Encapsulation of *α*-lipoic acid into chitosan and alginate/gelatin hydrogel microparticles and its *in vitro* antioxidant activity

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Abstract

Alpha-lipoic acid is an organosulphur compound well-known for its therapeutic potential and antioxidant properties. However, the effective use of *α*-lipoic acid depends on biological plasma half-life and its preserving stability, which could be improved by encapsulation. In this study, *α*-lipoic acid was incorporated into chitosan microparticles obtained by reverse emulsion crosslinking technique, as well as into microparticles of alginate/gelatin crosslinked with zinc ions. Encapsulation of *α*-lipoic acid in both cases was carried out by swelling of synthesized dried microparticles by their dipping in a solution of the active substance under strictly controlled conditions. Encapsulation efficiency of *α*-lipoic acid obtained in this study was up to 53.9%. The structural interaction of *α*-lipoic acid with the carriers was revealed by Fourier transform infrared spectroscopy. *In vitro* released studies showed that controlled release of α-lipoic acid was achieved through its encapsulation into chitosan microparticles. The results of *in vitro* antioxidative activity assays of released *α*-lipoic acid indicated that antioxidant activity was preserved at a satisfactory level. These obtained results suggested that chitosan microparticles could be suitable for modeling the controlled release of *α*-lipoic acid.

Keywords: alpha-lipoic acid, microparticles, chitosan, sodium alginate/gelatin, antioxidant activity.

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Alpha-lipoic acid (LA), also known as 1,2-dithiolane-3-pentanoic acid ($C_8H_{14}O_2S_2$) is a yellow solid organosulphur compound [1]. Having an asymmetric carbon atom, LA exists as two enantiomers: the R-(+)-LA and S-(–)-LA. Only the R-isomer is endogenously synthesized and is an essential cofactor for mitochondrial enzyme complexes that catalyze reaction responsible for oxidative glucose metabolism and cellular energy production. Synthetic racemic LA, a 50/50 mixture of R-and S-enantiomers, has been extensively used as a therapeutic agent in the treatment of diabetic neuropathy and as an antioxidant supplement in European countries and the United States [2].

Exogenously supplied LA is readily absorbed, transported to tissues and rapidly taken up by cells. Within the cells LA is reduced to dihydrolipoic acid (DHLA), then rapidly removed from cells and metabolized. Several lines of evidence indicated that both LA and its reduced form, DHLA, exerted potent antioxidant activities, both *in vitro* and *in vivo*. In addition to direct free radicals scavenging and metal chelating properties, LA/DHLA redox couple appears to be able to regenerate other endogenous antioxidants such as vitamins C and E, and glutathione, as well as to modulate the redox signaling pathways [3–5]. There is evidence that administration of LA might have a beneficial role in a variety of oxidative stress conditions such as ischemia-reperfusion injury, type 2 diabetes and associated complications, neurological disorders, ischemia-reperfusion injury [1].

Human pharmacokinetic studies have found that oral administrated LA is characterized by rapid absorption within 1 h, short plasma half-lives, extensive hepatic metabolism and low and varying bioavailability [6]. The short half-live of LA (about 30 min) may inhibit effective use [7]. On the other hand, LA is unstable under light and heat and gradually decomposes at room temperature. Temperatures greater than its melting point (59–62 °C) cause immediate polymerization and render it unusable, as polymerized LA is insoluble in almost all solvents [8]. Furthermore, the decomposition of LA is accompanied by an unpleasant odor due to the sulphur content [9].

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In the past several years, a forms of stabilized and controlled released LA were designed with the assumption that the efficacy of LA could be improved by slowing the absorption and keeping blood levels of LA above baseline for longer periods after ingestion [10]. However, the efficacy and safety from sustained-release dosage of α-lipoic acid forms have not been clear.

Active substances such as minerals, vitamins, proteins, enzymes, peptides, probiotics, phenolic compounds, etc., are widely used in the food, pharmaceutical and cosmetic industry nowadays [11]. These substances are very sensitive to extreme temperatures, presence of oxygen, microorganisms and moisture that often come in contact with, thereby losing their activity, both during storage and application conditions. Therefore, in the last few decades, a great attention has been put to mode the administration of active substances which should also provide the maintenance of their activities and their optimum (concentration) effect in specific parts of the body.

One of the most common ways to overcome these problems is the encapsulation of these substances into various carriers. Encapsulation can mask the unpleasant taste or smell, but also provides control of the release of the active substance. The main goal of encapsulation is to protect the substance from adverse conditions (light, moisture and oxygen) thereby contributing to the increase of the shelf life and, at the same time, controlling the release and increasing the *in vivo* half-life of encapsulated product [12]. In addition to the choice of the appropriate method of encapsulation, it is also important to choose the appropriate carrier. The ideal matrix for encapsulation must not have the degradation effect to the active substance, and at the same time it should be non-toxic and relatively easy to synthesize. Nowadays, various systems for encapsulation of active substances, most often consisting of carbohydrates, proteins, natural or synthetic polymers (chemically or physically crosslinked) are used among which hydrogels have become very popular as carriers in the various industry fields [13].

