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Formulation and characterization of novel liposomes containing histidine for encapsulation of a poorly soluble vitamin

Petar M. Batinić, Verica B. Đorđević, Sanja I. Stevanović, Bojana D. Balanč, Smilja B. Marković, Nevena D. Luković, Dušan Ž. Mijin, Branko M. Bugarski

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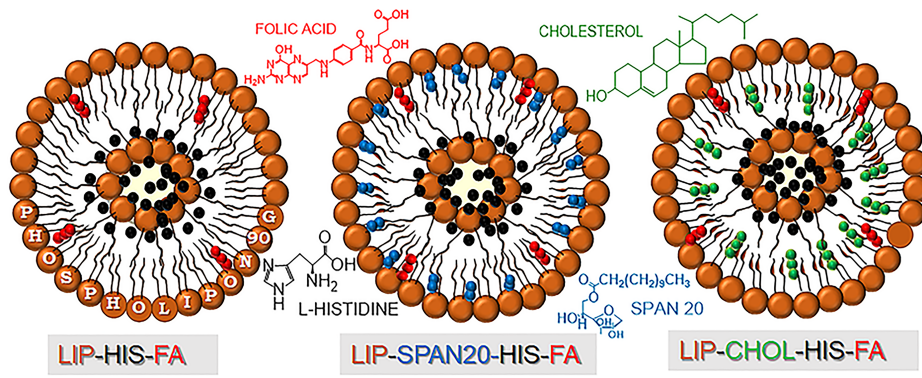**Sanja I. Stevanović** Investigation, Writing - original draft

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FOLIC ACID

CHOLESTEROL

PHOSPHOLIPO G90 N O

L-HISTIDINE

SPAN 20

LIP-HIS-FA   LIP-SPAN20-HIS-FA   LIP-CHOL-HIS-FA

Formulation and characterization of novel liposomes containing histidine for encapsulation of a poorly soluble vitamin

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Abstract

In this study, development of folic acid-loaded liposomes using a basic amino acid, histidine as a solubilizing agent for folic acid was presented, which tackled the poor solubility of this vitamin. The effect of the liposomal membrane modifiers, cholesterol and SPAN 20 on the characteristics of the final formulations was examined. Liposomes prepared from a commercially available purified soybean lecithin (Phospholipon 90G) by proliposome method were between 503 and 877 nm in average diameter, where cholesterol induced enlargement and SPAN.20 reduction of vesicles. High encapsulation efficiency of 84% and drug loading of 0.123 mg g^{-1} were achieved, irrespective to the composition. According to AFM images, folic acid-loaded liposomes of a fraction with a nano size were flattened compared to globular empty liposomes. FTIR analysis revealed possible interactions between phospholipids and histidine, while DSC study suggested interactions between folic acid and lipids during heating. Release study done by a Franz diffusion cell showed prolonged release of folic acid from liposomes and the release rate was determined by folic acid solubility.

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1. Introduction

Folic acid (UIPAC name (2S)-2-[(4-{[(2-amino-4-hydroxypteridin-6-yl)methyl]amino}-phenyl)formamido]pentanedioic acid) is a member of the Vitamin B family and necessary for the healthy function of a variety of bodily processes. The chemical structure of folic acid is shown in Figure 1A. Folic acid deficiency can induce variety of diseases like megaloblastic anemia, neural tube defects and cancer. Nowadays, the prevalence of folate deficiency has decreased since many countries in the western hemisphere introduced a mandatory folic acid food fortification program starting in the late 1990s. The recommended daily intake is 400 µg for an adult and an additional 800 µg for pregnant women. Currently, there are no established risks for adverse consequences resulting from existing mandatory folic acid fortification programs that have been implemented in many countries (Field and Stover, 2018). In addition, folic acid can be used against dermal intrinsic aging as it improves viability of UV-damaged skin cells by modulation of DNA repair mechanism (Ammar et al., 2016; Debowska et al., 2005). However, there is more than one problem associated with folic acid consumption. Firstly, folic acid is easily oxidized by oxygen or air at a relatively high temperature and humidity during the processing and storage (Qin et al., 2014). Secondly, folic acid has low solubility, 1.6 mg l^{-1} in water and the biological milieu. Consequently, after administration folic acid can display poor absorption and poor bioavailability often below the therapeutic threshold. Thirdly, when administrated topically, low uptake happens due to the barrier function of the stratum corneum and the absorption to the systemic circulation is low. Therefore, scientists have been developing new formulations of folic acid to enhance its solubility and bioavailability and/or to overcome the skin barrier and increase drug transport across the skin (Acavedo-Fani et al., 2018; Ammar et al., 2016; Barat et al., 2011; Jiao et al., 2018; Kapoor et al., 2018; Penalva et al., 2014). Among these few formulations, liposomes based on GRAS-labeled soya phosphatidylcholine seem to be most promising in delivery of folic acid. This study is aimed at taking a further step in developing liposomal delivery systems of folic acid. As a divergence from previous reports, proliposome method is applied for liposomes preparation, as an alternative to other conventional methods (e.g. thin lipid method), since it can be easily adopted to large scale production. Secondly, a commercial phospholipid mixture is used as alternative to pure lipids since mixtures containing impurities have far lower prices thus are suitable for large scale productions (Jovanović et al., 2018). Thirdly, in order to overcome a problem of low solubility of folic acid, a basic amino acid, histidine (Figure 1B), was used to have a role of

solubilizing agent. Histidine is an essential amino acid used by the body for manufacturing histamine, which is responsible for a wide range of physiological processes. People use histidine as medicine for treatment of rheumatoid arthritis, allergic diseases, ulcers and anemia caused by kidney failure or kidney dialysis (Fawzy et al., 2014). Its deficiency can cause poor hearing. Doses of up to 4 grams per day have been used in research without causing noticeable side effects.
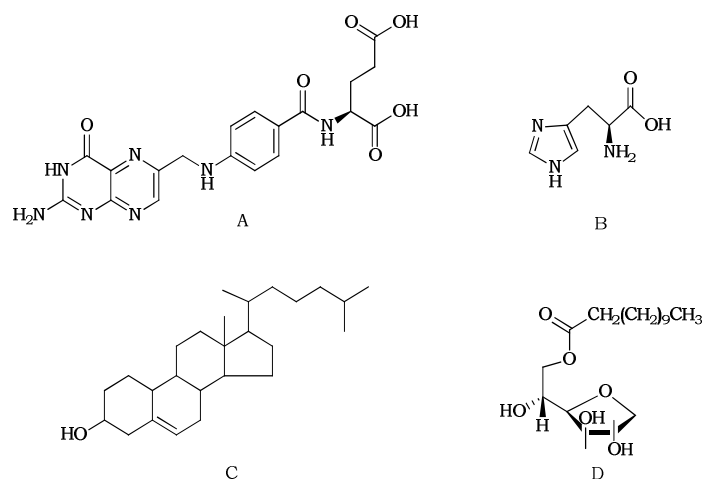

Figure 1. Chemical structures of folic acid (A), histidine (B), cholesterol (C) and SPAN 20 (D)

The aim of this study was to investigate possibilities for adjusting the composition of bilayer membrane by using cholesterol or a surfactant (SPAN 20) as a tool to control delivering efficiency for folic acid. Namely, cholesterol (Figure 1C) is used in liposomes to modulate membrane fluidity and enhance stability of phospholipids bilayer (Jovanović et al., 2018). On the other hand, surfactants such as SPAN 20 (Figure 1D) contribute to elasticity of liposomal bilayers and their transdermal delivery efficiency (Chou, 2015; Malvey et al., 2019). However, it should have in mind that the international use of Span is limited due to safety concerns. Precisely, in European Union and Switzerland Span 20 is one of commercially utilized common surfactants (food emulsifiers and stabilizers) with an E-number 493 and acceptable daily intake of up to 25 mg/kg bodyweight. However, it is banned in some parts of world (Australia), since surfactants do have some toxicity (they harm the enzyme activity and thus disturb the body's normal physiological function) and may accumulate in the human body (Yuan et al., 2014). Therefore, it is important to investigate whether Span would enhance (and to what extent) liposomal delivery system of FA, or would be better to avoid it. In this study the effects of cholesterol and SPAN 20 on liposomes size, morphology and release properties were investigated. In complex systems such as liposomes containing both, folic acid and histidine, and modified by cholesterol or a surfactant, interactions between such many constituents may

have effects on the end properties. Therefore, the interactions were investigated by ATR-IR and DSC analysis.

