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Milk protein-based formulations as controlled delivery systems for tolfenamic acid

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Casein-based gels were examined as potential drug carrier for a model drug, namely tolfenamic acid (TA). TA is widely applied as anti-cancer agent along with its ability to induce degradation of specific tumor proteins and decrease metastasis in liver in the case of pancreatic cancer. Casein-based spheres were formulated at high pH by ionotropic gelation in the presence of crosslinker $CaCl_2$. To optimize their chemical content and structure, casein concentration, TA concentration and casein/crosslinker ratio were varied. Sizes and morphology of casein gels loaded with TA were examined. The structure's phase state was tested by differential scanning calorimetry. ATR-FTIR was used to establish the crosslinking process between casein and $CaCl_2$. The efficiency of the loading process of drug was calculated. Studies on the drug release kinetics were conducted under simulated physiological conditions.

Keywords*:* casein, nanoparticles, tolfenamic acid, drug delivery system

INTRODUCTION

Proteins are macromolecules which play significant role in human life. From the nutrition aspect, through their specific functions in the human body all the way to different medical applications, proteins are practically irreplaceable on a daily basis in our life. Some of the most important advantages of the proteins are the facts that depending on the pH conditions they can be both negatively or positively charged, and they are able to interact with both hydrophobic and hydrophilic compounds [1]. Other significant characteristic of proteins is their affinity to bind ligands, hence they can be applied in specific targeted therapies. Proteins are also able to reduce the drug toxicity and extend the drug half-life [1]. These features make them desired potential drug carriers for different non-invasive paths of drug delivery [2]. One of the most examined proteins is the main milk protein, namely casein. It consists of 94% phosphoproteins (αS_1-, αS_2-, β-, and κ-casein in different ratios), which contain hydrophilic and hydrophobic domains, and 6% low-molecular weight compounds [3]. Casein is GRAS (generally recognized as safe) and it is quite often used in the preparation of different micro – and nanostructures due to its high stability, low price and low toxicity. Casein possesses other important physicochemical properties such as self-assembly, ability to bind with low-molecular compounds and ions. It is capable to form stable strong gels and bind large amounts of water. These properties are quite useful when casein-based structures are applied in the food and medical industries. The structures attracting highest interests are pH-responsive gels and particles, result of physical, chemical or even enzymatic crosslinking, that show great potential as sustainable or controlled drug release systems. Casein can screen UV radiation due to its strong adsorption properties in the range between 200 nm and 300 nm [3]. The biggest drawback of this compound is the possible immune or allergic reaction against it in lactose intolerant patients.

Tolfenamic acid is a non-steroidal anti-inflammatory drug acting as an inhibitor of cyclooxygenases (COX) which are primarily involved in inflammation processes. This compound has shown great potential in treatment of different types of cancer by inhibiting their specific proteins leading to shrinkage of the tumor tissue, hindering the synthesis of surviving cancer cells and inducing faster apoptosis with lower chances of metastasis [4]. However, it has two major disadvantages, namely low solubility and causing irritation in the gastrointestinal tract (GIT) after administration for long time [5]. Both of these shortcomings can be overcome with one step – encapsulation the active substance in a drug delivery system. In this way its solubility and bioavailability will be significantly increased and the presence of polymeric "package"

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will prevent the irritation of GIT.

In the present study the features of a drug delivery system for tolfenamic acid on the basis of casein were examined. The effect of the concentrations of the protein, the crosslinker and the active compound on the carrier surface morphology, loading efficiency and release rate were investigated.

EXPERIMENTAL

Used material

Sodium caseinate (casein sodium salt from bovine milk) was delivered by Sigma Aldrich. Tolfenamic acid was bought from Cayman Chemical. Calcium dichloride, ethanol and sodium hydroxide used were with analytical grade. Ultra-pure water was obtained with the system Adrona Crystal B30 Bio with conductivity 0.055 μS/cm.

Preparation of casein particles loaded with tolfenamic acid

The casein gels are formulated as a result of the electrostatic interactions between the negatively charged parts of the casein micelles and the positive divalent counter-ions of calcium chloride.

