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Polymer-lipid matrice based on carboxymethyl cellulose/solagum and liposomes for controlled release of folic acid

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Summary

Liposome-encapsulated folic acid was incorporated into the films made from sodium carboxymethyl cellulose (CMC) (2 mas%) and a mixture of carboxymethyl cellulose and solagum (9:1 w/w) using the film-forming cast solution method. Histidine was used to increase solubility for folic acid in liposomes (1-5 mg/ml), and propylene glycol was used as a film plasticizer (2.6 mas%). The obtained films (50-60 μm tick) containing 3.12-20.19 mg of folic acid per gram of film are envisaged to be used as patches for transdermal delivery of folic acid. Therefore, some physical, mechanical, release and structural attributes of the films were scrutinized. Folic acid gave yellow color to the films and contributed to stronger chemical bonds which resulted in improved strength of the film. Liposomes prolonged the release of folic acid from films to 24 h without adverse effects on mechanical properties of the films, but degraded homogeneity of the films, which could be ascribed to its agglomeration within the film matrix as revealed by AFM. According to the release at pH 5.5, the film formulation based on a blend of CMC and solagum containing 3 mg/ml liposome-encapsulated folic acid is recommended from the point of view of release kinetics determined by its solubility.

Practical application: Folic acid is effective in reducing oxidative stress levels in the skin and neutralizing the harmful free radicals and is also essential for various metabolic reactions in the body. However, the limited solubility of folic acid linked with its poor absorption in an organism, low storage stability, short half-life upon oral consumption, specific food preferences of some people, extensive liver metabolism, and pregnancy-induced vomiting point to a large potential in transdermal usage of folic acid. This has motivated us to design new multicomponent polymer-lipid systems as an alternative solution to overcome some of these drawbacks. The results obtained for these multicomponent films pointed to their potential for prolonged release of folic acid to 24 h, which can also be useful for scientists

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interested in encapsulating similar poorly soluble compounds in CMC patches. The finding can be also valuable information for pharmaceutical manufacturers and scientists worldwide.

Keywords: biopolymer films, structural analysis, thermal stability, polymer-lipid interactions, study of controlled release.

Introduction

Folic acid is a vitamin important for a range of functions in the body. This vitamin plays a significant role in DNA synthesis and prevents skin changes induces by UV radiation.[1,2] Depending on the concentrations it improves the viability of the primary human fibroblast and stimulates its proliferation.[3] Furthermore, folic acid is effective in reducing oxidative stress levels in the skin and neutralizing the harmful free radicals that are present in the environment.[4] Also, this vitamin is essential for various metabolic reactions in the body and its deficiency is associated with significant reproductive risks such as fetal structural defects and infertility.

Folate fortification programs are run in various countries, and these mainly imply oral consumption of folic acid. However, there is a need to develop transdermal formulations of folic acid. The limited solubility of folic acid, [5] linked with its poor absorption in an organism, low storage stability at room temperature,[6] short half-life (about 1,53±0,46 h) upon oral consumption,[7] specific food preferences of some people, extensive liver metabolism, and pregnancy-induced vomiting all point to a large potential in transdermal usage of folic acid. Different technologies have been proposed for delivering nutrients systemically through the skin. One is to use liposomes, as these vesicles are biocompatible, safe (non-toxic and non-immunogenic), and fully biodegradable.

However, transdermal strategies using liposomes have some limitations, such as low stability of liposomes and low transdermal permeability. To overcome these drawbacks, different excipients/additives are applied to liposomal dispersions, such as gelling agents, co-solvents, penetration enhancers, surfactants, etc.[8-10] An example related to folic acid is a cosmetic base baring nanosized liposomes and this system has been proved to be highly efficient in folic acid delivery through the skin: 11-fold increase in plasma folate in rats within 2 h, significantly higher plasma levels compared to oral delivery and safety after 4 weeks daily application even with 750-fold higher doses.[11] Another interesting approach to modify the complex barrier nature of the skin is to use a penetration enhancer biopolymer. Sodium carboxymethyl cellulose (CMC), which has excellent mucosa-adhesive strength,[12] can be used as such a polymer. This polysaccharide is applied as a water-soluble, bioadhesive, biocompatible, nontoxic, and hypoallergenic excipient with a GRAS status. Alone or as a blend or cross-linked with other materials, this polymer has been evaluated in many scientific reports for the fabrication of various extended-release dosage forms (patches, films, fibers, gauzes, microneedles) for skin delivery of bioactive molecules (antibiotics, anticancer drugs, antibacterial agents) and some of the papers recently dated affirm the state-of-the-art of CMC application.[13-23]

Various approaches are taken to improve the mechanical properties of CMC by using different organic and inorganic materials.[24] Blending CMC with gums has been applied to improve some of the film properties. The addition of Arabic gum decreased the water vapor permeability, light transmission and improved the thermal film properties.[25] In respect of controlled drug release behavior, xanthan gum matrices displayed some important pharmaceutical advantages over cellulose matrices, in specific, over hydroxypropyl methylcelluloses, such as higher drug-retarding capacity, absence of initial burst release, and the possibility of zero-order release kinetics.[26, 27] Additionally, xanthan is used as a

thickening ingredient that provides creams and lotions with nice skin feel during and after treatment. These findings have motivated us to explore the possibility of improving CMC film properties by blending with solagum (xanthan gum coated with Arabic gum). Precisely, we developed CMC and CMC-solagum complex films (containing propylene glycol), which incorporate folic acid that is initially encapsulated in liposomes, and these films are envisaged to be used as patches for transdermal application.

Based on the above-stated background, in this study, new CMC films embedding liposomes with folic acid are synthetized by relying on the folic acid-loaded liposomes recently developed [28] in which basic amino acid, histidine, was used as a solubilizing agent for folic acid. Herein, the new liposome in film systems are characterized by Fourier transform infrared analysis (FTIR), atomic force microscopy (AFM), and differential scanning calorimetry (DSC); in addition, mechanical properties, and release at pH 5.5 were also investigated.