2. Materials and methods

2.1. Materials

Phospholipon 90G – P90G (commercial lipid mixture which contains pure phosphatidylcholine (PC) 94.0-102.0%; lysophosphatidylcholine 4.0%; tocopherol 0.3% stabilized with ascorbyl palmitate 0.1%) was purchased from the Phospholipid GmBH, Germany. Folic acid (FA) and phosphate salts for preparing phosphate buffer were obtained from the Sigma Aldrich, Germany. Acetonitrile, HPLC grade was purchased from Chem-Lab NV, Zedelgem, Belgium. SPAN 20 (surface active compound – SPAN 20), cholesterol (CHOL) and amino acid L-histidine (HIS) were obtained from Sigma Aldrich, USA.

2.2. Methods

2.2.1. Preparation of liposomes

Liposomes were prepared using proliposome method defined and developed by Perret's model, with few modifications (Perret et al., 1993). P90G, ethanol and water in the mass ratio 1:1:2 were stirred at 600 rpm for five minutes at 60 . After that, the mixture was cooled to room temperature (20). Further, the mixture was hydrated using the solution of folic acid (FA) dissolved in histidine (HIS) aqueous solution (8 mg ml^{-1}) at concentration of 0.16 mg ml^{-1}. The hydration step was performed by adding 4.7 ml of hydrating medium to 0.1 g of lipids at room temperature (20) with stirring at 800 rpm for 60 minutes. In this way liposomes containing histidine with incorporated folic acid were prepared (LIP-HIS-FA). Liposomes containing only histidine (LIP-HIS) and empty liposomes (LIP) were prepared by the same method, by using histidine aqueous solution (without folic acid) and distilled water, respectively, as a hydrating medium.

For the preparation of folic-acid loaded liposomes containing cholesterol (LIP-CHOL-HIS-FA), cholesterol was added to the initial mixture in molar ratio 1:4 (CHOL:P90G).

For the preparation of folic-acid loaded liposomes containing SPAN 20 (LIP-SPAN 20-HIS-FA), the surfactant was added to the initial mixture in molar ratio 1:4 (SPAN 20:P90G).

2.2.2. Liposomes size and stability

Liposomes suspensions were analyzed using Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom). The average size of liposomal particles, index of polydispersity (PDI) and zeta potential were measured immediately after the preparation of

liposomes. Liposomes suspensions were diluted with water fifty times before each measurement. Also, the liposomes suspensions stability was monitored at the 1st, 7th, 20th and 30th day after the first measurement (Isailović et al., 2013). During the period, liposomes suspensions were stored at 4 , in the refrigerator.

2.2.3. Determination of encapsulation efficiency and loading capacity

The encapsulation efficiency of FA loaded liposomes was determined similarly as described by Rawia Khalil et al. (2017). In brief, one milliliter of liposome suspension was transferred into Eppendorf cuvette and centrifuged in three cycles for 15 minutes (4°C, 17000 rpm). The encapsulation efficiency was determined by measuring concentration of FA in the supernatant. The FA concentration was defined spectrophotometrically at a wavelength of 280 nm. The encapsulation efficiency was calculated using the equation 1.

$$EE\ (\%) = \frac{(m_I - m_{SN})}{m_I} \cdot 100 \tag{1}$$

where the m_I was initial amount of FA used for liposomes preparation, while the m_{SN} was the amount of FA in supernatant after centrifugation [30]. The loading capacity was expressed as loaded drug/lipid ratio (mg g^{-1}).

2.2.4. ATR-IR measurements

For the ATR-IR measurements, liposome suspension (1 ml) was placed on the glass plate, (76x26x1mm) and dried in nitrogen atmosphere for 1500 minutes (Krilov et al., 2014). A small tube was constructed which provided a constant flow of nitrogen. The glass plate, with the sample was fixed inside the tube, so the nitrogen could easily flow over the surface of the plate and directly dry the liposome suspension. When the sample was dried, a small amount of the sample was subjected to ATR-IR analysis (Thermo Fisher Scientific, Madison, USA, model: NICOLET iS10).

2.2.5. Atomic force microscopy (AFM)

The surface morphology was investigated by atomic force microscopy with Nano Scope 3D (Veeco, USA) microscope operated in tapping mode under ambient conditions. Etched silicon probes with spring constant 20-80 Nm^{-1} were used. Before the morphological examinations, mica substrate was mechanically polished with adhesive tape. 10 µl of operated suspensions of liposome were deposited on polished mica substrate and dried on air for 20 minutes.

2.2.6. Differential scanning calorimetry (DSC)

DSC analysis was used in order to examine possible interactions between constituents. Certain amount (2-6 mg) of each lyophilized sample (pure folic acid, histidine, plain liposomes, liposomes containing histidine and folic acid-loaded liposomes) was weighted and put in the aluminum pans. Aluminum pans were hermetically sealed. DSC analysis was performed using DSC 131 EVO (SETARAM Instrumentation, Caluire France). As the reference sample an empty aluminum pan was used. The pan with sample, as well as the reference one, were placed in DSC chamber and heated in the temperature range from -20 to 350 °C, with the exception of pure folic acid and histidine which were heated from room temperature to 350 °C. The heating rate was constant, about 10 °C per minute, while the flow of nitrogen gas was 20 ml per minute. Also, the baseline was adjusted by using two empty aluminum pans, under the same conditions.

2.2.7. Release studies of FA from liposomes

The release studies were done using a Franz diffusion cell. The volume of 2 ml of liposome suspension (0.016 mg ml^{-1}) was placed in donor section of the cell, and 20 ml of phosphate buffer (0.1 mol l^{-1}, pH 5.5 or 7) was used to fill acceptor section of the cell. Between the sections, cellulose-acetate membrane was placed (the transfer area was 4.91 cm^2). During the experiments, samples were taken in predetermined time intervals (up to 300 minutes) from the sampling pot connected to the acceptor section of the cell and the sample volume was 0.5 ml. Concentration of FA in all samples were analyzed using HPLC analysis.

2.2.8. HPLC analysis

The concentration of FA in samples collected during release studies was determined using high performance liquid chromatography (Nexera X2, LC 30 series, with RID 20A detector, Shimadzu) with C18 column (4.6x250 mm, 5 μm) maintained at 25 °C, UV–detector series at 280 nm. The mobile phase composition was consisted of acetonitrile and water (50:50) and 0.1% of formic acid. The mobile phase flow rate was 1.0 ml min^{-1} and injection volume was 20 μl. The calibration curve was made using the series of dilution of FA in HIS. The extinction coefficient of FA in HIS aqueous solution (pH 7.6) at 280 nm was determined to be 28.3 mol dm^{-3} cm^{-1}.

