PH – OSETLJIVI HIDROGELOVI NA BAZI POLI(METAKRILNE KISELINE), KAZEINA I LIPOZOMA ZA CILJANO OTPUŠTANJE SLABO VODORASTVORNIH AKTIVNIH SUPSTANCI

PH-SENSITIVE HYDROGELS BASED ON POLY(METHACRYLIC ACID), CASEIN AND LIPOSOMES FOR TARGETED DELIVERY OF POORLY WATER-SOLUBLE ACTIVE SUBSTANCES

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Kod razvoja polimernih nosača za ciljano otpuštanje slabo vodorastvornih aktivnih supstanci (SVAS) postoje dva ograničenja. Prvo, inkapsulacija SVAS zavisi od strukture nosača. Drugo ograničenje je vezano za naglo oslobađanje leka. Uprkos činjenici da je naglo oslobađanje leka, tzv. burst efekat poželjno kod nekih sistema, negativni efekti naglog oslobađanja leka u većini slučajeva mogu biti farmakološki opasni i ekonomski neefikasni. U ovom radu je prikazana nova strategija za inkapsulaciju i ciljano otpuštanje SVAS – kofeina bez burst efekta. Hidrofilni nosač na bazi poli(metakrilne kiseline) je modifikovan amfifilnim supstancama – kazeinom i centrifugiranim lipozomima kako bi se postigla inkapsulacija kofeina. Kofein je inkapsuliran u lipozome koji su zatim inkorporirani u mrežu nosača tokom sinteze nosača. Analiziran je uticaj morfologije nosača i interakcija koje su se uspostavile između njegovih komponenti na kinetiku otpuštanja kofeina. Zatim je analiziran uticaj stepen neutralizacije metakrilne kiseline na bubrenje nosača i otpuštanje kofeina u dve sredine različitih pH vrednosti koje su simulirale pH sredinu u gastrointestinalnom traktu čoveka. Pokazano je da sintetisani nosači imaju veliki potencijal za inkapsulaciju i ciljano otpuštanje SVAS.

Ključne reči: *poli(metakrilna kiselina); kazein; lipozomi; slabo vodorastvorne aktivne supstance; ciljano otpuštanje*

Polymeric carriers for targeted delivery of poorly water-soluble active substances (PWSAS) are facing two challenges. First, encapsulation of PWSAS depends on structure of the carrier. Second limitation is caused by the phenomenon of burst drug release. Despite the fact that the fast release of drug in a burst stage is utilized in certain drug administration strategies, the negative effects brought about by burst can be pharmacologically dangerous and economically inefficient. This study presents a novel strategy for encapsulation and targeted delivery of PWSAS - caffeine without the possibility of burst effect. Hydrophilic carrier based on poly(methacylic acid) was modified by amphiphilic substances - casein and centrifuged liposomes to ensure caffeine encapsulation. The caffeine was encapsulated in liposomes which further were incorporated into the carrier networks during the synthesis of the carriers. The release kinetic of caffeine was analyzed with respect to morphology of the carriers and interactions that could be established between the components of the carrier. The swelling of carriers and release of caffeine were further investigated depending on the neutralization degree of methacrylic acid in two media with different pH simulating the path of the carrier through the upper and lower human gastrointestinal tract. Synthesized carriers showed significant potential for encapsulation and targeted delivery of PWSAS.

Key words: *poly(methacrylic acid); casein; liposomes; poorly water-soluble active substances; targeted delivery*