The studied systems in the current research were prepared according to the following procedure. Stock solution of sodium caseinate with 2% w/v concentration was prepared. The pH of the stock was adjusted to 11 with 1M NaOH and it was kept the same throughout the whole preparative process. This stock was diluted in a manner that the final concentrations of casein at the end of the mixing process were 0.5% and 1%. Tolfenamic acid was dissolved in pure ethanol at two different concentrations – 8 mg/ml and 4 mg/ml. 1 ml of this solution was added to each 4 ml of casein solution and the protocol was followed for both concentrations of each solution. The resultant mixture was stirred for 30 min at room temperature and after that time the crosslinker was added. 5% w/v $CaCl_2$ solution in three different ratios with respect to the concentration of the casein, namely 3:1, 5:1 and 10:1 was used as a crosslinker. The crosslinking process was left to be done for 2 hours. Then the reacting solutions were centrifuged for 15 min at 14000 rpm. The precipitate was washed twice and the obtained particles were freeze-dried and stored for further use. The samples were named as follows: Cas concentration (0.5% or 1%), Cas:$CaCl_2$ ratio (3:1; 5:1 or 10:1), amount of TA (20 mg or 40 mg).

Characterization of non-loaded and loaded casein particles

The size and the size distribution of the particles were examined using a NANOTRAC WAVE Particle Size, Zeta Potential, and Molecular Weight Analyzer (Microtrac). The hydrodynamic diameter of the particles was presented as the z-average size.

The shape, size and aggregation phenomena of both loaded and non-loaded particles were investigated by atomic force microscopy (AFM) AFM NANOSURF FLEX AFM (SWITZERLAND) and scanning electron microscopy (SEM) PRISMA E SEM, Thermo Scientific (USA). The particles, examined by AFM, were redissolved in distilled water and the sample suspension was deposited on a freshly cleaned microscopic glass. One minute after the deposition the surface was rinsed with distilled water. The sample was left to dry for 24 hours. The images were collected in tapping mode of the AFM using standard cantilever Tap190Al-G with 10 nm tip radius. The resultant picture showed 2.5 μm × 2.5 μm area from the sample surface with viewing field of 256 × 256 pixels collected for 1 s scan time. The samples for SEM were deposited onto double-face adhesive carbon tape and left to dry at room temperature (25°C). The SEM images of both non-loaded and loaded particles were obtained at 15 kV and 20 kV acceleration voltage.

In order to confirm both the crosslinking and the encapsulation processes, ATR-FTIR spectra were taken. A comparison between native and crosslinked casein, as well as between empty particles and loaded particles was done. The equipment for this experiment was Nicolet iS 10 FTIR spectrometer (Thermo Fisher Scientific, Pittsburgh, PA, USA), equipped with a diamond attenuated total reflection (ATR) accessory. The operating range for the spectra collection was 600 cm^{-1} to 4000 cm^{-1} with a resolution of 4 nm and 64 scans. The obtained spectra were analyzed with OMNIC® software package.

Determination of the yield of the gelation process

The yield of the gelation process was estimated as the ratio between the dry mass of the particles (after the freeze-drying) and the total dry mass in the formulation. The calculation was done according to the equation presented below:

$$Yield\ (\%) = \frac{dry\ mass\ of\ the\ particles}{total\ dry\ mass\ in\ the\ formulation} \cdot 100 \quad (1)$$

Calculation of the loading efficiency of casein particles

To establish the encapsulated amount of tolfenamic acid into the casein structures, their loading efficiency was calculated. After the particle formation process, they were centrifuged at 14000 rpm for 15 min and the resultant supernatant was measured at 287 nm with UV-VIS spectrophotometer. The encapsulated amount of TA was calculated by the following equation:

$$E\% = \frac{(c_0 - c)}{c_0}.100 \quad (2)$$

where c_0 is the total amount of tolfenamic acid and c is the non-loaded tolfenamic acid.