The aim of this study was to investigate the possibility of encapsulation of LA into chitosan and alginate/gelatin microparticles and to investigate its release as this has not been studied extensively [10,14,15], as well as to investigate the antioxidant activity of released LA compared to the activity of free LA and its active metabolite DHLA. First part of the study was the attempt to encapsulate LA into chitosan microparticles obtained by reverse emulsion crosslinking technique, as well as into microparticles of alginate/gelatin (of various gelatin content) crosslinked using zinc ions. The synthesized microparticles were characterized by FT-IR and SEM analyses. Further on, the release of LA in pH simulated GI tract conditions were monitored followed by the experiment regarding *in vitro* antioxidant capacity of released LA expressed as Trolox equivalent antioxidant capacity (*TEAC*) and ferric reducing/antioxidant power (*FRAP*). The samples of plasma from healthy subjects were used for *in vitro* determination of antioxidant activities of released LA from selected investigated samples.

MATERIALS AND METHODS

Materials

Chitosan (Ch), sodium alginate (A) and gelatin (G) used for microparticles preparation were obtained from Sigma, Japan, while itaconic acid (2-methylidenebutanedioic acid) was purchased from Sigma–Aldrich, Germany. Glutaraldehyde (GA, pentane-1,5-dial), paraffin oil (light liquid paraffin) and zinc chloride were obtained from Centrohem, Serbia. Tween 80 (polyoxyethylene (20) sorbitan monooleate) used as an emulsifier was obtained from Riedel-de Haën, Germany. α-Lipoic acid (LA) (purity > 98%) was obtained from "Ivančić i sinovi" Belgrade, Serbia. Distilled water was used for the preparation of buffer solutions of pH 2.20±0.01 (KCl/HCl, Merck) and pH 6.80±0.01 (Na_2HPO_4/ /NaH_2PO_4, Lach-Ner). Folin–Ciocalteu reagent, ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid diammonium salt), potassium persulphate, TPTZ (2,4,6--Tris(2-pyridyl)-*s*-triazine), chloramine T trihydrate and peroxidase type I from horseradish were purchased from Sigma-Aldrich, Germany. Malonaldehyde bis(dimethyl acetal), Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) and TMB powder (3,3',5,5'-tetramethylbenzidine) were purchased from Acros Organics. 2-Thiobarbituric acid, trichloracetic acid, dimethylsulfoxide (DMSO) and hydrogen peroxide were purchased from Merck, Germany. All other chemicals and reagents used were of analytical reagents grade quality and obtained from standard commercial suppliers. Dihydrolipoic acid (DHLA) was prepared by treating LA with $NaBH_4$ using a method described in a previous report [16]. Purity was confirmed by NMR, comparing with literature data.

Preparation of chitosan and alginate/gelatin microparticles

Chitosan microparticles, with 1.5% (*w*/*V*) of chitosan content crosslinked with glutaraldehyde of various contents (0.1–0.4 vol.%) were prepared as previously described [17]. The ratio of oil-to-water phases in all emulsion preparations was 1:5 with the addition of 2.0 vol.% Tween 80 (1 cm^3) as an emulsifier. Hydrogels of Na-alginate and gelatin (various ratios) in the form of microparticles were synthesized, as well, using Zn^{2+} as the crosslinking agent, while keeping the concentration of crosslinking agent constant. Namely, Na-alginate was

added to 14 cm^3 of distilled water and allowed to dissolve using magnetic stirring at 500 rpm at 50 °C. After on, gelatin was added to the solution to reach the total mass of the reactants of 0.8000 g. Then the stirring speed was reduced to 100 rpm in order to remove air bubbles. For the synthesis of microparticles Syringe pump (New Era Pump Systems Inc., model NE-1000) was used. The mixture of 10 cm^3 was added drop wised at the rate of 75 μL/min to 100 cm^3 0.1 M $ZnCl_2$ solution. During the instillation immediate formation of hydrogel microparticles was evident. Hydrogel microparticles were left for 24 h in a solution of zinc chloride in order to achieve complete crosslinking. After that, a solution of zinc chloride has been removed, and the hydrogel particles were washed with water three times and maintained in 100 cm^3 of water for 24 h in order to remove unreacted substances. Then the synthesized microparticles were immersed in acetone for 30 min, knowing that acetone would ease the drying process not allowing the coalescence of microparticles. The particles were then removed from acetone solution and allowed to dry to constant weight. The compositions of all investigated samples are given in Table 1.

Table 1. The composition of the synthesized hydrogel microparticles

Sample	Content, wt.%			Crosslinking agent concentration, mol/dm^3
	Ch	A	G	
S1	2.0	–	–	0.08
S2	0.5			0.02
S3	1.0			0.04
S4	–	100	–	0.10
S5		66.7	33.3	
S6		50.0	50.0	
S7		33.3	66.7	

Fourier transform infrared spectroscopy

A Bomem MB 100 FT-IR spectrophotometer was used to record infrared spectra of the microparticles, applying the KBr disc method. Sample/KBr (ratio 1/50) was mixed and grounded and then compressed into a pellet under an 11 tones weight, for one minute, using Grase by Specac, model 15.011. FT-IR spectra were obtained in the wavenumber range 4000–400 cm^{-1}, at 25 °C and at 4 cm^{-1} spectral resolution.