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1 Introduction

Soft polymeric networks based on poly(methacrylic acid) (PMAA) are well known pH sensitive, biocompatible and non-toxic hydrogels which are extensively used in the systems for targeted delivery of active substances. Their hydrophilic nature is derived from the presence of the large number of ionisable carboxylic groups (-COOH) in their polymer network. When the pH value of the external medium is higher than the pKa of methacrylic acid (MAA) (pKa=4.6) ionization of carboxylic groups occurs causing the generation of permanent negative charges along the polymers chains and repulsion of polymeric chains [1]. As a consequence PMAA hydrogel swells and releases encapsulated active substance. When the pH value of the external medium is lower than the pKa value of MAA, PMAA hydrogel collapses. Beside the all favorable characteristics of PMAA hydrogels, these carriers are able to deliver only hydrophilic substances due to their highly hydrophilic nature. In this paper, PMAA was modified with amphiphilc substances protein – casein and centrifuged phospholipidic nanoparticles – liposomes, which enabled encapsulation of poorly water-soluble active substance - caffeine, its prolonged release and better control of caffeine release rate without the possibility of burst effect (sudden release of encapsulated active substance)[2]. Casein is non-toxic, temperature stable and pH sensitive milk protein. Also, the addition of casein improved mechanical characteristic of PMAA [1]. All these characteristics make casein a good candidate for targeted delivery of poorly water-soluble substances. Addition of liposomes in polymer network enables better control of release rate of encapsulated active substance without the possibility of its burst release, because the shell of liposomes presents additional barrier[2]. Centrifugation of liposomes stabilize liposomes particles, so these particles could be uniformly distributed in the hydrogel network during its synthesis and therefore enable better control of caffeine release [2]. In this paper, carriers based on poly(methacrylic acid), casein and centrifuged liposomes with encapsulated caffeine (PMAC/Lc) were synthetized and the interactions between the components of the carrier and morphology of synthetized carriers were investigated in detail. The swelling behavior of PMAC/Lc carriers and caffeine release from the carriers in two media with two different pH values (which simulated human gastrointestinal tract) were investigated depending on the change in the neutralization degree of MAA.

2 Materials and methods

2.1 Materials

Methacrylic acid (MAA) (99.5%) was purchased from Merck (Germany). Sodium caseinate (CSNa) powder, containing 88.9 wt% of protein (the rest being lactose, lipids, attached moisture, and ashes), was supplied from Lactoprot Deutschland GmbH (Germany). NATIPIDE®II containing phospholipids from soybean >20% (with 3-sn-phosphatidylcholine 76+ 3%) was supplied from Lipoid (Germany). N, N'-Methylenebisacrylamide (MBA) (p.a.) and sodium hydroxide (p.a.) (NaOH) were supplied from Aldrich Chemical Co. (USA). The initiator, 2, 2'-azobis-[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) (99.8%) was purchased from Wako Pure Chemical Industries (Japan). Caffeine was purchased from Merck (Germany). Hydrochloric acid (37%) was supplied from Zorka Pharma (Serbia). Monobasic sodium phosphate (anhydrous) (NaH_2PO4) and dibasic sodium phosphate (anhydrous) (Na_2HPO4) were supplied from Centrohem (Serbia). All chemicals were used as received.

2.2 Preparation of PMAC-Lc carriers

PMAC/Lc carriers was synthesized via free-radical polymerization by the adaptation of the procedure that was previously described by M. Markovic et al [2].

Firstly, liposomes with the encapsulated caffeine were prepared by pro-liposomes method [3]. The caffeine solution in distilled water was added to NATIPIDE®II (10wt% with respect to final liposomal formulation) during the constant stirring. Caffeine concentration in prepared liposomal formulation was 20 mg/ml. Prepared liposomal formulation was centrifuged and used for synthesis of PMAC/Lc carriers.

Secondly, the PMAC/Lc carriers were prepared via the following procedure. 4 cm^3 of MAA were dissolved in distilled water and centrifuged liposomes were added drop-wise under continuous stirring at 25°C. The volume ratio of centrifuged liposomes and distilled water were 50:50. The certain amount of aqueous solution of sodium hydroxide was added in synthesis of the samples with certain neutralization degree of MAA (50% and 100%). In the synthesis of the sample with non-neutralized MAA, NaOH was not added. Afterwards, the temperature of the reaction mixture was elevated to 40°C and 4 g of casein was added and dissolved using a magnetic stirrer. After casein dissolution, the crosslinker - MBA (0.4mol% with respect to MAA) was added. The reaction mixture was stirred for 15 minutes and the initiator VA-044 (0.9 cm^3 of 1wt% aqueous solution) was added. The prepared reaction mixtures were instantly poured into the glass moulds (plates, 17x17 cm, separated by a 3 mm thick PVC hose) and left in the air oven at 60°C for 5h. Then, the disc-shaped samples (7 mm in diameter) were cut, dried at 25°C and stored in an exicator.