2. Materials and methods

2.1. Standards and reagents

Folic acid (FA), carboxymethyl cellulose sodium salt (medium viscosity, 400-800 cP, a molar mass of 250 kDa, approximately, degree of substitution between 0.65-0.9), phosphate salts for phosphate buffer preparation were purchased from Sigma Aldrich, Germany. Solagum® - AX (SG) (a combination of gum acacia (*Acacia Senegal*) and xanthan gum) was supplied by Seppic, Germany. Essential amino acid L-histidine (HIS) and sodium hydroxide were purchased from Sigma Aldrich, USA. Phospholipon 90 G (P90G), (phosphatidylcholine 94-102%; lysophosphatidylcholine 4%, alpha-tocopherol 0.3%, stabilized with ascorbyl palmitate) was purchased from the Phospholipid GmBH, Germany. Acetonitrile, methanol,

and acetic acid, HPLC grade, were obtained from Chem-Lab NV, Belgium. Propylene glycol was purchased from Acros Organic, USA.

2.2. Methods

2.2.1. Preparation of liposomes

Liposomes were prepared using the proliposome method developed by Perret et al.[29] with some modifications. P90G (0.4 g), ethyl alcohol (0.4 g), and deionised water (0.8 ml) were stirred at 600 rpm in an open vessel for five minutes at 60 °C; during this step the lipids dissolved and the solvent was mostly removed. This lipid phase was left to cool to room temperature. Meanwhile, a solution containing HIS (8 mg/ml) and optionally folic acid (FA) at three different concentrations of FA (1,3 or 5 mg/ml) was prepared as the aqueous phase. A small amount (0.5 vol%) of NaOH (0.1 mol/l) was added to the aqueous solution to improve dissolution. The hydration step was performed by gradually adding 18.8 ml of an aqueous solution to the previously prepared lipid phase at room temperature (20 °C) with stirring at 800 rpm for 60 minutes. This procedure was used for the preparation of liposomes with folic acid (LIP-FA), as well as for the preparation of empty liposomes (LIP), which were used as control. The concentrations of FA are noted in the subscript of each sample name. The obtained liposomal formulations were purified by centrifugation at Optima XPM-100 ultracentrifuge (Beckman Coulter USA) in three cycles for 10 min at 40 000 rpm.

2.2.2. Preparation of liposome-loaded films

CMC (0.4 g) and propylene glycol (0.52 g) were added to a liposome suspension (20 ml) and mixed using a magnetic stirrer at 500 rpm for 24 h at room temperature. The obtained blend was poured out into Petri dishes and dried in a laboratory oven (Emmeret UN 160, Emmeret GmbH + Co. KG, Germany) at 50 ºC for 24 hours. Finally, the biopolymer films were

conditioned for three days in a desiccator containing magnesium sulphate, at room temperature. In this way, carboxymethyl cellulose films embedding liposomes with three different concentrations of folic acid (CMC-LIP-FA), 1, 3, and 5 mg/ml were prepared, denoted as CMC-LIP-FA$_1$, CMC-LIP-FA$_3$ and CMC-LIP-FA$_5$, respectively. The film type CMC-SG-LIP-FA$_x$ (with the x number is subscript expressing FA concentration in liposomes) was made by the same procedure but using a polymer blend consisting of 0.36 g CMC mixed with 0.04 g SG. This ratio (9:1) between two polymers is the most suitable and gives consistent, transparent and fairly strong biopolymer films which can easily be manipulated and tested. The control samples free of liposomes were prepared similarly when CMC, propylene glycol, and SG were added to HIS aqueous solution (8 mg/ml) of FA obtaining CMC-FA and CMC-SG-FA film samples. The pure CMC film is prepared by dissolving 0.4 g of CMC in 20 ml of deionized water. The dried films were peeled off from the glass plate and prepared for characterization. The content of folic acid in the final formulations was calculated taking into account the weight of folic acid added to the formulation and the total weight of the film after drying. The drug content was expressed as mass of folic acid per mass of the film after drying.

2.2.3. Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopy analysis was used for the characterization of the chemical interactions between film components. The FTIR spectra were recorded in the range of 400 to 4000 cm^{-1} using a Nicolet iS10 ATR-IR spectrometer (Thermo Scientific, Sweden) with a scanning resolution of 1 cm^{-1}. Biopolymer films were cut into small plates (1x1 cm) for the FTIR measurements and fixed on the Diamond chassis.

2.2.4. Atomic force microscopy (AFM)

The surface morphology of the folic acid-loaded liposomes incorporated in the film matrix was investigated by atomic force spectroscopy (AFM) with NanoScope 3D (Veeco, USA) microscope. AFM images were obtained in tapping mode under ambient conditions. Etched silicone probes with a spring constant of 20-80 N/m were used for testing. Before the morphological analysis, the samples of biopolymer films containing folic acid-loaded liposomes were cut into small plates (1x1 cm) and deposited on a polished mica substrate. After that, the AFM analysis was performed.

2.2.5. Differential scanning calorimetry (DSC)

DSC thermograms were recorded on a differential scanning calorimeter DSC131 Evo (SETARAM Instrumentation, Caluire-et-Cuire, France). The sealed pan with samples was placed in a DSC chamber, against an empty one used as a standard, and heated at a temperature range of -50 to 350 °C. The heating rate was constant, about 10 °C/min, while the flow of N_2 was 20 ml/min. The obtained data were processed using Calisto thermal analysis software.