3. Results and discussion

3.1. Encapsulation efficiency and drug loading

In this study a basic amino acid–histidine was used as a solubilizing agent for folic acid. Namely, folic acid is a poorly soluble drug at neutral conditions with the recorded value of solubility 1.6 mg l^{-1} in water at 298 K (ONeil, 2006). Due to this limitation some commercial

folic acid supplements failed in the disintegration and dissolution performance on USP standards and the vital reason was pH dependency of solubility of FA (Wu et al., 2010; Younis et al, 2008). In addition, scientists struggle to prepare liposomes and lipid nanoparticles incorporating FA, either by using for a hydrating medium of a lipid film a water solution of FA above solubility limit which brings a question of homogeneity of the final formulation or by using a lipid-based excipients to dissolve FA although FA is a water soluble vitamin (Ammar et al., 2016; Jiao et al., 2018; Kapoor et al., 2018) In this study FA was dissolved in aqueous solution of histidine (pH=7.6) at concentration of 160 mg l^{-1} which is about 20 times higher than the saturation point in the absence of histidine at the given pH (Younis et al., 2008). Similarly, Penalva et al. (2014) used an aqueous solution of lysine (8 mg ml^{-1}) (one of three amino acids that have basic side chains at neutral pH) to dissolve FA at concentration as high as 300 mg ml^{-1}. Our hypothesis is that FA molecules, as behave as weak acids in alkaline solutions, having a negative charge, develop electrostatic interactions with positively charged α-amino group (which is in the protonated –NH$_3^+$ form, pK$_a$=9.17) and with a partially protonated imidazole side chain, (pK$_a$=6.04), the last one present in the solution in a small fraction, since neutral form of histidine prevails at a given pH of 7.8 (Figure 1B). Bourassa et al. (2017) revealed that FA forms conjugates with human serum albumin (and other serum proteins) via hydrophilic, hydrophobic and H-bonding contacts and their docking results disclosed histidine residue as one of the binding sites. The final liposomal formulations resulted with encapsulation efficiency of 84% and drug loading of 0.123 mg g^{-1}, irrespective to the composition. This maybe a result of two conflicting consequences of cholesterol presence; on the one hand cholesterol causes increased hydrophobicity, increased stability and decreased permeability of the bilayer which leads to more efficient trapping of a drug. Counteracting this, cholesterol may compete with FA for packing space within the bilayer therefore excluding the drug as the amphiphiles assemble into liposomes.

3.2. Liposomes size and stability

The results of hydrodynamic size, polydispersity index (PDI) and zeta potential of liposomes, are listed in Figure 2. The parameters were measured on the 1st, 7th, 20th and 30th day of preparation. The average size of folic acid-free liposomes was 385 nm which is nearly the same as the size of the liposomes prepared from the same lipid mixture by the same (proliposome) method using a buffer solution for hydration instead of aqueous solution of histidine. This is an indication that histidine *per se* was not embedded inside the bilayers, which is expected since histidine is a hydrophilic amino acid. On contrary, folic acid induced enlargement of vesicles with average diameter between 503 and 877 nm on the first day depending on the formulation. A possible explanation is that folic acid impairs the PC bilayer

due to the aromatic part which has an affinity for the lipid hydrophobic environment while the presence of carboxyl and amino groups indicates a preference for a polar environment. Therefore, it is likely that FA molecules are positioned closer to the lipid-aqueous interface than in the inner part of the bilayer.

SPAN 20-containing liposomes were the smallest among FA-loaded liposomes. Namely, SPAN 20 would significantly decrease the interface tension of the membranes, so that PC might tend to form condensed lipid layers when the surfactant was present but cholesterol was absent. In addition, there was less aggregation of LIP-SPAN 20-HIS-FA liposomes due to the high surface hydrophilicity as evidenced by decreasing PDI values, below 0.5, which is a proof for a monodisperse and homogenous dispersion. One can find in literature adverse effects of surface-active molecules on the size and polydispersibility of vesicles, depending on the formulation, surfactant type and concentration and the method of liposomes preparation (Bnyan et al., 2018; Liu et al., 2013; Pravilović et al., 2017; Tai et al., 2017). Liu et al. (2013) also observed a SPAN 20-induced size reduction of PC liposomes with embedded flavonol (quercetin) manufactured by ethanol injection method. In another way behaved liposomes of Phospholipon 90G with encapsulated hydro soluble polyphenols, such that SPAN 20 caused 2.5-fold increase in hydrodynamic radius (Pravilović et al., 2017).

On the other hand, when cholesterol was involved in the formation of the liposomal membranes, the average particle size of FA-loaded liposomes was observed to increase dramatically. The reason is that cholesterol inserted into the bilayers played a significant role in the structure formation. More specifically, sterol presence provokes interactions between lipid chains close to the head group of phospholipids, development of inter-lipid space and membrane expansion (Jovanović et al., 2018; Zhao et al., 2015).

Regarding the zeta potential, all liposome dispersions possessed a negative surface charge, in the range between -37 and -20 mV (Figure 2B), which is characteristic for the liposomes made of PC and thus can be considered as stably dispersed due to the electric repulsion between the particles. Cholesterol is generally known to increase the absolute zeta-potential and electrostatic repulsion between PC liposomes, but this effect is limited at concentrations below 30 mol% (Jovanović et al., 2018), as also happened here (Figure 2). The formulation with SPAN 20 had a lower negative (in absolute) zeta potential (~-20 mV) than un-modified liposomes (~-30 mV), which is in agreement with Liu et al. (2013) who observed such effect of non-ionic surfactants (and the opposite influence of anionic surfactants).

Concerning the stability monitored during time, a decrease in size of all liposomes over time was observed, statistically significant after one-month storage at 4 °C (Figure 2A). This decrease corresponds to a rising trend of zeta potential absolute values only in case of LIP-SPAN 20-HIS-FA liposomes. A possible reason for the size reduction could be a leakage of FA from observed over time from all thee formulations since loading capacity reduced for about

22-24 % even after one week (Figure 2C). Namely, leakage of the embedded agents is an undesired phenomenon frequently associated with storage of liposomes, especially MLVs (Bai et al., 2015). Another possible effect which could contribute to size reduction is that of ethanol residues present in the liposome suspensions (produced by proliposome method) (Isailović et al., 2013; Lopez-Pinto et al., 2005). There is a proposition that ethanol may trigger a modification of the net charge of the system and provide steric stabilization to some degree which finally leads to a decline in mean particle size (Lopez-Pinto et al., 2005). Our results opposed to stability evaluation of FA-loaded (soya PC, PC-oleic acid and PC-stearic acid) liposomes recently done by Kapoor et al. (2018) who claimed about unaltered content of FA, together with either agglomeration or unchanged size at the best, after 6 months at 4°C. However, all their liposomes were nanosized (100–200 nm) and unilamellar aimed at transdermal FA delivery.