Swelling degree studies

In order to simulate the gastrointestinal (GI) tract temperature and pH conditions as well as the average time spent in GI tract, the swelling studies were carried out at 37 °C, by immersing the microparticles for 2 h in a buffer solution of pH value of 2.20±0.01 and subsequently in a buffer solution of pH 6.80±0.01 for the next 22 h. After immersion, microparticles' weight was measured in pre-determined time intervals. The degree of swelling was calculated according to the following equation:

$$q = w_t/w_0 \quad (1)$$

where w_t corresponds to the weight of swollen microparticles at time t and w_0 to the weight of dried microparticles. The swelling process was monitored gravimetrically.

Scanning electron microscopy

The surface morphology of microparticles was evaluated using scanning electron microscopy (Tescan Mira3 XMU, Cranberry Twp, PA, USA). Prior to SEM analysis samples were coated with gold/platinum alloy (15/85) under vacuum conditions, using Polaron SC502 vacuum sputter coater. Samples were analyzed in two different forms: dry microparticles and rehydrated microparticles.

Determination of encapsulation efficiency of α-lipoic acid

Alpha-lipoic acid was encapsulated by immersing ready-made hydrogels into solution of α-lipoic acid that consists of 1 g of active substance dissolved into 75 cm^3 mixture of ethanol and 2 vol.% acetic acid solution (ratio 1:2). The pH of the obtained solution was 2.70 offering the good environment for LA encapsulation. The LA encapsulation efficiency was determined spectrophotometrically. Encapsulation efficiency was calculated as the ratio of the actual loading, $(\text{Total LA content})_e$, of the hydrogel particles with α-lipoic acid and the theoretical loading, $(\text{Total LA content})_i$, using the following equation:

$$EE(\%) = (\text{Total LA content})_e/(\text{Total LA content})_i \quad (2)$$

$(\text{Total LA content})_e$ was calculated as the difference between the total amount of LA in the initial solution used for encapsulation and the free amount of LA left in the solution after encapsulation process).

Calibration curve of LA

A calibration curve is required for the determination of encapsulation efficiency and release rates of the microparticles. A known concentration of LA in buffers of pH values of 2.20 and 6.80 were scanned in the range of 200–500 nm by using UV/Vis spectrophotometer. Prominent peaks at 329 and 332 nm were noticed. The concentration of LA was obtained from the calibration curve prepared by measuring the absorbance (329 and 332 nm) of different concentrations of LA.

***In vitro* antioxidant activity of the released LA**

The anti-oxidant capacity of released LA following *in vitro* digestion was examined by FRAP and ABTS assays.

The ferric reducing/antioxidant power (FRAP) assay was carried out according to the method described by Benzie and Strain [18]. The working FRAP reagent was prepared by mixing 300 mmol/L acetic buffer (pH 3.60), 10 mmol/L TPTZ in 40 mmol/L HCl and 20 mmol/L $FeCl_3 \cdot 6H_2O$ in a ratio of 10:1:1. Then 200 µl of sample from release test medium was added to 3.80 cm^3 of FRAP reagent. After 4 min of reaction, the absorbance at 593 nm was read. The FRAP values were expressed, taking into account a standard curve of $FeSO_4 \cdot 7H_2O$ and the results are expressed as µmol Fe(II)/$g_{particles}$. All measurements were performed in triplicate.

Determination of antioxidant activity using the ABTS method was performed according to the method described by Re *et al.* [19]. The free radical-cation $ABTS^{\bullet+}$ was generated by the oxidation reaction of the ABTS with potassium persulphate in the dark condition for 12–16 h at room temperature. After 24 h, the absorbance was adjusted with PBS pH 7.4 up to 0.70 units, at 732 nm. For the evaluation, 20 µL of the sample was added to 1880 µL of the $ABTS^{\bullet+}$ and the absorbance readings were taken after exactly 6 min. The results were expressed as *TEAC* (trolox equivalent antioxidant capacity) by the construction of a standard curve, using several concentrations of the Trolox antioxidant. All measurements were performed in triplicate.

Evaluation of antioxidant activity of LA in human plasma

After night-time fasting, blood samples from healthy, non-smoking volunteers were collected into EDTA-containing sample tubes according to the ethical review board approved protocols. The plasma was pooled and used for all subsequent analysis. Tested solutions of native LA, its active metabolite (DHLA) and released LA from chitosan microparticles were added to the samples of plasma (0.7 cm^3) and incubated for 1 at 24 h at 37 °C. The control plasma samples (without tested solutions) were prepared. To evaluate the antioxidant activity of the test solutions against copper induced oxidative stress, plasma samples were also incubated with copper sulphate (2 mmol/L) plus tested compounds and released LA added in combination. Incubation of plasma samples was stopped by cooling the samples in an ice-bath.

The levels of lipid peroxidation products in plasma were assayed in plasma according to Girroti *et al*. [20]. This assay is based on the formation of a complex between thiobarbituric acid and malondialdehyde, an end product of lipid peroxidation, which absorbs at 535 nm. Total anti-oxidant capacity (*TAC*) was measured in plasma according to Erel's method [21]. This assay is based on the bleaching of the characteristic color of a stable 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) by antioxidants present in plasma. The TAC value of the samples tested is expressed as µmol Trolox equivalent/L. Prooxidant–antioxidant balance (*PAB*) was measured according to the previously published method [22], slightly modified in our laboratory. The assay is based on 3,3',5,5'-tetramethylbenzidine and its cation, used as a redox indicator participating in two simultaneous reactions. The standard solutions were prepared by mixing varying proportions of (0–100%) of 250 µmol/L hydrogen peroxide with 10 mmol/L uric acid. *PAB* is expressed in arbitrary HK units, which represent the percentage of hydrogen peroxide in the standard solution.