Investigation of the release kinetics of tolfenamic acid from casein particles

The release kinetics of the active compound from the resultant casein structures was examined on the base of the diffusion method in a dialysis bag. A certain amount of loaded particles (equivalent to 10 mg of tolfenamic acid) was suspended in 1 ml of PBS (pH = 7.4) and placed in a dialysis membrane. Then, the membranes were placed in 25 ml of release medium. For the first two hours the pH of the medium was 1.5 (hydrochloric acid buffer), for the next two pH = 4 (acetate buffer) and for the next time period the pH was kept at 7.4 (PBS). During the whole process the temperature was kept 37° C and the stirring speed was 100 rpm. For the spectrophotometric analysis, samples at each hour for a consecutive 8-hour period were taken and an aliquot amount of the same buffer was added back to the solution. All the samples were examined in triplicate. The samples were analyzed at 287 nm.

Investigation of the tolfenamic acid physical state

The TA phase state was examined by the method of differential scanning calorimetry. It was performed on a TA Discovery 250 (TA instruments, New Castle, DE, USA) at a heating rate of 10 °C/min for a temperature ranging from 25 to 350 °C in an argon environment with a purging rate of 30 mL/min. Aluminum T-zero containers were used to seal the samples. The onset temperature, peak temperature and normalized enthalpy (calibrated against indium melting enthalpy standard) were analyzed by TA TRIOS software 5.1.1.46572.

RESULTS AND DISCUSSION

In the present research, structures of casein micelles crosslinked with calcium chloride were successfully formulated at different concentrations of the polymer and the crosslinker by the method of ionotropic gelation. Their size and size distribution were investigated by dynamic light scattering technique. The average sizes of the structures are presented on Figure 1. No sizes were detected for the sample with 0.5% casein concentration and polymer:crosslinker ratio 10:1, possibly due to the low yield and concentrations. In the process of examination, a bimodal distribution was observed for all detected samples. About 70% of the particles possessed sizes in the nano-range - between 140 nm and 370 nm depending on the casein concentration and Cas:$CaCl_2$ ratio. The other structures were in the μm range and were most likely aggregates of nanoparticles.

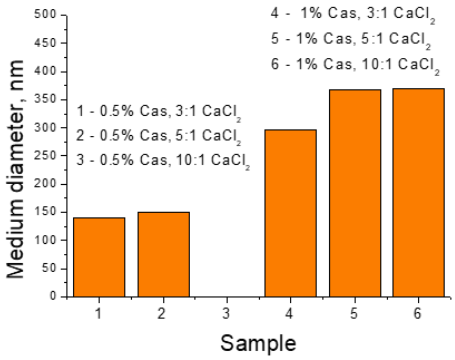

Fig. 1. Medium diameter of the formed casein structures with different concentrations of the polymer and the crosslinker.

The particle sizes strongly depend on both polymer and crosslinker concentrations. They are 140 nm (Cas:$CaCl_2$=3:1) and 150 nm (Cas:$CaCl_2$=5:1) when 0.5% casein solution is used, and grow to 296 nm (Cas:$CaCl_2$=3:1), 368 nm (Cas:$CaCl_2$=5:1) and 370 nm (Cas:$CaCl_2$=10:1) at polymer concentration of 1.0%. As it is shown on the graph, for the same concentrations of casein, smaller sized particles are formed with the highest amount of $CaCl_2$. This result is a consequence of the mechanism of micelle crosslinking – the higher the concentration of the crosslinker, the denser micelle structure is formed.

It could be concluded, based on the size examination, that the concentration of casein influences more significantly the size distribution of the particles than the crosslinker concentration.

Couple characteristics of the particles like shape, size, morphology and aggregation were examined with AFM and SEM techniques. A microphotograph of empty casein particles and their cross-section profile is presented on Fig. 2. As can be seen from the figure, some of the particles are stuck together,

resulting in bigger aggregates. This fact, together with the cross-section image, confirms the results from the DLS. The particle's shape is not quite spherical, but closer to irregular oval one. Possible reason for this could be the result of the internal characteristic distances reported from a SAXS experiment. Similar images were observed by other authors [6]. The formed structures are uniformly distributed in the investigated area, suggesting that the particles' solution is homogenous.