2.2.6. Determination of film mechanical properties

The tensile strength (TS, MPa), break force (BF, N), and elongation at break (EB, %) of the films were analyzed using a Universal Testing Machine (Shimadzu Corporation, Kyoto, Japan) equipped with a load cell of 100 N (Fig. 1). The rectangular film strips (25x80 mm) were stretched using stainless steel grips at 10 mm/min crosshead speed. The gage length was set at 50 mm. Measurements were performed in triplicates. The mechanical properties of the films were determined from engineering stress-strain and force-displacement curves at the breaking point. The film thickness was measured by a digital nonius depth

caliper (0-Industrial&Scientific, Pittsburgh, USA, 0-150 mm), while the film weight was measured on the analytical balance (Mettler, USA, Type AE 200; ±0.0001 g).
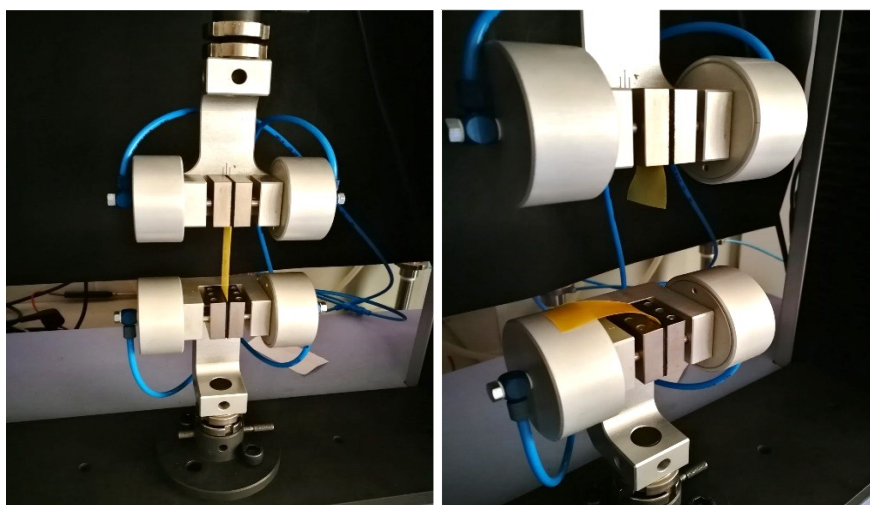

Figure 1. Tensile testing of the CMC-based films using Universal Testing Machine; sample between the grips before testing (left) and after the fracture (right)

2.2.8. Release study

Studies of controlled release of folic acid from liposome-loaded films were performed using the Franz diffusion cell (PermGear, Inc, Hellertown, USA). Franz cell is made of two compartments, the donor and the acceptor, separated by a cellulose acetate membrane (diffusion area: 4.91 cm^2, pore size of 0.2 μm). The films were placed in the donor compartment (~0.05 g, d=2.5 cm). The receptor compartment was filled with release medium (phosphate buffer, pH = 5.5, c = 0.1 mol/l), and constantly mixed at 280 rpm using magnetic stirring. The samples were taken in appropriate time intervals for 24 h hours. For the quantification of folic acid in these samples, Waters ACQUITY ultra-performance liquid chromatography (UPLC) system coupled with a UV detector controlled by Empower software was used. The system was equipped with an ACQUITY UPLC BEH C$_{18}$ column with the dimensions 1.7 μm, 100 mm x 2.1 mm (Waters). The analysis of folic acid was

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performed under isocratic conditions with a mobile phase consisting of 5% acetic acid in water (5:95) and methanol (v/v). The eluent flow rate was 0.2 ml/min and the injection volume was 6 µl. Quantification of folic acid was determined on the wavelength of 285 nm.

2.2.9. Statistics

The data presented in the text are the mean values of two or three independent experimental measurements. The statistical analysis was performed by using the analysis of variance (one-way or two-way ANOVA) followed by Duncan's post-hoc test within the statistical software, STATISTICA 7.0. The differences were considered statistically significant at $p < 0.05$, $n = 3$. Also, the data in table and diagrams are presented as mean ± standard deviation.

3. Results and Discussion

Folic acid was encapsulated in liposomes in three different concentrations (1, 3 and 5 mg/ml) with high encapsulation effeicency of ~84%. The obtained liposomes had an average size of ~220 nm and zeta potential values of -~30 mV, irrespectively of the folic acid content, and were stable over a one month period of storage at 4 °C in the presence of propylen glycol (Supplementary). Such liposomes were used for obtaining films based on CMC and blend of CMC and SG.

3.1. Physical characteristics

All the CMC-based patches were found to be smooth and flexible while transparency changed depending on the formulations (Fig. 2), thus as the CMC blank sample was the only one completely transparent. Photos of CMC-LIP formulations show that liposomes have been inhomogeneously distributed within the polymer matrix and the presence of liposomes has

The thickness values ranged between 50 and 60 µm (Table 1), although the volume for each
film forming solution cast on each Petri dish was cautiously controlled during the casting
process to provide the same thickness. As a consequence of different concentrations of folic
acid used for preparations and different content of bonded water in different formulations,
drug loading expressed as the content of folic acid per mass unit of the film also differs, in
the range from 3.1 to 20.2 mg/g (Table 1). When analyzing CMC-LIP-FA$_1$ vs. CMC-LIP-FA$_5$
folic acid appeared to increase film compactness so that the increase in drug content is not
proportional to the increase in FA content used for samples preparation. Drying kinetics,
expressed as water loss (relative to initial water content) over time, confirmed that folic acid
boosted water loss in samples (Supplementary). Similarly, the film containing solagum has
increased drug load compared to the film made solely from CMC, although both formulations
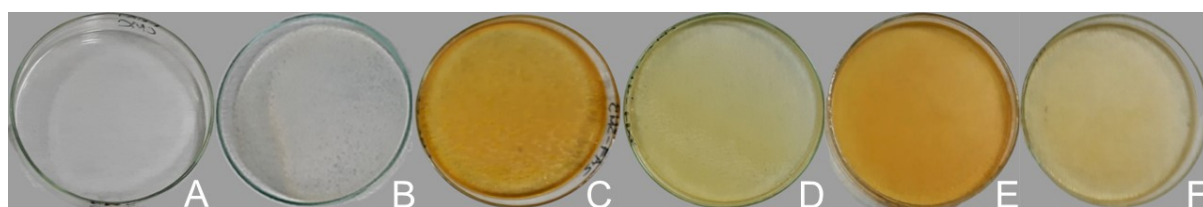have been prepared with the same solid content.