The carriers were denoted as PMAC/Lc-xN, where xN was the symbol adopted for the neutralization degree of MAA (0%, 50% i 100%).

2.3 Methods

The FT-IR spectra of xerogel disks of the PMAC/Lc carriers were recorded in transmittance mode for the wavelength range of 400–4000 cm^{-1} with a resolution of 4 cm^{-1}, using Nicolet™ iS10 FTIR Spectrometer.

The SEM analyses of the PMAC/Lc carriers were performed using a Tescan MIRA 3 XMU field-emission gun scanning electron microscope (FEG-SEM) with an acceleration voltage of 20 kV. Prior to SEM analysis, the PMAC/Lc carriers swollen to equilibrium were freeze-dried in order to conserve the structure without collapsing. The carriers were then fractured in half in frozen state. Before analysis, cross-sections of the carriers were Au-Pd coated using a POLARON SC502 sputter coater.

The degree of caffeine encapsulattion in liposomes, which were used for preparation of PMAC/Lc carriers, was determined using the UV-Vis Shimadzu UV-1800 spectrophotometer. The supernatant, which was obtained after the liposomal formulation with caffeine was centrifuged, was used for determination caffeine encapsulation degree in liposomes. The caffeine encapsulation degree (ED) in the liposomes (%) was calculated by applying the Eq. 1[2]:

$$ED=((m_{ct} - m_{csn})/m_{ct}) \times 100 \quad (1)$$

In Eq. (1), m_{ct} (g) is the total caffeine weight in the liposomal suspension and m_{csn} is the measured caffeine weight in the supernatant (g).

Swelling measurements were carried out at 37°C in two media during a 24h period: 0.1M hydrochlorid acid (0.1M HCl) and phosphate buffer with pH value of 6.8 (PB 6.8), as a simulation of pH environment of human stomach and intestines, respectively[4]. Dry PMAC/Lc carrier disks with known weight (m_0, g) were entirely immersed in specified medium and left to swell. At predetermined time intervals disks were removed from the medium and weighted (m_t, g) until equilibrium was reached (m_{eq}, g). The swelling measurements were done in triplicate and the mean values are presented in the Results and discussion section. The swelling degree (SD) of the PMAC/Lc carriers was calculated by applying equation (2)[5] and the equilibrium swelling degree (SD_{eq}) of the PMAC/Lc carriers was calculated using equation (3)[6]:

$$SD= (m_t - m_0)/m_0 \quad (2)$$

$$SD_{eq}= (m_{eq} - m_0)/m_0 \quad (3)$$

The controlled caffeine release from the PMAC/Lc carriers was carried at same experimental conditions as was the swelling of the PMAC/Lc carriers. The absorbances of the solutions were measured at predefined time intervals at 273 nm using the UV-Vis Shimadzu UV-1800 spectrophotometer. To provide perfect sink conditions during the caffeine release, each synthesized carrier was put into 100 ml of a prepared media. At predefined time intervals, 3 ml of each solution were withdrawn, analyzed by UV-Vis spectrophotometer and then returned back into the medium. Each

experiment was conducted in triplicate and the mean values are presented in the Results and discussion section.