2a

2b

2c
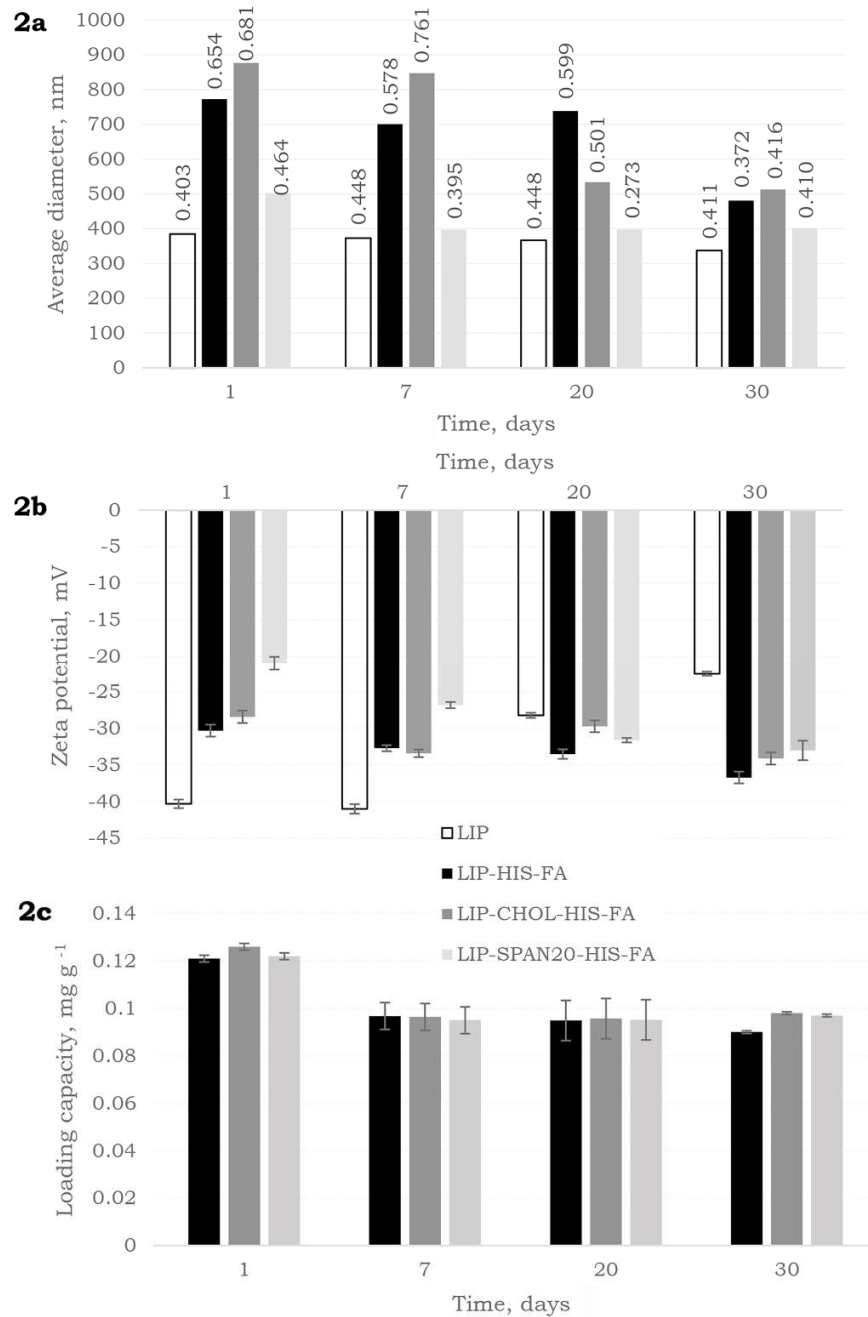
Figure 2. Stability of liposomes: a) The average size (bars) and polydispersity index (numbers above bars) b) Zeta potential, c) Loading capacity.

3.3. AFM measurements

The atomic force microscope poses many advantages in comparison to conventional optical microscopes and electron microscopes especially in studies of biological samples. First, preparing samples for AFM experiments do not required freezing, metal coating or exposure to

conditions of vacuum so there is a no disruption of samples during preparation for imaging. Also, the atomic force microscope is capable of operating in air as well as in liquid, so samples can be scanned in their physiological solutions with a very high resolution. With such a high level of resolution, images of single vesicles that cannot be seen by other imaging techniques can be obtained. The images resolve individual liposome (spherical particles) on a flat background, which is mica surface.

AFM measurements clearly revealed the difference of surface-modified FA-loaded vesicles compared to unmodified vesicles and FA-loaded compared to unloaded vesicles. The geometric diameter of the particles, measured based on tapping mode image section analysis, differs from one sample to another: 120 nm for unloaded liposomes, 209 nm for LIP-HIS-FA, 216 nm for LIP-CHOL-HIS-FA and 130 nm for LIP-SPAN 20-HIS-FA liposomes (presented in selected figures as representative images: Figures 3 and 4). The effects of FA, SPAN 20 and cholesterol on liposome size are consistent with the conclusions based on dynamic light scattering measurements, but the values of size are obviously far lesser. The reason lies in fact that light scattering technique gives the average bulk values of the size distributions in a solution, which means that extra-large vesicles present in the solution will affect the size distribution severely. On the other hand, AFM measures only adsorbed the vesicles on mica surface, while not every vesicle–surface interaction would ended with adhesion upon adsorption. In addition, large liposomes may rupture upon adsorption and they are more easily disrupted during AFM scanning (Liang et al., 2004). Therefore, the conclusions derived from AFM measurements should be taken with precaution since they refer only to a fraction of small liposomes. The mean height was 7-12 nm for LIP-HIS-FA, 4-8 nm for LIP-CHOL-HIS-FA and 9-11 nm for LIP-SPAN 20-HIS-FA samples, respectively. Empty liposomes exhibited a regular globular shape (Figure 3) while FA-loaded liposomes were flattened and collapsed (certainly, the LIP-CHOL-HIS-FA sample is more than other two formulations (Figure 4)).
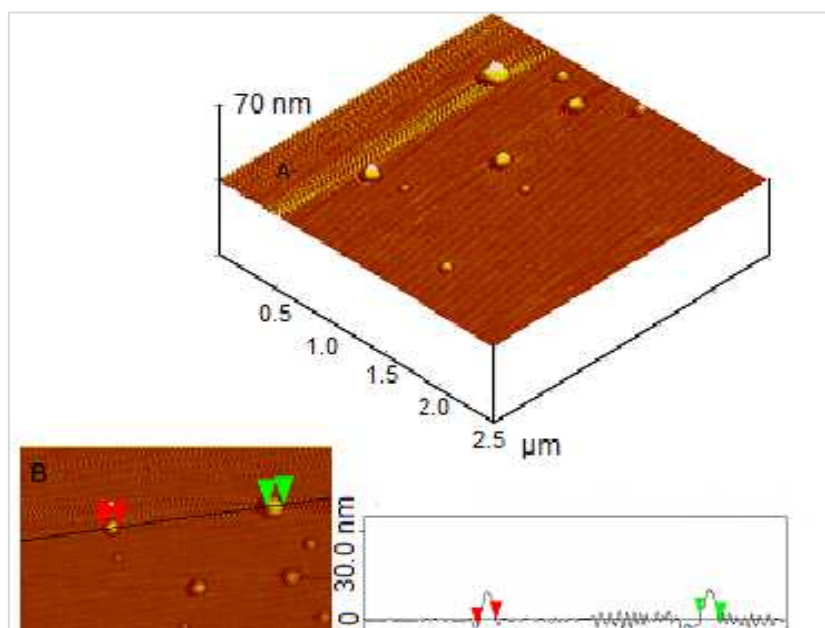
Figure 3. Offline image processing of an AFM fluid-tapping mode image capturing a representative liposomes (control) made from Phospholipon 90G: (A) the three-dimensional surface plot and (B) the section analysis
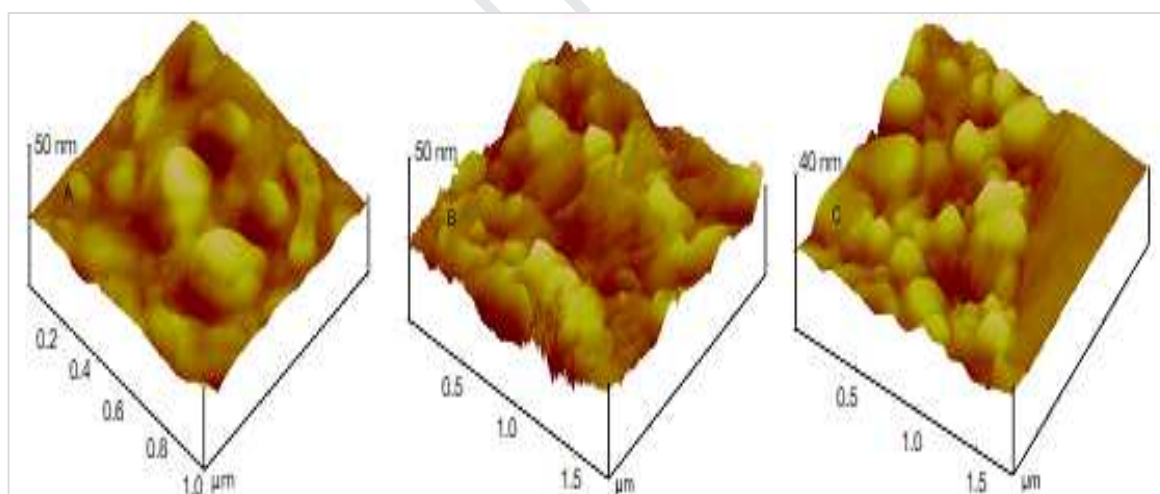