Figure 2. Photographs of the patches: A. blank carboxymethyl cellulose film (CMC); B.
carboxymethyl cellulose film with embedded blank liposomes (CMC-LIP); C. carboxymethyl
cellulose film with folic acid in concentration of 5 mg/ml (CMC-FA$_5$); D. carboxymethyl
cellulose film with embedded liposome-loaded folic acid in concentration 1 mg/ml (CMC-
LIP-FA$_1$); E. carboxymethyl cellulose film with embedded liposome-loaded folic acid in
concentration 5 mg/ml (CMC-LIP-FA$_5$); F. carboxymethyl cellulose-solagum film with
embedded liposome-loaded folic acid in concentration 5 mg/ml (CMC-SG-LIP-FA$_5$).

3.2. Mechanical properties

Mechanical properties, tensile strength (TS), break force (BF), and elongation at break (EB) of the CMC films are summarized in Table 1.

Folic acid made the CMC film stronger and more flexible (CMC vs. CMC-FA$_5$ and CMC-LIP-FA$_1$ vs. CMC-LIP-FA$_5$) due to the molecular interactions between folic acid, histidine and polymer chains, as revealed by FTIR analysis given below. Interestingly, the addition of liposomes did not induce significant changes in the mechanical behavior of the CMC film regarding TS, BS, and EB values. This can be a result of some opposite effects. Namely, liposomes would be expected to disrupt the CMC matrix leading to its weakening. On the other hand, it has been reported that the tensile strength of CMC films was increased by commercial lecithins (mixtures of several phospholipids and fats) when added alone or combined with other surfactants.[30, 31] The explanation was that positively charged choline groups of phospholipids could interact with CMC anion groups, leading to the formation of stronger chemical bonds which resulted in improved strength of the film. Furthermore, Rodríguez et al.[31] claim the synergistic effect between a plasticizer and surfactants (lecithin, tween and span). It is worthy to notice that films containing liposomes expressed a very high variability of values of mechanical parameters (standard deviations between 19% and 48%) pointing to an inhomogeneous structure.

The highest elongation at break (5.07%) was noticed for CMC-FA$_5$ films, possibly due to intermolecular forces between the CMC chains and folic acid. The significance of molecular interactions between the film components on the material flexibility was also noticed by Dayarian et al.[32] The liposome addition in CMC-based films decreased film elongation probably due to interaction between choline groups of phospholipids and CMC anion groups, leading to lower flexibility.

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Comparing CMC with CMC-SG film formulation, no difference was observed, which was surprising. Namely, Arabic[25] and xanthan gums [33] usually reduce the tensile strength of CMC films, and this effect has been explained by a weakening in the intermolecular forces caused by an increase in the OH group. It seems that interactions between many constituents of this complex structure affect the behavior of the films upon stress.

3.3. FTIR analysis

The FTIR spectra of blank liposomes (LIP), folic acid-loaded liposomes (LIP-FA), LIP-FA coated with CMC (CMC-LIP-FA), or with a mixture of CMC and SG (CMC-SG-LIP-FA) are shown in Fig. 3a-d. Also, the supplementary shows the IR spectra of pure compounds (Fig. S4a-f). The coating LIP-FA with CMC or a blend of CMC/SG induced some changes in the intensities or disappearance of some peaks in a few different regions. After coating, the disappearance of peaks at 3542, 3410, and 3319 cm^{-1} which originate from folic acid is noticeable. Also, there are some changes in the intensities of the LIP and LIP-FA at 3010, 2924 and 2850 cm^{-1} comparing to the spectra of CMC-LIP-FA and CMC-SG-LIP-FA (Fig. 3a-d). The mentioned changes represent =C-H and C-H stretching vibrations in the vinyl, and methyl/methylene groups of the phospholipid molecule and molecule of FA. Comparing the spectra of LIP with the other three spectra (LIP-FA, CMC-LIP-FA, and CMC-SG-LIP-FA), the intensity of the peaks in the region 3010-2850 cm^{-1} is the highest in LIP, followed by the LIP-FA, while between the latest two spectra (CMC-LIP-FA and CMC-SG-LIP-FA) there are no significant differences. The peak at 1736 cm^{-1} observed in the spectra of LIP and LIP-FA can be assigned to the C=O of the ester group from the fatty acids of phospholipids.[34] It disappears in the formulation of CMC-LIP-FA and CMC-SG-LIP-FA, indicating some physical interactions between liposomes and polymer matrix. The bands at 1630 and 1558 cm^{-1} in the spectrum of LIP-FA can be assigned to the coupling of NH bending and C-N stretching of amide I and amide II as well as aromatic skeletal vibration from FA and HIS