3 Results and discussion

3.1 Characterization of PMAC/Lc carriers

The FTIR spectra of the PMAC/Lc carriers are shown in Fig. 1. The FTIR spectra of PMAC-50N and PMAC/Lc-50N are not presented in Fig. 1. because they could overburden the Figure's content making its contribution even unfavorable for the interpretation of the results. The FTIR spectra of the PMAC, PMAC-0N, PMAC-50N and PMAC-100N carriers have been analyzed in detail in our previous work [1]. With increase in degree of neutralization of MAA the intensity of the peak at 1540 cm^{-1} (symmetric stretching C(=O)-O^- vibration) increased, whereas the intensity of the peak at 1645 cm^{-1} (symmetric bending C(=O)-OH vibration) decreased. This could be a consequence of the presence of larger number of $–COO^-$ groups than the number of –COOH groups. The addition of centrifuged liposomes in the PMAC-0N, PMAC-50N and PMAC-100N carriers caused the shift of PMAC-0N, PMAC-50N and PMAC-100N peaks detected at 1235 cm^{-1} and 1452 cm^{-1} to 1243 cm^{-1} and 1444.48 cm^{-1}, respectively. These discrete shifts could be a consequence of poor, physical interactions established between casein and phospholipids [2, 7]. The FTIR spectra of the PMAC/Lc-0N, PMAC/Lc-50N and PMAC/Lc-100N carriers revealed the same characteristic peaks deriving from the carrier's components. The FTIR spectra of caffeine, liposomes and the liposomes with the encapsulated caffeine are also presented in Fig. 1. The characteristic peaks of caffeine at 1653 cm^{-1} (corresponds to asymmetric and symmetric C=O stretching vibration in pyrimidine ring), at 1543 cm^{-1} (corresponds to amide II), at 1358 cm^{-1} (C-H stretching) and at 743 cm^{-1} (corresponds to N_2-CH_3 vibration in plane imidazole ring) are present in the FTIR spectrum of the liposomes with the encapsulated caffeine [8-10]. Presence of the characteristic peaks of the encapsulated active substance confirmed a successful entrapment of caffeine [2]. Comparison of the FTIR spectrum of the liposomes with the encapsulated caffeine with the FTIR spectrum of each component of this system (Fig. 1.) showed no significant difference as a sign of interaction between the liposomes as a carrier and caffeine as an active loading [2]. It can be concluded that encapsulation of caffeine was due to physical entrapment during the liposomes preparation [2].

The SEM micrographs of the synthesized PMAC/Lc carriers are presented in Fig. 2. PMAC/Lc-0N carrier had grained structure with closely packed casein micelles between which liposomes particles were presented (Fig. 2. a)). SEM micrographs of PMAC/Lc-50N carrier showed that large aggregates of casein micelles were not presented, as they were presented in PMAC/Lc-0N carrier network (Fig. 2. b)). Individual micelles of casein were distributed within the PMAC/Lc-50N network and these micelles did not exist in the structure of the PMAC/Lc-100N carrier. Namely, total neutralization of MAA caused the dissociation of the casein micelles and consequently PMAC/Lc-100N carrier formed regular porous structure (Fig. 2. c)) [1, 11]. It can be concluded that the change of neutralization degree of MAA caused the change in the form of casein micelles in the carrier structure. The micrographs of the cross-sections of PMAC/Lc carriers clearly indicated the presence of the liposomes in the carriers [12]. It could be observed that liposomal particles appeared as individual particles and were uniformly distributed within the network of the PMAC/Lc-50N carrier, as well as within the network PMAC/Lc-100N carrier (Fig. 2. b) and c)). It could be concluded that no physical degradation of the liposomes occurred during the synthesis of the carriers and exposure of liposomes to the relatively high temperatures (Fig. 2. a), b) and c)) [2, 13].

3.2 Degree of encapsulated caffeine in the samples

The degree of encapsulated caffeine in the centrifuged liposomes, which were used for synthesis of the PMAC/Lc carriers, was determined according to the procedure described in the Section 2.3. The calculated value of the caffeine encapsulation degree in liposomes was 84.8%. Encapsulation degree of the caffeine was relatively high according to the results that were published in scientific literature [11].

3.3 Swelling of PMAC/Lc carriers and caffeine release from PMAC/Lc carriers

The swelling curves of PMAC/Lc carriers in PB 6.8 and 0.1M HCl are presented in Fig. 3. a) and b), respectively. The influence of the neutralization degree of MAA was clearly reflected through the values of the equilibrium swelling degree of PMAC/Lc carriers: with increase in neutralization degree of MAA increased the value of SDeq of the carriers (Table 1.). Also, the SDeq values of PMAC/Lc carriers were around three times higher in PB 6.8 than in 0.1M HCl (Table 1.). This could be explained by the generation of negative charges on the carboxylic groups [14-16], favored at pH of this medium (pH=6.8) which is above the pKa of PMAA (4.6) and the isoelectric point of casein (pI=4.6) [1].