Figure 4. Surface Plot Analysis (3D) of AFM fluid-tapping mode images for FA-loaded liposomes: (A) LIP-HIS-FA, (B) LIP-CHOL-HIS-FA, (C) LIP-SPAN 20-HIS-FA

3.4. ATR-IR analysis

ATR-IR spectra of liposomal formulations, including control liposomes (LIP) and FA-loaded liposomes (LIP-HIS-FA, LIP-CHOL-HIS-FA and LIP-SPAN 20-HIS-FA) are shown in Figure 5. In order to establish possible interactions upon encapsulation, spectra are compared with that of the physical mixture of Phospholipon 90G, histidine and FA (blended in the same

ratio as in liposomal formulation LIP-HIS-FA), also shown in Figure 5. The FTIR spectra of the individual components have been provided as supplementary data (Figures 1S-5S, Supplementary). All spectra presented in Figure 5 contain the pair of peaks around 2800-3000 cm^{-1} which represents symmetric and asymmetric stretching modes of C-H bonds in CH_2 and CH_3 groups in the alkyl chains. The absorption vibration peak at 1734 cm^{-1} in spectra of all liposomal formulations indicates C=O stretching band originates from P90G (Figure S3, supplementary). However, the intensity of this peak (relative to the neighboring at 1630 cm^{-1}) changes from one spectrum to another (marked by circles in Figure 5) possible due to the formation of a hydrogen bond between -OH groups (present in all compounds) and the carbonyl groups of the lipid. According to the literature, the relative intensity of the C=O bands is the key parameter to monitor since a change in this value indicates a change in relative population of free and hydrogen bonded C=O groups (Arsov and Quaroni, 2007; Chen and Tripp, 2012). Namely, cholesterol is incorporated into phospholipid bilayers in such way that its small hydrophilic 3β-hydroxyl head group is located in the vicinity of the lipid ester carbonyl groups and the hydrophobic steroid ring orients itself parallel to the acyl chains of the lipid. The peak at 1630 cm^{-1} can be ascribed to the C=C stretching vibrations, also present in spectra of the raw phospholipid (Figure S3, Supplementary) and histidine (Figure S2, Supplementary), but also to C=N stretching in FA (Figure S1, Supplementary). Methylene deformation band (δCH_2) at 1464 cm^{-1} can be ascribed to fatty acid chains of phospholipid and SPAN molecules and to sterol molecules as well (Figures S3-S5, Supplementary). The peak at 1416 cm^{-1} (control LIP-HIS and LIP-HIS-FA) or at 1410 cm^{-1} (LIP-CHOL-HIS-FA and LIP-SPAN 20-HIS-FA) can be ascribed to NH_2 bending vibrations of both, FA and histidine (Figures S1-S2, Supplementary). The stretching region of the PO_2^- groups of phospholipids was found at 1140-1250 cm^{-1} (observed also in the spectrum of raw phospholipids, Figure S3, Supplementary) and it seems to be insensitive to the presence of either bilayer modifiers or FA. According to Cieslik-Boczula et al. (2009) this particular band is most sensitive to the state of hydration of the phospholipid bilayer. A P-O-C stretching band was recorded at 1060 cm^{-1} in all spectra of liposomes (also in spectrum of the raw phospholipids, Figure S3, Supplementary) but the ring deformation of cholesterol also appeared at the same wavelength (Figure S4, Supplementary). The two peaks (3008 and 1338 cm^{-1}) in all spectra in Figure 5 indicate the presence of histidine (Figure S2, Supplementary). When comparing the spectra of liposomes and physical mixture, the alterations is noticed in the region 965-919 cm^{-1} (marked by circles in Figure 5), assigned to C-C=O stretching of phospholipids (Figure S3, Supplementary) and to $CH_2=CH_2$ of histidine (Figure S2, Supplementary). These changes suggest possible interactions between head group of phospholipids and histidine, likely via its imidazole ring (highly polar) or α-amino group. When comparing spectra of different liposomal formulations of FA, a divergence is visible between 623 cm^{-1} (assigned to O-CO-C of phospholipids) and 535 cm^{-1} (due to O=C=O rocking

(Lambert et al., 1987) in histidine), appearing as a new small peak between the two, clearly recognizable in spectra of LIP-CHOL-HIS-FA and LIP-SPAN 20-HIS-FA (marked by circles in Figure 5). At this point it is rather difficult to state with certainty about nature of these interactions, but they seem to be more intensive in a more tightly packed bilayer. In the spectrum of LIP-SPAN 20-HIS-FA there is a peak at 3300 cm^{-1} which indicates the N-H stretching characteristic for SPAN molecule (Figure S5, Supplementary).
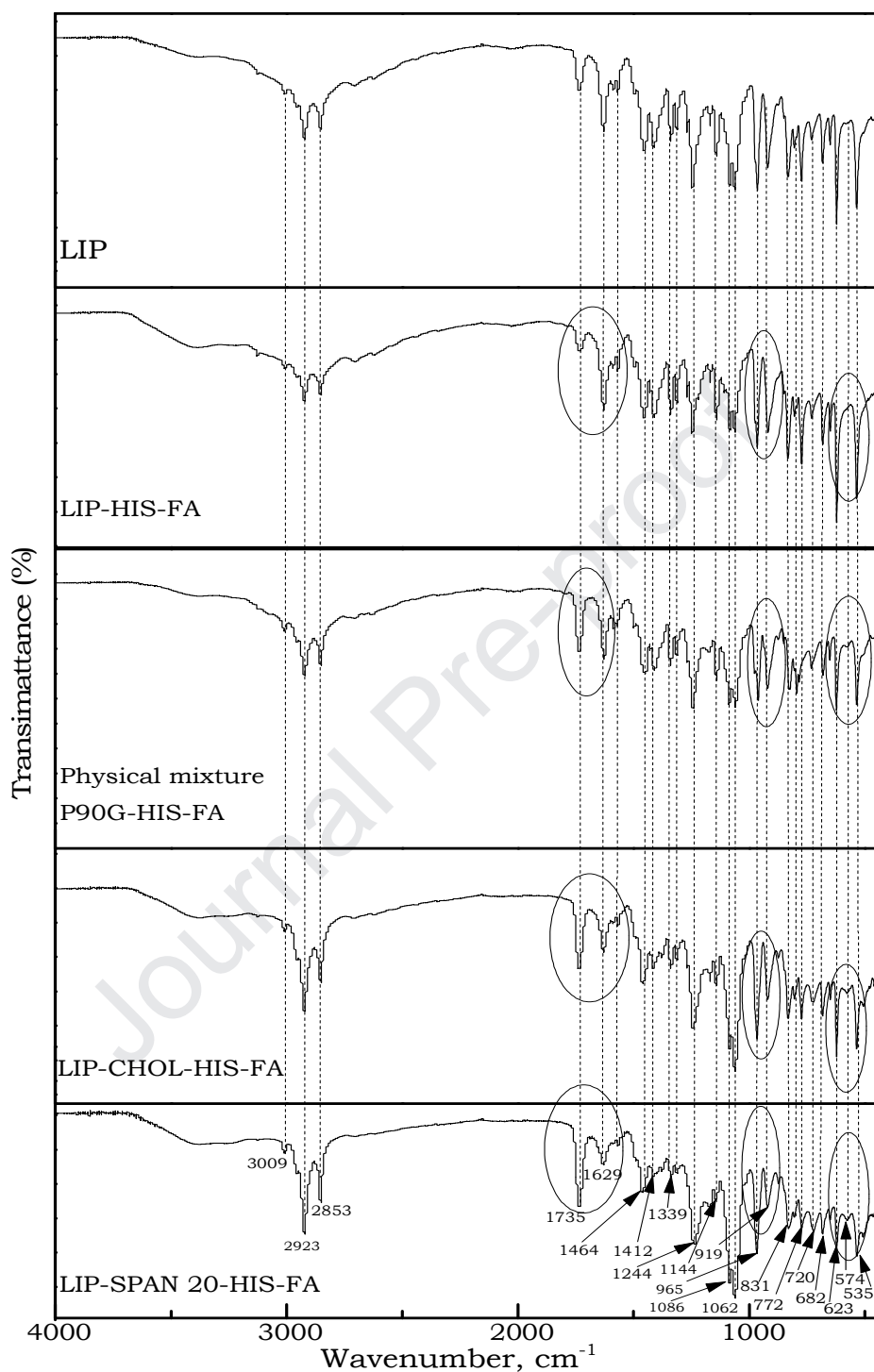
Figure 5. ATR-IR spectra of the physical mixture (Phospholipon 90G, histidine and folic acid) and liposomal formulations – control liposomes (LIP-HIS), folic acid-loaded liposomes (LIP-HIS-FA), folic acid-loaded liposomes modified with cholesterol (LIP-CHOL-HIS-FA) and folic acid-loaded liposomes modified with SPAN 20 (LIP-SPAN 20-HIS-FA)