Statistical analysis

All the values were expressed as mean ± *SD*. The data were analyzed with the one-way repeated measures analysis of variances using SPSS version 18 (Chicago, IL, USA).

RESULTS AND DISCUSSION

FT-IR analyses

FT-IR spectra of pure chitosan microparticles were discussed in our earlier paper [17]. FT-IR spectra of the samples S4–S7 and pure gelatin are shown in Figure 1.

From Figure 1 in the spectrum of gelatin the following characteristic bands occur: 3425 cm^{-1} (NH stretching vibrations), 1641 cm^{-1} (amide I band which comes mainly from the C=O stretching vibration) and 1562 cm^{-1} (amide II band that comes from NH deformation vibration in the plane coupled with CN stretching vibration). Amide III band at 1300 cm^{-1} comes from the CN stretching vibration coupled with NH deformation vibration) [23,24]. In the FT-IR spectrum of the sample S4 the most important are the following bands: 3436 cm^{-1} (OH stretching vibration), 1625 cm^{-1} (COO asymmetrical stretching vibration) and 1420 cm^{-1} (COO symmetrical stretching vibration) [24]. In the spectrum of the hydrogels containing gelatin, the absorption bands corresponding to the OH stretching vibration of alginate (about 3430 cm^{-1}) is shifted towards lower wave numbers as the content of gelatin within the samples increases. This is due to the formation of hydrogen bonding between gelatin and alginate. Also, COO asymmetric stretching vibration of alginate at 1625 cm^{-1} is coupled with the C=O stretching vibration of gelatin, which caused displacement of the band corresponding to this vibration to higher wavenumbers as the gelatin content increases. At the same time, there was a shift of the absorption bands corresponding to the COO symmetric vibrations towards lower wave numbers. All the above mentioned shows the existence of intermolecular interactions between alginate and gelatin, caused by the formation of hydrogen bonding between the $–COO^-$, –OH, –C=O and $–NH_2$ groups of the alginate, and gelatin [23].

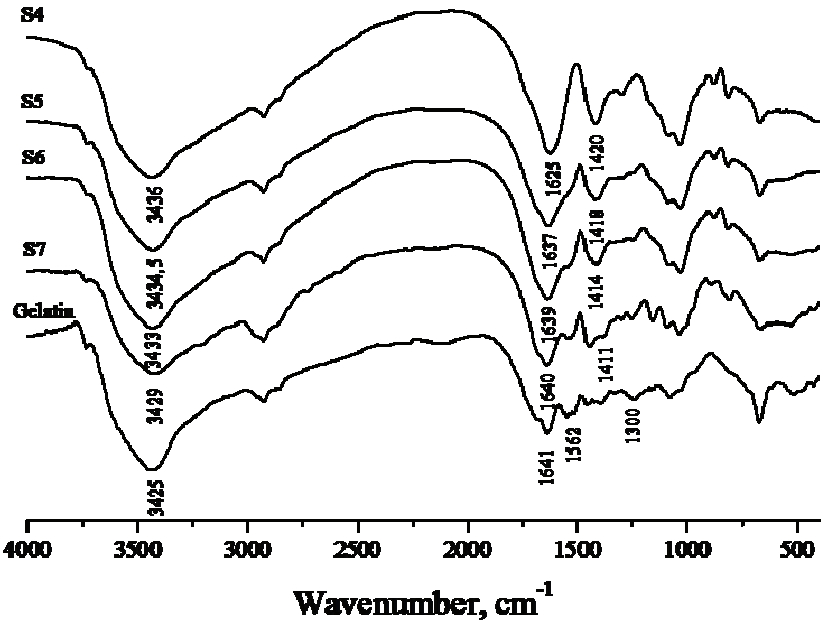

Figure 1. FT-IR spectra of alginate/gelatin samples (S4–S7) and pure gelatin.

FT-IR spectra of selected microparticles after encapsulation of α-lipoic acid were given in Figure 2.

The spectra of encapsulated LA were analyzed in two major regions; 3600–2800 cm^{-1} and 1800–445 cm^{-1}. The characteristic high-intensity bands for $–CH_2$ stretching of LA are evident around 2933 cm^{-1}). Other characteristic bands of LA at 1717 cm^{-1} (C=O stretching), already reported in the literature [25] were assumed to be shifted upon LA encapsulation within alginate/gelatin and chitosan carriers. Namely, (C=O) has been shifted from 1717 to 1720 or 1713 cm^{-1}, respectively. The shift to lower wavenumbers can be attributed to the interruption of strong hydrogen bonding in LA upon encapsulation with alginate/gelatin carrier, showing relatively weak affinity for this matrice. However, shifting to somewhat higher wavenumbers after encapsulation of LA into chitosan matrices suggests that there is interaction between LA and chitosan carrier implying