Table 1. SDeq values of PMAC/Lc carriers in 0.1M HCl i PB 6.8

Sample	SD_{eq}	
	0.1M HCl	*PB 6.8*
PMAC/Lc-0N	1.36	9.11
PMAC/Lc-50N	5.32	13.5
PMAC/Lc-100N	15.1	38.3

The curves of caffeine release from the PMAC/Lc carriers in PB 6.8 and 0.1M HCl are presented in Fig. 4. a) and b), respectively. As a consequence of specific pH dependent swelling behavior of PMAC/Lc carriers, the highest concentration of caffeine was released from PMAC/Lc-100N. Also, around two times higher values of caffeine concentrations were released from PMAC/Lc carriers in PB 6.8 than in 0.1M HCl. As it could be seen in Fig. 4. a) and b) addition of the centrifuged liposomes prevented the burst released of caffeine and provided prolonged release of caffeine during a 24h. Centrifugation of liposomal formulation prevented agglomeration of liposomes particles [2], so they appeared as individual particles uniformly distributed in the carriers network (as SEM analysis showed). Therefore, the control of caffeine release rate and targeted delivery without burst effect was achieved.

4 Conclusions

In this study, carriers based on poly(methacrylic acid), casein and centrifuged liposomes for targeted delivery of poorly water-soluble active substance - caffeine were synthetized and analyzed. Results showed that these carriers have great potential for encapsulation and targeted delivery of poorly water-soluble active substances.

The contribution of this study is reflected through the formation of complete picture on behavior of hydrophilic hydrogels used as carriers for poorly water soluble substance (PWSS): swelling of the carriers and release of PWSS (such as caffeine) in simulated human gastrointestinal tract including the improvement of the PWSS targeted delivery. The obtained results could be also used as a guideline for the successful overcoming of the drawbacks related to the usage of caffeine. It is well known that caffeine is used as psychoactive substance which improves cognitive ability and reduces fatigue. However, after 5h of caffeine consumption, human may feel sudden stimulation after which they feel exhausted. Carriers synthesized in our study could mask caffeine bitterness and enable prolonged and controlled release of caffeine and therefore prevent sudden stimulation of nervous system. Intestines in human body have the largest surface area for absorption of the active substance. Their membranes are more permeable than those in stomach and they potentiate relatively fast distribution of the active substance from intestines to systemic circulation. Therefore, carriers synthesized in our study could be also used for targeted delivery and controlled release of caffeine in intestines where its absorption could be the fastest.

5 Figures

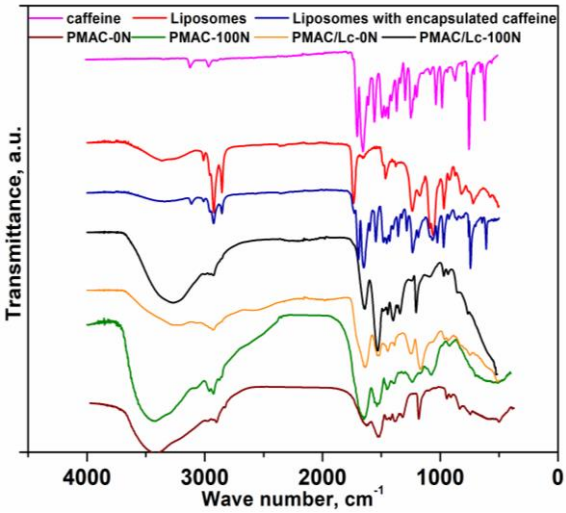

Figure 1. The FTIR spectra of PMAC-0N, PMAC-100N, PMAC/Lc-0N, PMAC/Lc-100N carriers and the FTIR spectra of caffeine, liposomes and liposomes with encapsulated caffeine