3.5. DSC analysis

Figure 6. presents DSC curves of pure folic acid, histidine, plain PC liposomes, PC liposomes containing histidine, and FA-loaded liposomes. Thermal degradation of FA occurs in three steps, as shown by three endothermic events, at 154, 200 and 260 °C. According to literature, the first one is accounted for glutamic acid moiety breaking away, followed by the degradation of pterin and p-aminobenzoic acid (PABA) moieties (Gazzali et al., 2016).
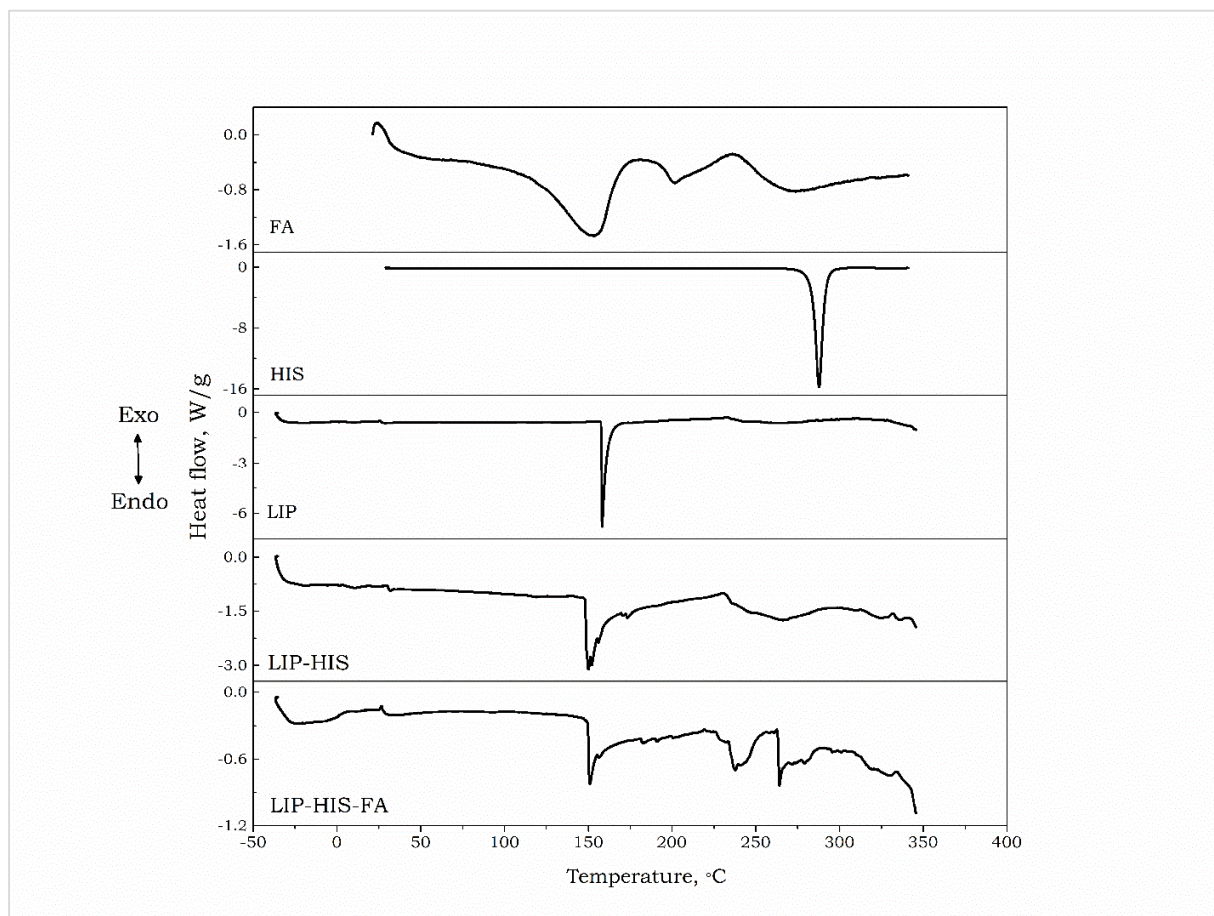
Figure 6. DSC thermograms for pure folic acid (FA), histidine (HIS), plain liposomes (LIP), liposomes containing histidine (LIP-HIS) and folic acid-loaded liposomes (LIP-HIS-FA)

At 200 °C, the amide and acid functionalities were totally lost and the crystalline folic acid became amorphous. The third DSC endotherm at 260 °C has been associated with the mass loss, while the first two DSC endothermic reactions occur without a mass loss (Vora et al., 2002). In the DSC curve of histidine, a single-sharp peak is recorded at 288 °C corresponding to its crystalline melting and decomposition of histidine (Neacsu et al., 2018). The thermograms of plain liposomes (LIP) and liposomes containing histidine (LIP-HIS) showed

two endothermic events, one centered at ~160 °C and degradation above 230 °C which is in accordance to literature data on DSC heating curve for Phospholipon 90G (Khurana et al., 2016). Compared to that, FA-loaded liposomes containing histidine (LIP-HIS-FA) show three endothermic events, which can be ascribed to degradation of both, phospholipids and folic acid. The second thermal event is shifted toward higher temperatures compared to that of folic acid, which is an indication that interactions occurred between folic acid and phosphatidylcholine, simultaneously implicating to higher thermal stability of PABA moiety. Furthermore, complete absence of melting point for histidine can be observed and this is a proof that histidine is in an amorphous state in the liposomes.

3.6. Release study

Folic acid release study was carried out using a Franz diffusion cell in phosphate buffer at two different pH values, 7 and 5.5 and the concentration of FA was measured by HPLC analysis. The release curves of LIP-HIS-FA, LIP-CHOL-HIS-FA and LIP-SPAN 20-HIS-FA are compared with the curves of free FA diffusion and presented in Figure 7.