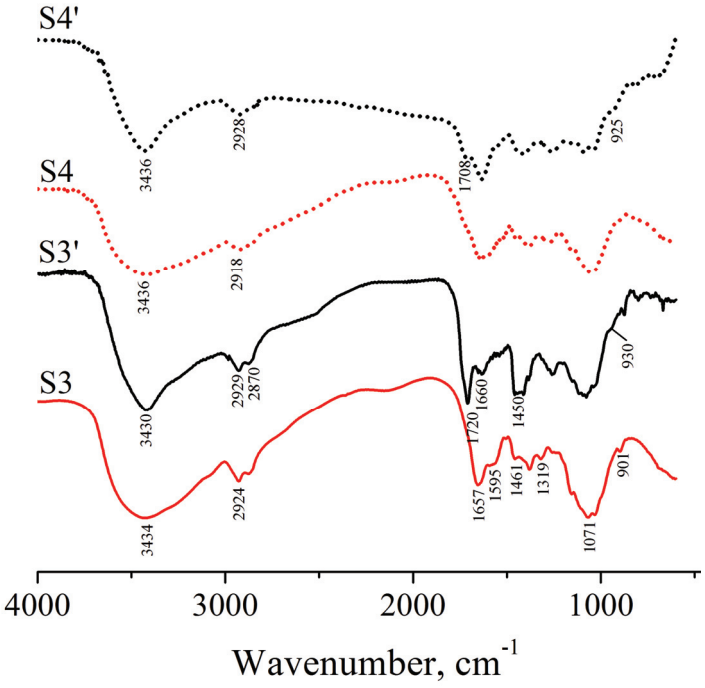

Figure 2. FT-IR spectra of chitosan microparticles without (S3) and with (S3') encapsulated α-lipoic acid; alginate/gelatin microparticles without (S4) and with (S4') encapsulated α-lipoic acid.

that carbonyl property of LA bounded to the chitosan support gets stronger than that of free LA. The bands at 930 and 925 cm^{-1} belong to O–H vibrations of LA. A small shoulder peak at 1660 cm^{-1} appears in the spectrum of the LA encapsulated chitosan sample due to the overlapping of the carboxylic acid group in LA with the amide group in chitosan [14].

The swelling kinetics

Figure 3 shows dynamic swelling behavior of all samples in simulated gastric fluid of pH 2.20±0.01 for 2 h and in intestinal fluid of pH 6.8±0.01 up to 24 h. In an acidic environment, the swelling rate increased since the chitosan amino groups were protonated (NH_3^+) and the positive charges induce repulsive forces between polymer chains [26]. As expected, the swelling rate was lower as the concentration of glutaraldehyde in chitosan microparticles was higher (less free amino groups available for protonation offer lower swellability of chitosan microparticles) in acidic medium. After being transferred to the medium of pH 6.80±0.01 chitosan microparticles shrunk, due to the hydrogen bonds tendency to associate by changing $–NH_3^+$ into $–NH_2$ groups, and after on the swelling of microparticles remained constant. This implies that swollen chitosan microparticles remained stable and can be used for delivery of low molecular weight molecules such as LA.

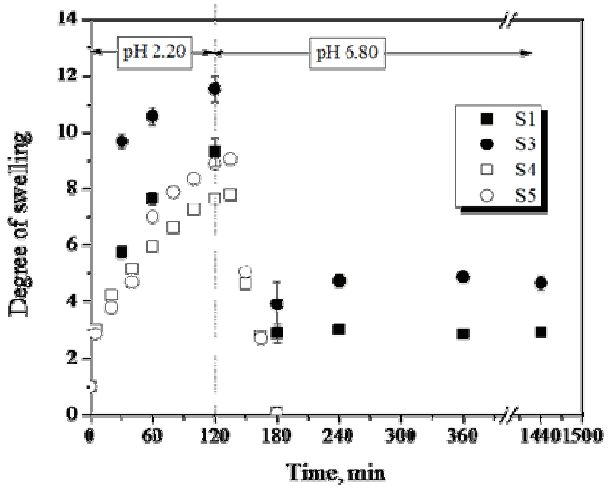

Figure 3. The swelling kinetics of chitosan microparticles (S1 and S3) and the effect of gelatin on the degree of swelling of alginate/gelatin microparticles (S4 and S5) in pH 2.20±0.01 and 6.8±0.01 at 37 °C.

Alginate is a natural polymer having carboxyl groups with pK_a values of 3.38–3.65. This means that the carboxyl groups of the alginate are ionized at pH values higher than the pK_a value, while at the pH below this value they appear in their non-ionized form. On the other hand, gelatin represents an amphiphilic component that is ionized at all pH values, except at its isoelectric point (4.8–5.5). Based on this, one would expect that the synthesized hydrogels showed significant swelling at higher pH values.

At pH 2.20±0.01 carboxyl groups of the alginate are in non-ionized form so that there is no electrostatic repulsion between the functional groups, and the degree of swelling of all the samples is minute. It can be observed that the smallest equilibrium degree of swelling has sample S4, while with increasing the gelatin content in the sample increases and the equilibrium degree of swelling. This can be explained by the fact that at this pH gelatin is below its isoelectric point and positively charged when it comes to the electrostatic repulsion between molecules of gelatin which causes an increase in hydrophilicity of the network and increases the volume of the hydrogel. Also, with the increase of gelatin concentration the possibility of aggregation within the hydrogel reduces since part of the functional groups of alginate are already used for the formation of hydrogen bonds with gelatin, which enhances the swelling of hydrogels.