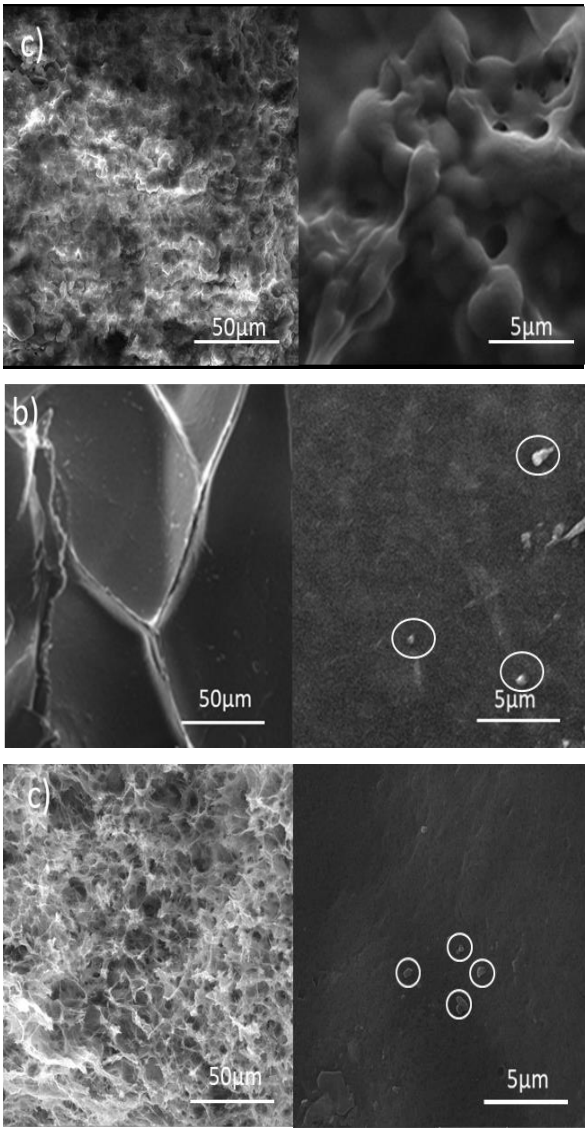

Figure 2. SEM micrographs of: a) PMAC/Lc-0N, b) PMAC/Lc-50N and c) PMAC/Lc-100N carriers (liposomal particles are marked with white circles)