The rate of FA diffusion and the extent of release from liposomes were affected by pH value which is expected since the vitamin solubility increases with pH (Wu et al., 2010; Younis et al., 2008). The release profiles indicate the sustained FA release from liposomes in a common drug release manner; after 300 minutes of incubation at pH 7, the amount of FA in the acceptor compartment was about 30% reduced due to liposomal barrier properties. Regarding the impact cholesterol and SPAN 20, they both contributed to a faster release compared to plain liposomes. Surface modified liposomes in most occasions provide higher diffusion resistance than un-modified liposomes and sorbitan monolaurate, as having the short saturated hydrophobic hydrocarbon chain, is especially effective in this role due to the small size of molecule and the high hydrophilic character (HLB value of 8.6). However, this kind of action failed to happen in our experiments. In fact, the release rate of FA was significantly higher from the formulation with SPAN 20. This result might arise from free surfactant molecules present at some concentration in the bulk solution which may enhance FA permeability through a hydrophilic acetate cellulose membrane of Franz diffusion cell. Ita et al. (2007) have dealt with statistically insignificant effect of surfactants on permeation of three low-soluble drug compounds (methotrexate, aciclovir and idoxuridine) from liposomes (Phospholipon 90G) across human epidermal membrane. Regarding the impact of cholesterol, it is known that it reduces permeability of phospholipid bilayer above Tm, but there are also experimental evidences in a number of publications showing the independence of drug release on cholesterol content or even the opposite phenomenon. Briuglia et al. (2015) have shown that cholesterol contributes to increased release rate of a hydrophilic drug from DPPC, DMPC and

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DSPC liposomes and the opposite conclusion was derived with a hydrophobic model drug. We assume that upon decreasing pH to 5.5, the histidine form with the positively charged imidazole ring bearing two NH bonds becomes dominant which emphasizes attraction with folic acid, so that release of folic acid may be prompted with the leakage of histidine.
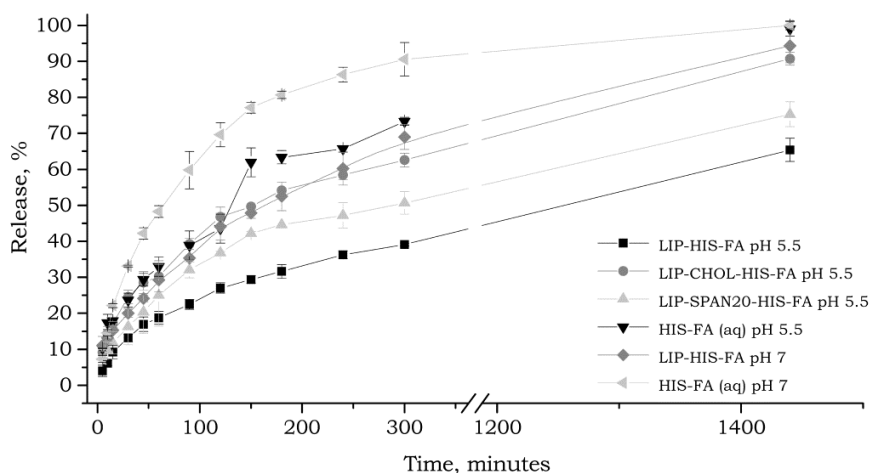

Figure 7. The diffusion release profiles of folic acid from the solution (FA(aq)) and liposome formulations at pH 5.5 and 7.0

4. Conclusions

Encapsulation of folic acid into liposomes was successfully done and drug loading of 0.123 mg g^{-1} was accomplished. The light scattering technique shows that molecules of cholesterol and folic acid lead to increase the size of MLV liposomes, while the SPAN 20 reduces their size. According to AFM measurements, folic acid loaded liposomes of a fraction with a nano size were flattened in contrast to globular empty liposomes. The absorption in FTIR spectra in the wavelength region 965-919 cm^{-1} and 623-535 cm^{-1} showed the interactions between histidine and liposomes. Liposomes provide the effect of prolonged release of folic acid, but cholesterol and SPAN 20 do not contribute to this effect. Recommendations for further research are modification of the liposomal systems by heteropolysaccharides, which would improve their performance of drug transfer through to the skin and control release in gastro-intestinal tract.
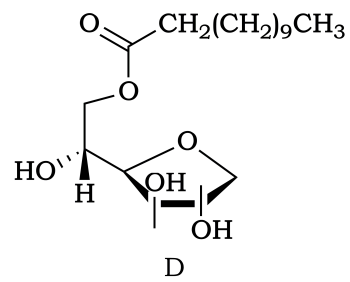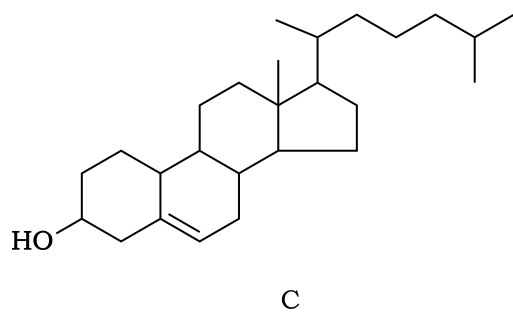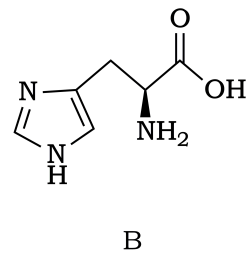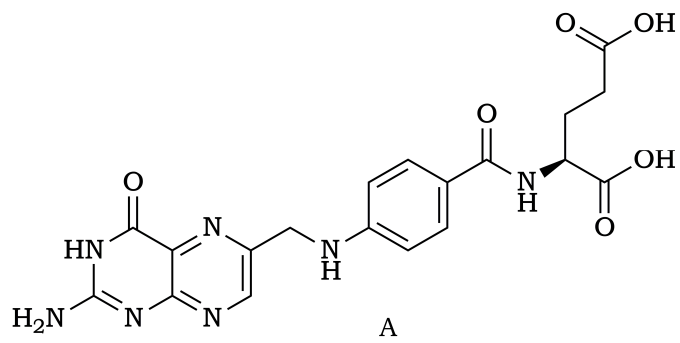
Acknowledgment