At pH 6.80±0.01, which is higher than the pK_a value of an alginate, carboxyl groups exist in their ionized form ($–COO^-$). When the sample of pure alginate, S4 is placed in a phosphate buffer of pH 6.80±0.01, it comes to the exchange of Na^+ present in the buffer solution with the Zn^{2+} bonded to the $–COO^-$ group in polymannuronic segments. The electrostatic repulsion between $–COO^-$ groups appears thereby increasing the hydrophilicity of the polymer chain and the swelling of the hydrogel. In the further process of swelling, there is an exchange of the Na^+ with Zn^{2+} bound to $–COO^-$ groups in polyguluronic segments. As these Zn^{2+} are responsible for crosslinking of Na-alginate and hydrogel formation, the established ionic interactions are broken, the physical crosslinking disappears and the sample transforms from the gel into solution.

In other samples it was found that there is no complete “dissolution” after 24 h. It can be seen that there has been a loss of mass in samples S5 (and S6, data not shown), indicating that some ionic interactions have been terminated to some extent due to exchange of sodium and zinc ions. Also, when increasing the content of gelatin the weight loss of the investigated microparticles is slower, increasing the stability of the gels. It is assumed that gelatin hinders the zinc ion exchange with Na^+, and microparticles dissolution. With the increase of gelatin content in the samples a slight increase in the equilibrium degree of swelling is achieved. This pH values, besides the ionization of the carboxyl groups of the alginate, allows negative charging of gelatin molecules, leading to additional electrostatic repulsion and microparticles volume increase.

SEM analysis

SEM analysis was performed in order to collect information about morphology of chitosan and algi-

nate/gelatin microparticles. The surface morphology is an important characteristic when particles come in contact with fluid, solid or gaseous environment and together with its internal structure, will influence its suitability for a targeted use [17].

Micrographs (Figure 4) reveal the surface of microparticles of the investigated oven-dried chitosan microparticles (Figure 4a), while Figure 4b shows the alginate/gelatin sample without encapsulated α-lipoic acid swollen to equilibrium in ethanol/acetic acid mixture at 25 °C. In order to investigate the inner structure of S4 (Figure 4c), microparticles were freeze-dried, immersed in liquid nitrogen and cut.

SEM analysis confirmed that the test particles have a porous surface and structure. As known that the appearance of the particles depends on the temperature of the medium in which the particles swell, the increase in temperature leads to a partial termination of the physical connection to the gelatin molecule due to the uncoiling of the triple helix, which reduces cross-linking gelation.

Antioxidant capacity

The encapsulation efficiency of α-lipoic acid into ready-made hydrogel microparticles was in the range between 11.6 (S2) and 53.9% (S3). Modeling of controlled release of active substances at different sites in the gastrointestinal (GI) tract is preferred approach for preventing its degradation, as well as to obtain the desired serum levels over the extended period of time [27]. Moreover, antioxidant capacity will depend on the release of LA *via* swelling and/or degradation of chitosan microparticles. In order to explore whether the encapsulation influences the antioxidant capacity of LA, ferric reducing/antioxidant power (*FRAP*) assay and ABTS radical cation discoloration assay were performed. *In vitro* release of LA and its antioxidant capacity was evaluated in simulated gastric fluid (pH 2.20) and simulated intestinal fluid (pH 6.80), separately. The amount of released LA was determined spectrophotometrically at 329 nm (pH 2.20) and 332 nm (pH 6.80). The obtained results are presented in Figure 5.

The results show sustained release of LA from chitosan microparticles (sample S3). The amount of LA rel-

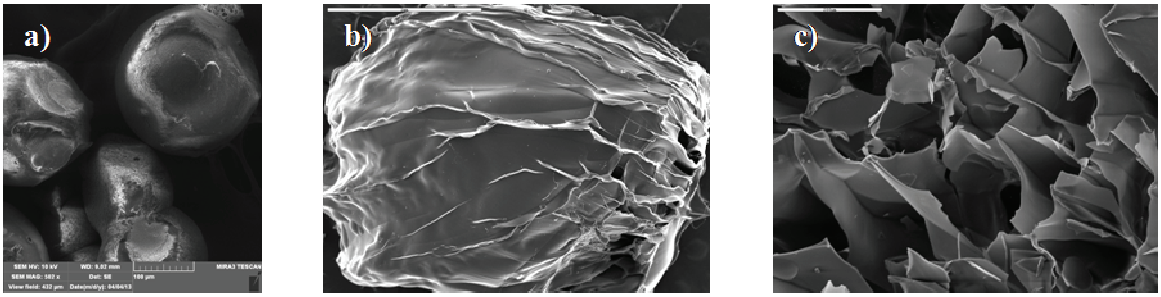

Figure 4. SEM micrographs of microparticles without encapsulated α-lipoic acid of: a) oven-dried chitosan microparticles (S3), b) whole alginate/gelatin microparticles (S4) upon reaching equilibrium degree of swelling in ethanol/acetic acid mixture at 25 °C and c) cross-section of the alginate/gelatin (S4) microparticles.