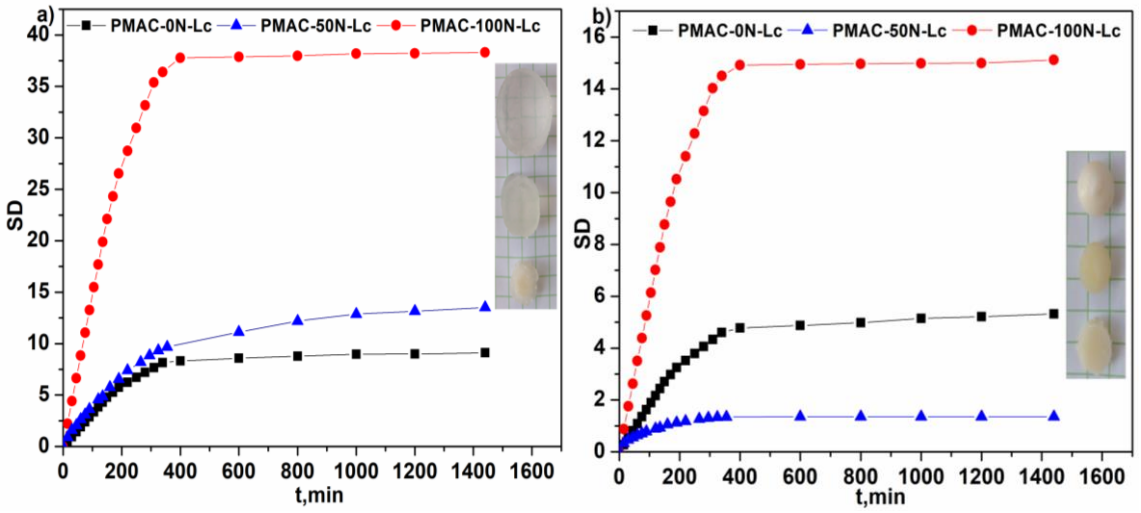

Figure 3. Swelling curves of PMAC/Lc carriers in: a) PB 6.8 and b) 0.1M HCl

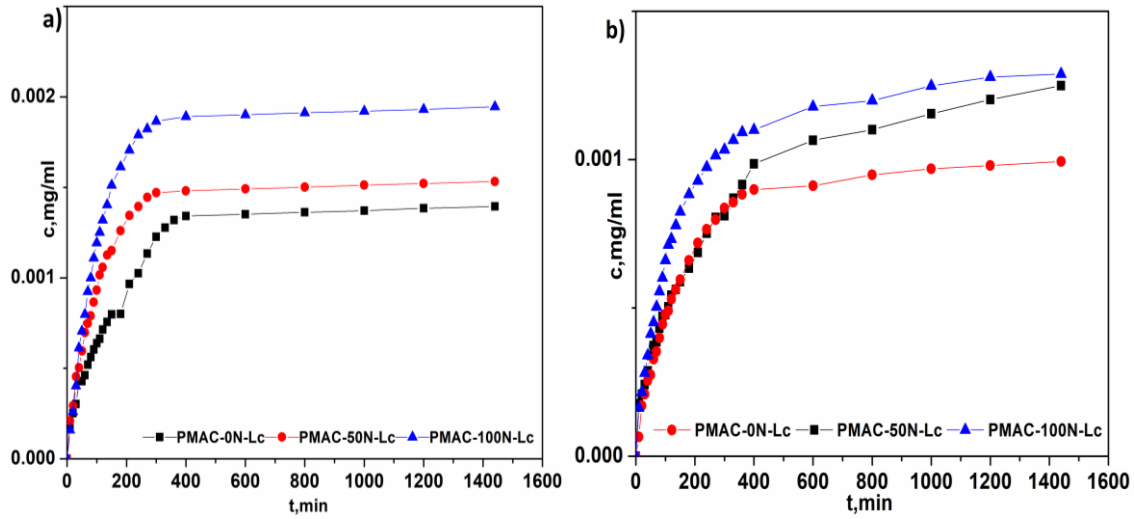

Figure 4. Curves of caffeine release from PMAC/Lc carriers in: a) PB 6.8 and b) 0.1M HCl

6 Abbreviations

ED	– caffeine encapsulation degree in liposomes (%)
FTIR	– Fourier Transform Infrared spectroscopy
m_0	– weight of dry hydrogel sample (xerogel) (g)
m_{csn}	– measured caffeine weight in the supernatant (g)
m_{ct}	– total caffeine weight in the liposomal suspension (g)
m_{eq}	– weight of equilibrium swollen hydrogel sample (g)
m_t	– weight of swollen hydrogel sample at time t (g)
MAA	– methacrylic acid
MBA	– N,N′-methylenebisacrylamide (crosslinker)
PMAA	– poly(methacrylic acid)
PMAC	– drug delivery carriers based on poly(methacrylic acid) and casein
PMAC/Lc	– PMAC carriers with incorporated centrifuged liposomes in which caffeine was encapsulated
PMAC/Lc-0N	– PMAC/Lc carriers with non-neutralized MAA
PMAC/Lc-50N	– PMAC/Lc carriers with 50% of neutralization degree of MAA
PMAC/Lc-100N	– PMAC/Lc carriers with 100% of neutralization degree of MAA

SD – swelling degree of the carriers
SD_{eq} – equilibrium swelling degree of the carriers
SEM – Scanning Electron Microscopy
t – time of the swelling of the PMAC/Lc carriers and time of caffeine release process (min)
VA-044 – 2,2′-Azobis-[2-(2-imidazolin-2-yl)propane]dihydrochloride (initiator)

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7 References

[1] **M.D. Markovic, P.M. Spasojevic, S.I. Seslija, I.G. Popovic, D.N. Veljovic, R.V. Pjanovic, V.V. Panic,** Casein-poly(methacrylic acid) hybrid soft networks with easy tunable properties, European Polymer Journal 113 (2019) 276-288.