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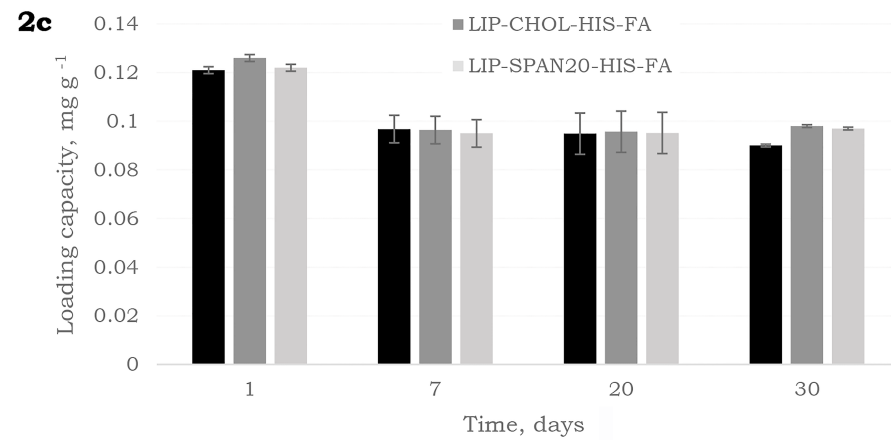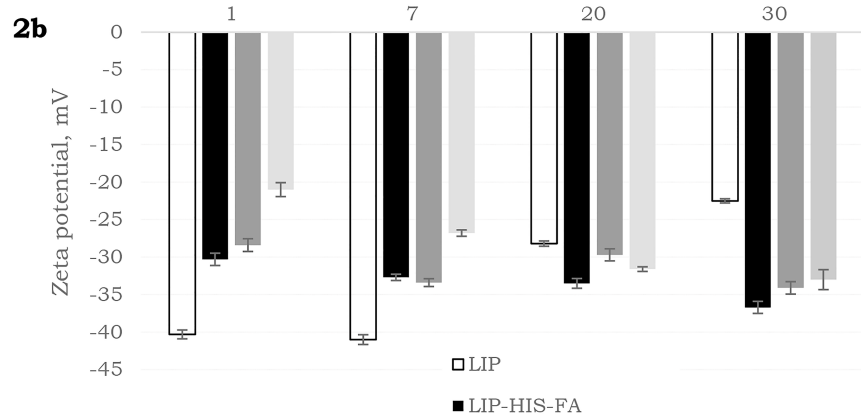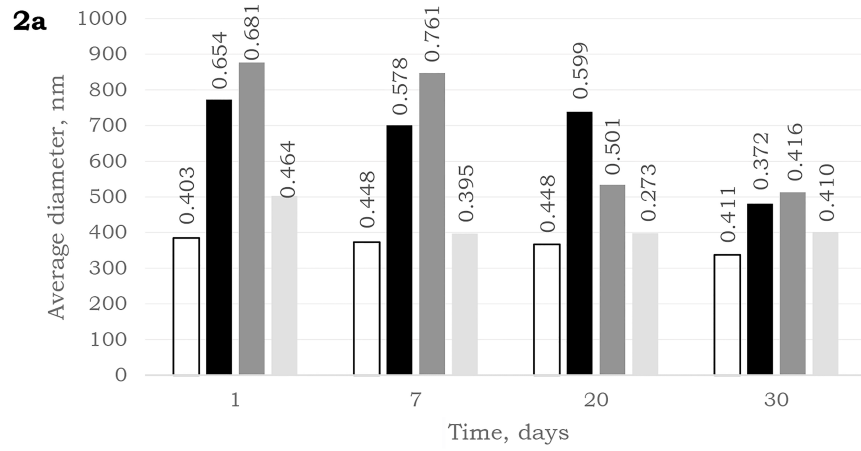
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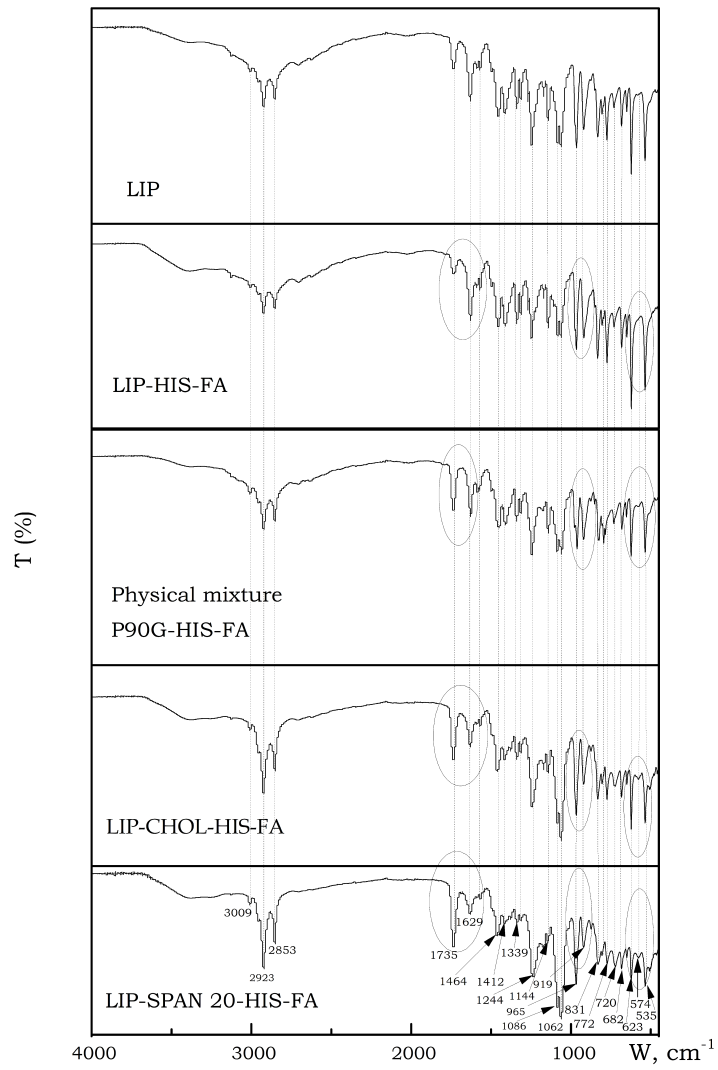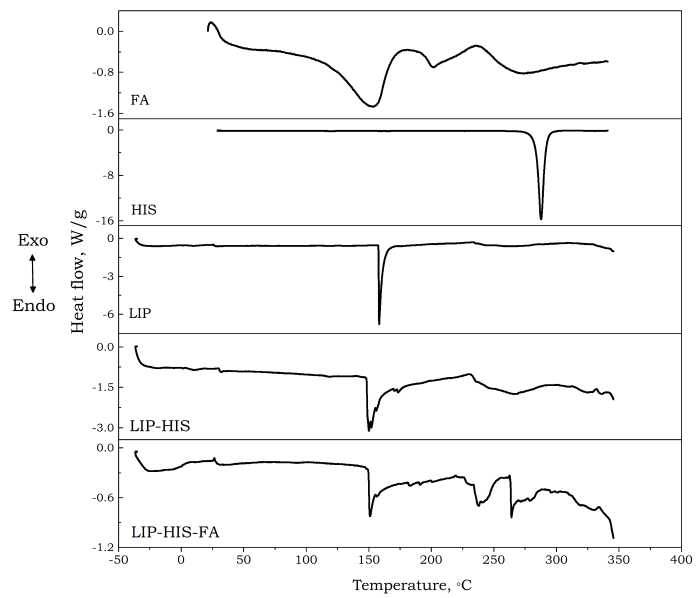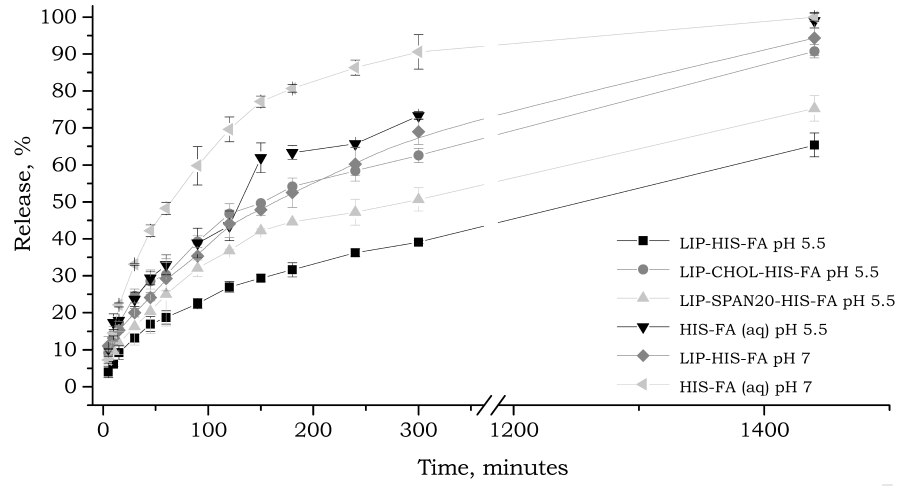
A

B

C

D

2a

2b

2c

Legend:
- ■ LIP-HIS-FA pH 5.5
- ● LIP-CHOL-HIS-FA pH 5.5
- ▲ LIP-SPAN20-HIS-FA pH 5.5
- ▼ HIS-FA (aq) pH 5.5
- ◆ LIP-HIS-FA pH 7
- ◀ HIS-FA (aq) pH 7

Highlights

- Liposomes were used as a model system for encapsulation of folic acid.
- A basic amino acid-histidine was used as a solubilizing agent for folic acid.
- Interactions between folic acid and phospholipids were determined by DSC analysis.
- Interactions between histidine and phospholipids were analyzed by ATR-IR.
- Cholesterol and SPAN 20 did not contribute to the effect of prolonged release.

Conflict of Interest

The authors have declared no conflict of interest

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