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(Fig. 3b; Fig. S4b and c). However, in the spectra of the film formulations, these two bands are united in one band which gives the maximum absorption at 1593 cm^{-1}. The signal at 1593 cm^{-1} in the CMC-LIP-FA and CMC-SG-LIP-FA indicates the presence of OH angular deformation vibrations overlapped with asymmetric stretching of the carboxylate group of pyruvate and glucuronic acid from CMC and SG.[35-37] In the region of 1458 cm^{-1} could be observed some asymmetric stretching vibrations of carboxylic groups of the uronic residues from solagum.[37] The presence of the band at 1417 cm^{-1} (Fig. 3) can be ascribed from two sources: first, from the C-C group of FA and HIS, and second from carboxylic groups of CMC (Fig. S1d). Also, some papers state that vibrations around 1420 cm^{-1} can be attributed to the O-H (in-plane) bending of the phenyl skeleton.[37] In addition, the assignment at 1313 cm^{-1} represents the C-C stretching vibration of the side chain of the histidine backbone and confirms its presence in all the examined analytes. At 1246 cm^{-1} the stretching vibration of C-O, C-O-C, and C-N of the aromatic structure originates from histidine molecule (Fig. S4c), and the P=O stretching from the phospholipid phosphate group (P90G, Fig. S4a). The expressed band at 1086 cm^{-1} in the LIP-FA represents C-O stretching of the carboxylic group, and at the same time a wide band in the CMC-LIP-FA and CMC-SG-LIP-FA, with an absorption maximum at 1052 cm^{-1} represents P-O-C stretching, which originates from HIS and P90G.[38, 39]
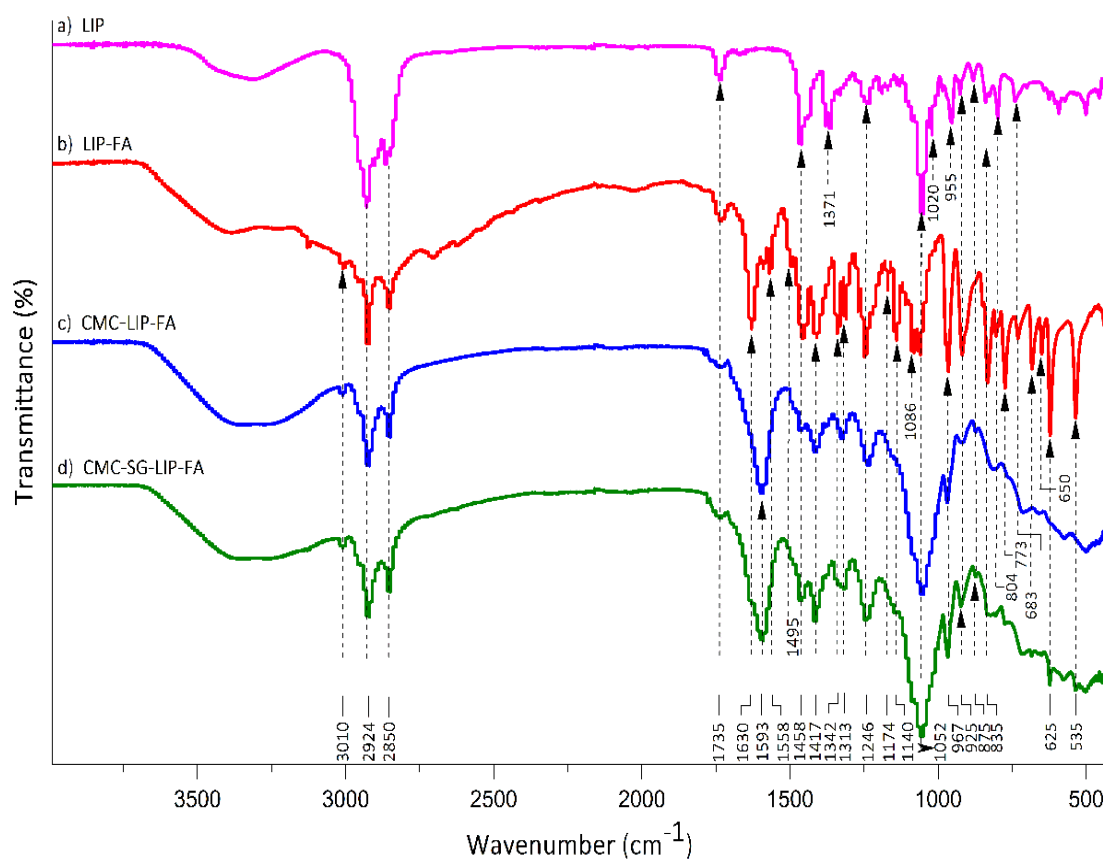
Figure 3. FTIR spectra of (a) blank liposomes – LIP, (b) liposomes loaded with folic acid – LIP-FA, (c) LIP-FA coated with carboxymethyl cellulose - CMC-LIP-FA, and (d) LIP-FA coated with mixture of CMC and solagum – CMC-SG-LIP-FA

3.4. Release study

The release profiles of the FA from four different formulations produced with 3 mg/ml of the initial FA concentration in a phosphate buffer (pH 5.5) at room temperature were monitored for 24 h and presented in Fig. 4A as a released percentage (amount of drug released at time t, m_t, divided by the amount of drug release at equilibrium, m_e, in percentage) over time. In general, at a pH higher than the pKa of carboxylic groups (pKa 4–5), carboxylic acid groups (COO^-) become deprotonated and the electrostatic repulsive forces between negatively charged sites (COO^-) enable swelling of CMC, which facilities FA release. The fraction of FA released increased with time, more rapidly if FA was added as a suspension in polymer

