflections very similar to those of NLCs. However, these peaks were found at short spacings of 0.414 and 0.374 and 0.455 nm for BW SLN, and 0.408 and 0.369 and 0.453 nm for PW SLN.

XRD data confirmed DSC results, showing a reduction in intensity of the main diffraction peaks of NLC formulations compered to their SLNs. Lower intensities indicate higher proportion of β′ polymorph and lower degree of crystallinity (Liu & Wu, 2010). This may be due to the incorporation of the oil among parts of the crystal lattice of the lipid, leading to more imperfections in the crystals and increasing the efficiency of carrier in maintaining the active ingredient (Muller et al., 2000). Previous study has also demonstrated that the NLC produced by incorporation of liquid oil into SLN has a less ordered microstructure in the lipid matrix (Liu & Wu, 2010)

As is depicted (Fig. 5c–d), the NLC particles had spherical and uniform shape which did not depend on the type of applied solid lipid. Many factors affect the size and shape of a nano particle, among them the velocity of lipid crystallization, the lipid composition and the shape of lipid crystals (Attama, Schicke, Paepenmüller, & Müller-Goymann, 2007). The spherical morphology confirms a less ordered crystalline structure of the lipid matrices since an ordered structure (β modification) results in elongated crystals (Manea, Vasile, & Meghea, 2014). The spherical or ovoid structure has also been reported for NLC in previous studies (Hejri et al., 2013; Hu et al., 2005; Zhu et al., 2015).

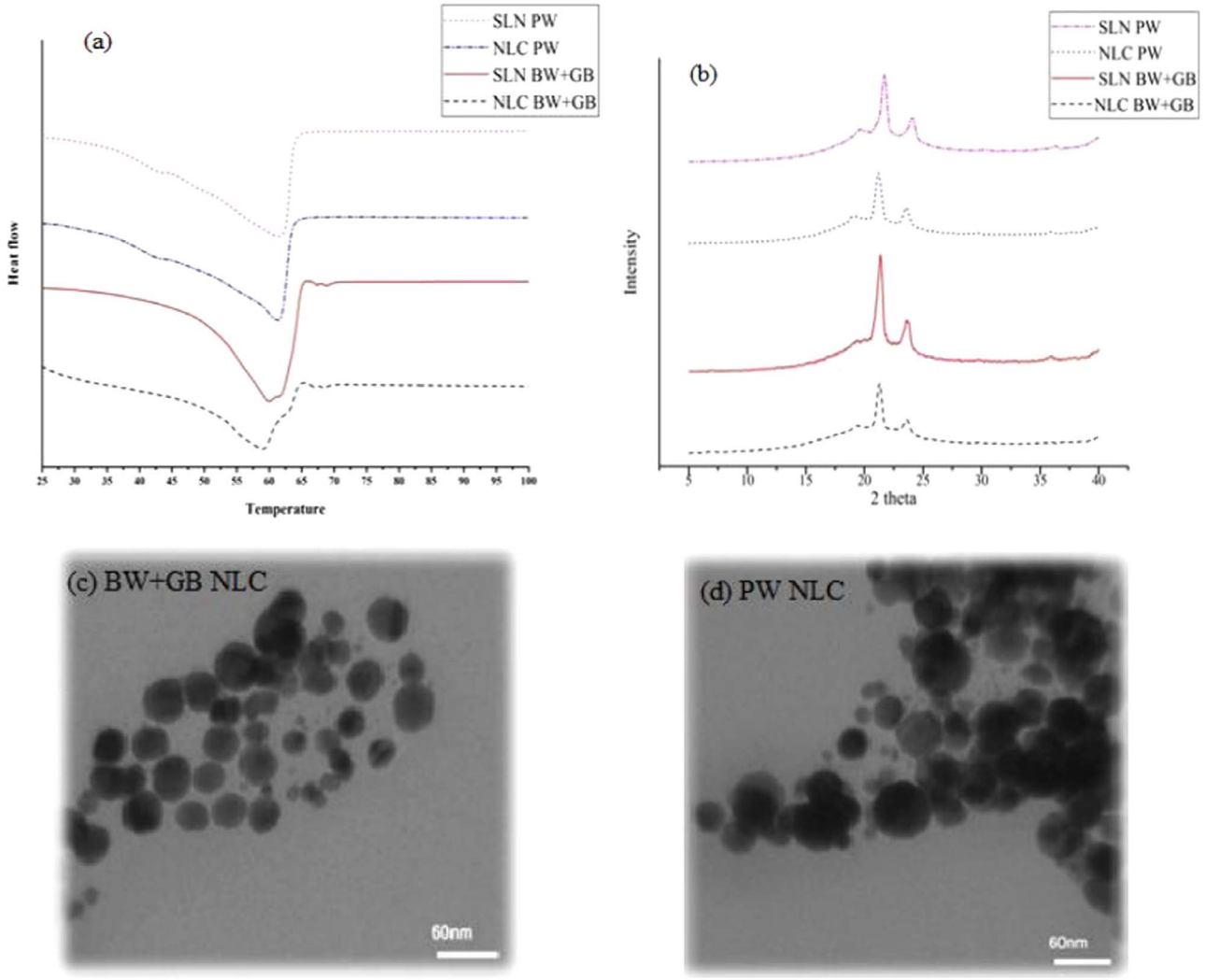

Fig. 5. DSC curves (a), X-ray diffraction pattern (b) and TEM morphology (c) of developed wax nanoparticles. PSO was replaced by respective solid lipids in formulation of SLNs. Nano carriers have been prepared using 6% surfactant mixture of Tween80/Lecithin (1:0.25).

4. Conclusions

The general objective of this research was to design and synthesize stable and functional lipid nanocarriers, based on the combination of natural waxes (beeswax and propolis wax) and health-promoting pomegranate seed oil, for further application in food industry. Data from PS analysis and encapsulation efficiency indicated high distribution of oil throughout the lipid carriers and good physical stability, as reflected by no creaming or phase separation. Increasing the surfactant concentration up to 6% resulted in a decrease in particle size, PDI and zeta potential of samples, regardless of oil content or solid lipid type. Interaction between oil content and lipid composition exhibited a reduction in PS of BW NLCs and an increase in PS PW NLCs with increase in oil concentration. Mixture of wax and GB yielded smaller particles in comparison to BW alone; however, PW showed an opposite trend.

Based on PCA analysis, the best formulations were PW and BW + GB samples containing 10% oil and 6% surfactant which produced smaller NLCs with narrow size distributions and high stability. TEM, DSC and XRD results indicated the spherical morphology and lower crystallinity of oil-loaded lipid mixtures compared with oil-free SLNs that would lead to higher drug incorporation efficiency.

Current results suggest that this type of novel lipid carrier could be an efficient, healthful and promising carrier system for developing food grade drug delivery systems: (i) by virtue of broad health-promoting properties manifested by PSO and PW (such as antioxidant and antibacterial capacity); (ii) by assuring an efficient encapsulation efficiency due to the complex composition of applied lipid matrix and (iii) by the superior physical stability that wax NLCs exhibit.

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