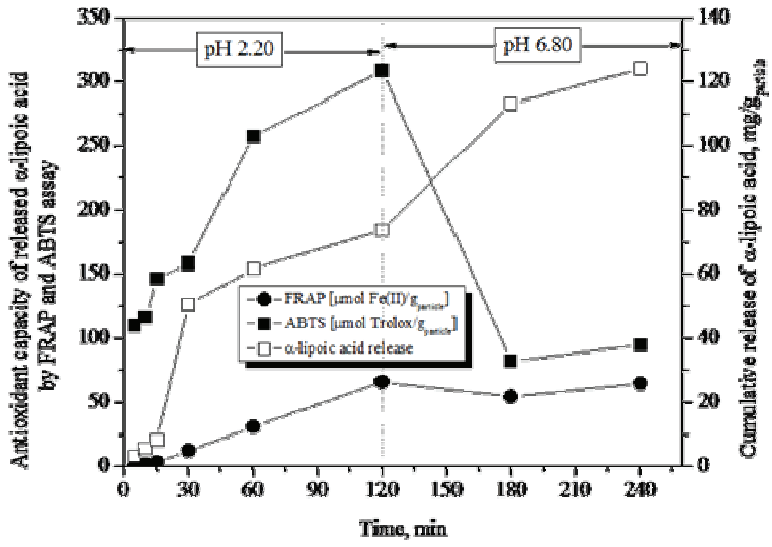

Figure 5. Antioxidant capacity of released α-lipoic acid by FRAP and ABTS assays and cumulative release of LA in simulated conditions of gastrointestinal tract (n = 3).

eased from the carrier was about 11.5 and 13.7% after 60 and 120 min, respectively. Changing the dissolution medium from pH 2.2 to pH 6.8 did not result in disintegration of the LA chitosan microparticles in contrast to alginate/gelatin hydrogel microparticles (release data not shown), and controlled release of LA was achieved. After 240 min, approximately 25% of the LA was released after simulated dissolution test at pH 6.80. The rate of release of LA from chitosan microparticles associated with observed changes in the antioxidant capacity measured by FRAP and ABTS assay.

Plasma lipid peroxidation was significantly increased in $CuSO_4$ treated plasma samples. This increment was abolished at least in part with LA and especially with DHLA preparation. This difference between LA and DHLA is more evident after 24 h of incubation. The same pattern of influence was clear from prooxidative–antioxidative balance, a parameter which showed the superiority of DHLA in antioxidative protection. At the same time, antioxidative plasma capability measured through the TAC level was abrogated upon Cu^{2+} influence and revitalized through the antioxidative LA and DHLA protection, which is the highest in the case of reduced LA formulation (DHLA).

In this study, we assessed the effect of released LA from chitosan microparticles, native LA and its reduced metabolite (DHLA) on the modification of oxidative/ /antioxidative balance induced by $CuSO_4$ *in vitro* by measuring the plasma level of lipid peroxidation, the prooxidant-antioxidant balance levels, as well as the total antioxidant capacity (Figure 6). In agreement with previous reports, we confirmed that incubation with the copper increased the oxidation of plasma phospholipids [28] that resulted in an elevation of levels of lipid peroxidation and depletion of vitamin E and other antioxidants in plasma [29]. The obtained results showed that the increase of lipid peroxidation is significantly lower in plasma supplemented with LAs and