solution forming films (CMC-FA and CMC-SG-FA) than if it was incorporated in films as liposomal formulation (CMC-LIP-FA and CMC-SG-LIP-FA). The liposomes-in-polymer system sustained FA release for 24 h while polymer alone for less than 6.5 h. Our results confirmed the key role of liposomes in increasing diffusional resistance and holding FA in the CMC and this coincides with earlier papers on liposomes-in-film formulations for the delivery of active ingredients.[40] Solagum also contributed to the diffusional resistance of the films, and the CMC-SG-LIP-FA sample exhibited the slowest release of folic acid.
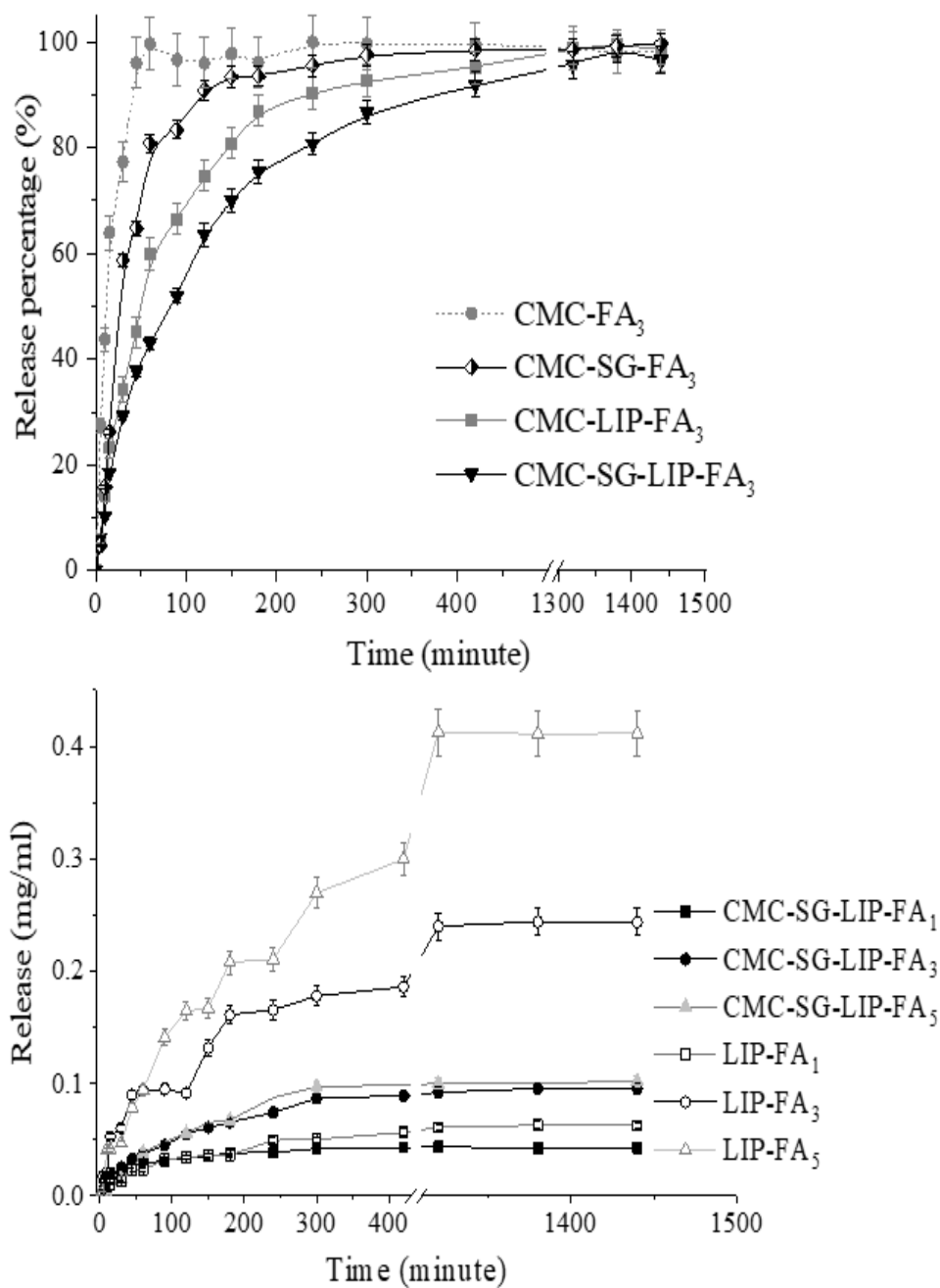
Figure 4. Release kinetics of folic acid from different film formulations: A. containing 3 mg/ml of folic acid; one-way ANOVA was performed to test for differences among the samples; B. CMC-SG-LIP film and LIP-FA formulations containing different concentrations of folic acid (1, 3 and 5 mg/ml); two-way ANOVA was performed to test for differences among the group samples: 1) samples with the same FA concentration with vs. without film

matrix and 2) samples of the same type of coating (LIP or CMC-SG) with various FA concentrations.

The impact of the FA concentration (1, 3 and 5 mg/ml) on its release from CMC-SG-LIP-FA-based films compared to LIP-FA formulations is illustrated in Fig. 4B. The CMC-SG matrix reduced the absolute drug released concentrations, in case of the samples with 1 mg/ml the difference was significant only starting after 300 min of release ($p \leq 0.05$), while in the case of the samples with 3 and 5 mg/ml the difference was significant from the very beginning. As expected, free liposomes released as more FA as more they contained. However, in case of polymer-lipid samples the absolute drug released concentrations substantially increased with the increase in the initial content from 1 to 3 mg/ml, but a further increase to 5 mg/ml did not cause this effect. It seems that FA solubility in film matrix appeared as a limiting factor.

3.5. Thermal stability

DSC analyses were used to identify thermal events and obtain further information on the interactions between film components. CMC films exhibited several thermal events (Fig. 5). The broad glass transition peak that appeared around 90 ºC significantly diminished in CMC and CMC-SG films as compared to raw sodium carboxymethyl cellulose. Reduced enthalpy of melting of CMC films means less gathering of CMC chains and final structures with lesser crystallinity. A pronounced endothermic peak of CMC films assigned to boiling of propylene glycol (PG) is broadened and shifted to lower temperatures compared to pure propylene glycol (a single sharp endothermic peak in thermogram of pure PG is at 190 ºC), especially in the case of CMC-SG films, indicating interactions between components of the blended film. DSC curves of both, films and raw CMC showed a distinct exothermic event appearing as one main exothermic peak, at around 284 ºC, which can be attributed to thermal degradation of the carboxylate ion and C-O-C ethers of the six-membered backbone chain of CMC.[41]