[2] **M.D. Markovic, V.V. Panic, S.I. Seslija, A.D. Milivojevic, P.M. Spasojevic, N.M. Boskovic-Vragolovic, R.V. Pjanovic,** Novel strategy for encapsulation and targeted delivery of poorly water-soluble active substances, Polymer Engineering & Science n/a(n/a).

[3] **B.D. Balanč, A. Ota, V.B. Djordjević, M. Šentjurc, V.A. Nedović, B.M. Bugarski, N.P. Ulrih,** Resveratrol-loaded liposomes: Interaction of resveratrol with phospholipids, European Journal of Lipid Science and Technology 117(10) (2015) 1615-1626.

[4] **G.P. Asnani, J. Bahekar, C.R. Kokare,** Development of novel pH–responsive dual crosslinked hydrogel beads based on Portulaca oleracea polysaccharide-alginate-borax for colon specific delivery of 5-fluorouracil, Journal of Drug Delivery Science and Technology 48 (2018) 200-208.

[5] **M. Tavakol, S. Dehshiri, E. Vasheghani-Farahani,** Electron beam irradiation crosslinked hydrogels based on tyramine conjugated gum tragacanth, Carbohydrate Polymers 152 (2016) 504-509.

[6] **G.A. Mun, Z.S. Nurkeeva, V.V. Khutoryanskiy, B.B. Yermukhambetova, S.M. Koblanov, I.A. Arkhipova,** Radiation synthesis of hydrogels based on copolymers of vinyl ethers of monoethanolamine and ethyleneglycol and their interaction with poly(acrylic acid), Radiation Physics and Chemistry 67(6) (2003) 745-749.

[7] **A. Mohan, D.J. McClements, C.C. Udenigwe,** Encapsulation of bioactive whey peptides in soy lecithin-derived nanoliposomes: Influence of peptide molecular weight, Food Chemistry 213 (2016) 143-148.

[8] **N. Noor, A. Shah, A. Gani, A. Gani, F.A. Masoodi,** Microencapsulation of caffeine loaded in polysaccharide based delivery systems, Food Hydrocolloids 82 (2018) 312-321.

[9] **R. Verma, L. Kumar,** Characterization of caffeine isolated from camellia sinensis leaves of sikkim himalayan region, 2010.

[10] **H. El Hamdani, M. El Amane, C. Duhayon,** Studies on the syntheses, structural Characterization, antimicrobial of the CO-CRYSTAL 1,10-phenanthrolin-1-IUM(1,10-phenH+)-caffeine(caf)-hexafluorophosphate, Journal of Molecular Structure 1155 (2018) 789-796.

[11] **F. Bignotti, S. Agnelli, F. Baldi, L. Sartore, I. Peroni,** Macroporous polyacrylamide hydrogels with tailored porosity and mechanical properties via microphase separation in the presence of hydroxyethylcellulose, Polymer Engineering & Science 57(7) (2017) 764-771.

[12] **A. Billard, L. Pourchet, S. Malaise, P. Alcouffe, A. Montembault, C. Ladavière,** Liposome-loaded chitosan physical hydrogel: Toward a promising delayed-release biosystem, Carbohydrate Polymers 115 (2015) 651-657.

[13] **M. Grit, D.J.A. Crommelin,** Chemical stability of liposomes: implications for their physical stability, Chemistry and Physics of Lipids 64(1) (1993) 3-18.

[14] **N. Higashi, M. Niwa,** Organized polyelectrolyte monolayers as a structural model of biopolymer, Colloids and Surfaces A: Physicochemical and Engineering Aspects 123-124 (1997) 433-442.

[15] **K. Durai Murugan, P. Natarajan,** Studies on the structural transitions and self-organization behavior of polyacrylic acids with complementary polymers in aqueous solution by laser flash photolysis method using the triplet state of covalently bound phenosafranine, European Polymer Journal 47(8) (2011) 1664-1675.

[16] **Y. Muroga, T. Yoshida, S. Kawaguchi,** Conformation of poly(methacrylic acid) in acidic aqueous solution studied by small angle X-ray scattering, Biophysical Chemistry 81(1) (1999) 45-57.