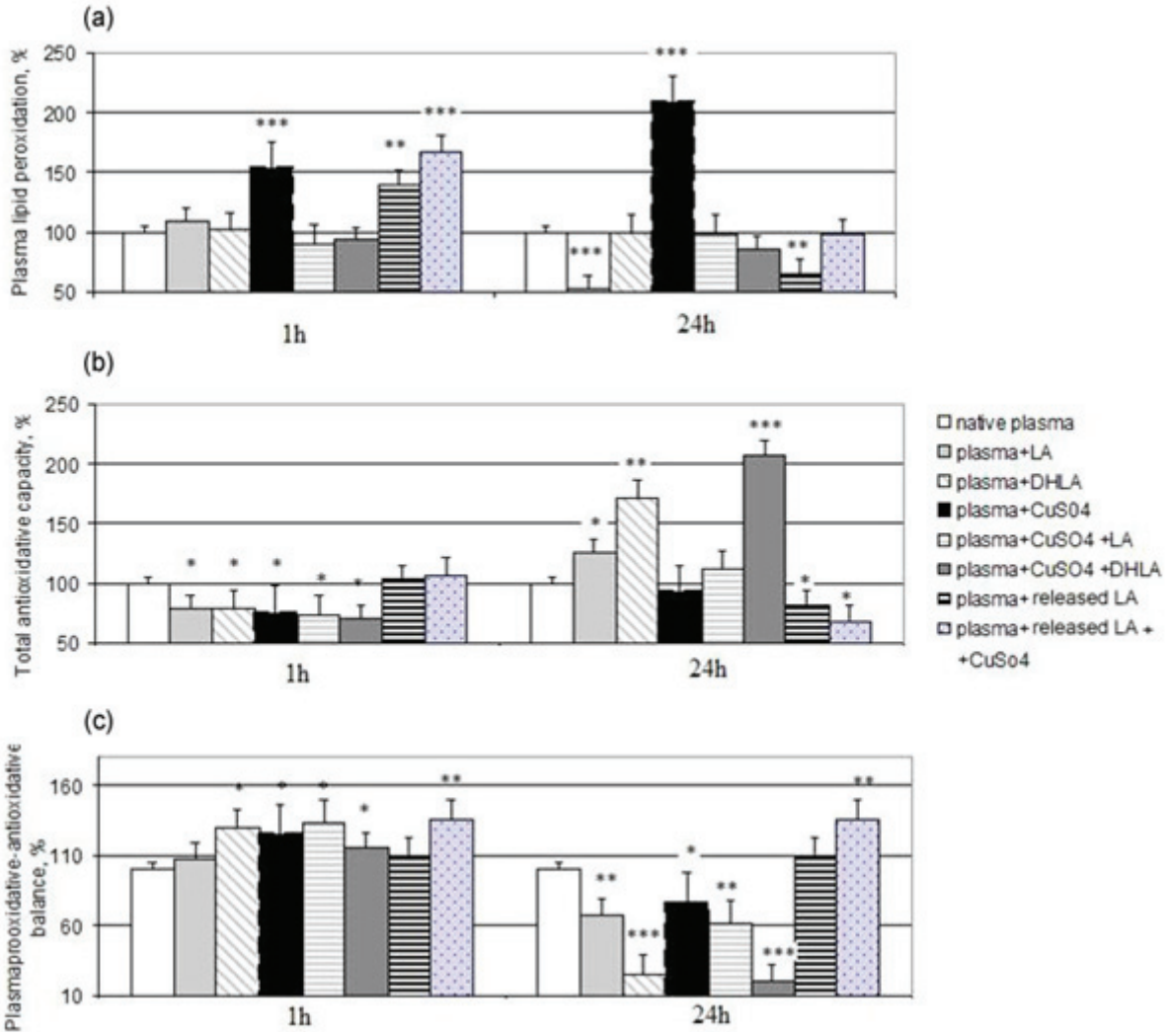

*Figure 6. The effect of LA, DHLA and LA released from chitosan microparticles on plasma lipid peroxidation level (a), total antioxidative capacity (TAC) (b) and prooxidative-antioxidative balance (PAB) (c) in human plasma treated with $CuSO_4$ as oxidative reactions catalyst (***p < 0.001, **p < 0.01, *p < 0.05 vs. native plasma according to repeated measures ANOVA test).*

DHLA in the presence of cupric ion, indicating antioxidant properties of LA. This effect is more evident after 24 h incubation of plasma samples. The same pattern of influence was noticed from prooxidant-antioxidant balance. At the same time, antioxidative plasma capability measured through the TAC level was abrogated upon Cu^{2+} influence and revitalized through the antioxidative LA and DHLA protection.

CONCLUSIONS

In conclusion, this study showed the alginate/gelatin hydrogel microparticles are unable to release LA in a controlled manner. However, the presented results indicated the potential benefit of using chitosan microparticles for controlled release of LA and sufficient antioxidative protection during the prolonged period of time.

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IZVOD

INKAPSULACIJA α-LIPONSKE KISELINE U HITOZANSKE MIKROČESTICE I MIKROČESTICE ALGINATA I ŽELATINA I *IN VITRO* ANTIOKSIDATIVNA AKTIVNOST

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(Naučni rad)

Alfa-liponska kiselina je organosumporno jedinjenje koje ima poznati terapeutski potencijal i izražena antioksidativna svojstva. Relativno kratko poluvreme eliminacije i hemijska stabilnost α-liponske kiseline prema faktorima iz spoljašnje sredine mogu se modifikovati njenom inkapsulacijom na čvrste nosače. Alfa-liponska kiselina iz egzogenih izvora se brzo apsorbuje, distribuira do tkiva, odnosno ćelija u kojima se redukuje u dihidroliponsku kiselinu, a potom brzo uklanja iz ćelija i eliminiše. Prema rezultatima *in vitro* i *in vivo* istraživanja α-liponska kiselina kao i njen redukovani oblik, dihidrolipoinska kiselina, imaju izražena antioksidantivna svojstva. U ovom radu izvršena je inkapsulacija α-liponske kiseline u hitozanske mikročestice dobijene reverznom emulzionom tehnikom. Takođe, ispitana je i mogućnost inkapsulacije α-liponske kiseline u mikročestice alginata i želatina koje su umrežene jonima dvovalentnog cinka. Inkapsulacija α-liponske kiseline u oba nosača izvršena je metodom bubrenja, odnosno potapanjem sintetisanih suvih čestica nosača u rastvor α-liponske kiseline pod strogo kontrolisanim uslovima. Interakcija α-liponske kiseline sa nosačima potvrđena je primenom infracrvene spektroskopije sa Furijerovim transformacijama. Nakon inkapsulacije α-liponske kiseline u ispitivane nosače *in vitro* studijom otpuštanja u simuliranim uslovima gastrointestinalnog trakta potvrđeno je kontrolisano otpuštanje α-liponske kiseline iz hitozanskih mikročestica. Efikasnost inkapsulacije α-liponske kiseline iznosila je do 53,9%. U *in vitro* eksperimentima pokazan je zadovoljavajući nivo antioksidativne aktivnosti otpuštene α-liponske kiseline iz hitozanskih mikročestica. Ovi rezultati ukazuju da hitozanske mikročestice mogu biti pogodni nosači za kontrolisano otpuštanje α-liponske kiseline.

Ključne reči: Alfa-liponska kiselina • Mikročestice • Hitozan • Natrijum-alginat/ /želatin • Antioksidativna aktivnost