When liposomes were incorporated in films, endothermic event broadened to a range of 140-200 ºC due to the overlapping of two peaks (from PG and liposomes, the second centered at ~160 °C in plain liposomes shown by Batinić et al.[28] while degradation of phospholipids at ~230 ºC was not observed, as enthalpy change of this event was too low. In order to establish possible interactions between the FA-HIS couple and CMC, a liposomes-free sample was prepared as the CMC film with entrapped FA-HIS (CMC-FA); its thermogram displayed a distinctive endothermic event centered at ~130 ºC which corresponded to that at 154 ºC of pure FA, and accounted for glutamic acid moiety cleavage[28] this shift toward lower temperatures is an indication for interactions between folic acid and CMC, implicating lower thermal stability of glutamic acid moiety. Furthermore, the complete absence of melting point for histidine (at 288 ºC for pure HIS) can be spotted confirming that histidine is in an amorphous state in the CMC-FA-HIS system as it was also in liposomes with entrapped FA-HIS.[28] As regards interactions between phosphatidylcholine and FA upon heating, these are reflected in the temperature range of PABA moiety degradation as shown by Batinić et al.[28]
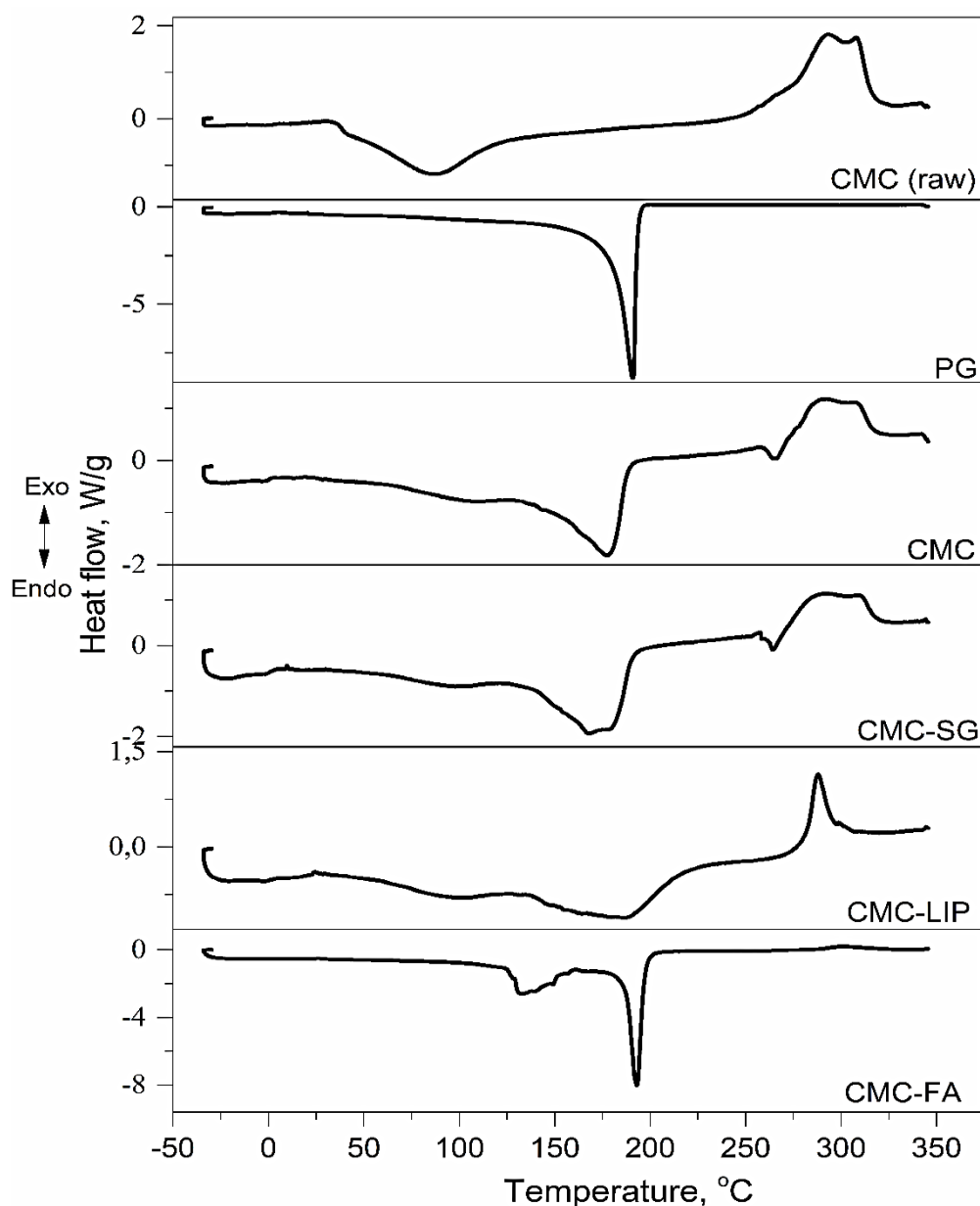
Figure 5. DSC thermograms for raw sodium carboxymethyl cellulose (raw CMC), propylene glycol (PG), carboxymethyl cellulose film (CMC), film made from a blend of carboxymethyl cellulose and solagum (CMC-SG), liposomes-loaded carboxymethyl cellulose film (CMC-LIP), and folic acid-loaded carboxymethyl cellulose film (CMC-FA).

3.6. AFM measurements

AFM images in Fig. 6. display the morphology of the films with integrated FA-containing liposomes. The structure of free (empty) and folic acid-liposomes has been previously documented by Batinić et al.[28] According to them, empty liposomes made from

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Phospholipon 90G by the proliposome method exhibited a regular globular shape while folic acid caused flattening from spherical structure.[28] In this study, the presence of the vesicles in CMC and CMC-SG films is evidenced by figures 6-L and 6-R. Physical stability of liposomes in the presence of propylene glycol as a plasticizer was preserved as confirmed by unchanged size, polydispersity index and zeta potential over a period of one month (Supplementary, Fig. S2). The liposomes embedded in the CMC film keep their integrity and appear as separated particles nicely distributed with an average diameter of 249.3 nm. Unlike those, liposomes in CMC-SG show a higher tendency to fusion and formation of agglomerates.
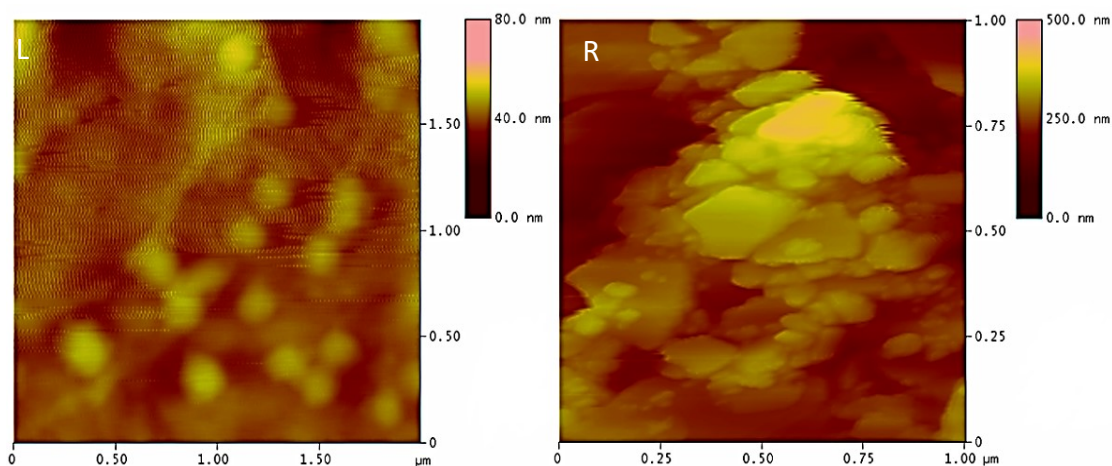

Figure 6. AFM top view image scanning in the tapping mode: LIP-CMC-FA (L), LIP-CMC-SG-FA (R)

Conclusions

The formation of films made from CMC or CMC-SG mixture with incorporated liposome-encapsulated FA has been performed using the film-forming cast solution method. Thin yellow films were obtained with improved strength due to FA presence. The release studies performed at pH 5.5 favor formulation based on a blend of CMC and solagum containing 3 mg/ml liposome-encapsulated folic acid, regarding limited folic acid solubility. The results obtained for these multicomponent films can also be useful for scientists interested in

encapsulating similar poorly soluble compounds in CMC patches. However, biological characterization is necessary to confirm the applicability of the films, including investigation of drug permeation across the skin, retention in the skin, and skin irritancy in ex-vivo and in-vivo experiments.

Acknowledgments

Conflict of Interest

The authors declare no conflict of interest.

Standard Abbreviations: P 90 G – phospholipon 90 G, FA – folic acid, DNA – deoxyribonucleic acid, SD – standard deviation; FTIR – Fourier-transform infrared spectroscopy; DSC – differential scanning calorimetry; AFM - Atomic force microscopy.

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Table 1. Physical and mechanical properties of different types of carboxymethyl cellulose-based films.

Type of sample	Film thickness (mm)	FA content (mg per gram of film)	Tensile strength (TS) (MPa)	Break force (BS), (N)	Elongation (EB), (%)
CMC	0.06	-	4.79±0.36	9.58±0.72	3.31±0.66
CMC-FA$_5$	0.05	20.19	7.19±0.43	8.99±0.53	5.07±0.98
CMC-LIP-FA$_1$	0.06	3.12	5.40±2.61	10.81±3.52	1.37±0.92
CMC-LIP-FA$_5$	0.05	19.54	6.98±1.34	13.97±2.68	0.97±0.30
CMC-SG-LIP-FA$_1$	0.06	6.37	5.41±1.43	10.82±3.36	0.94±0.22

FILM CASTING METHOD
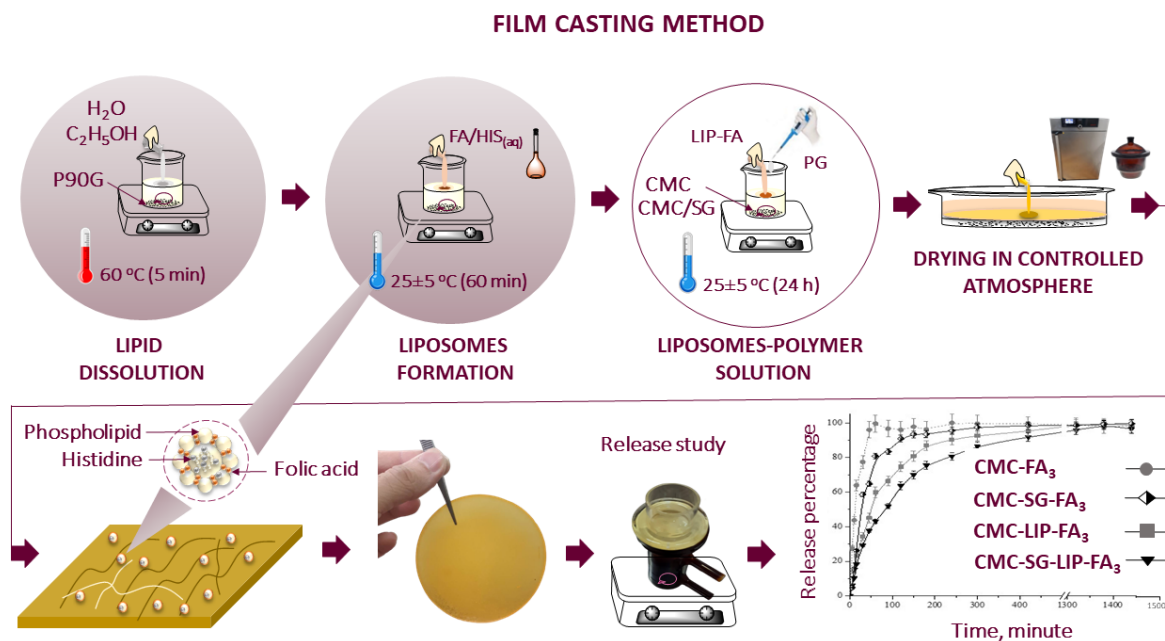
The research's findings provide a thorough understanding of the mechanism of folic acid release from liposomes incorporated into natural polymers. All the formulations under test were created using the film casting technique, and their physical, mechanical, structural, and release characteristics were examined. This study makes a contribution to the field of transdermal administration of active compounds with poor solubility.