Similar irregular oval shape of the empty particles is also observed in the SEM images, which are shown on Fig. 3. After the encapsulation of tolfenamic acid, there is a variation of the morphology. Probably, as a result of the entrapment of the drug and its crystallinity state, the appearance of the micelles changed from oval to elongated rod-like shape. In addition to this, loaded particles also aggregated into branch-like islands of particles. This turns out to be a common behavior of casein micelles loaded with crystalline drug [7, 8].

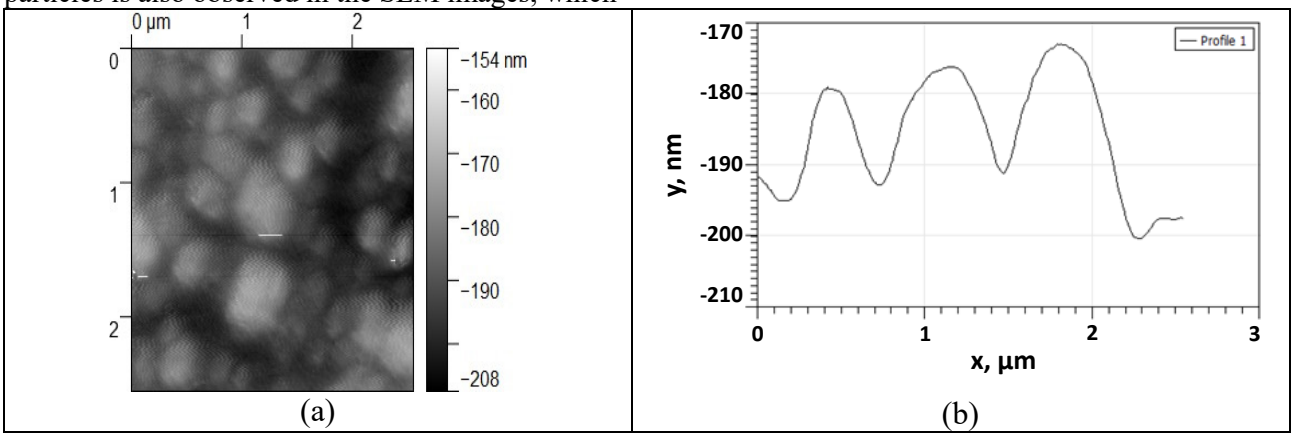

Fig. 2. AFM image of empty casein particles (a) and cross-section (b)

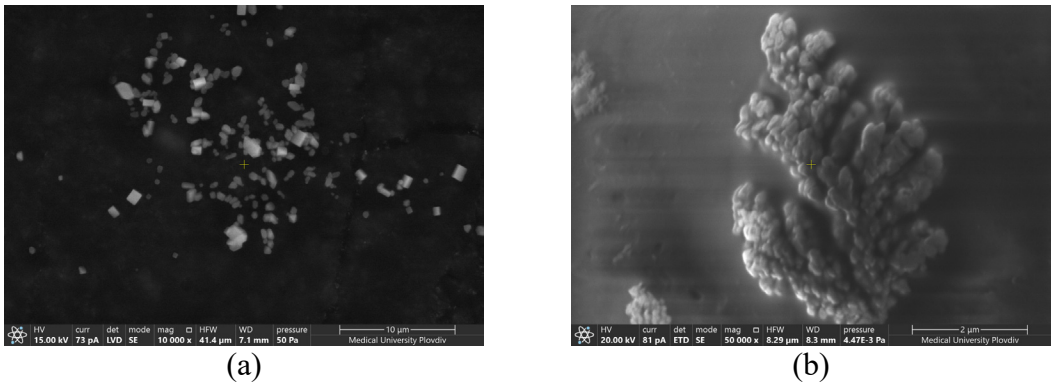

Fig. 3. SEM images of non-loaded (a) and loaded (b) casein particles

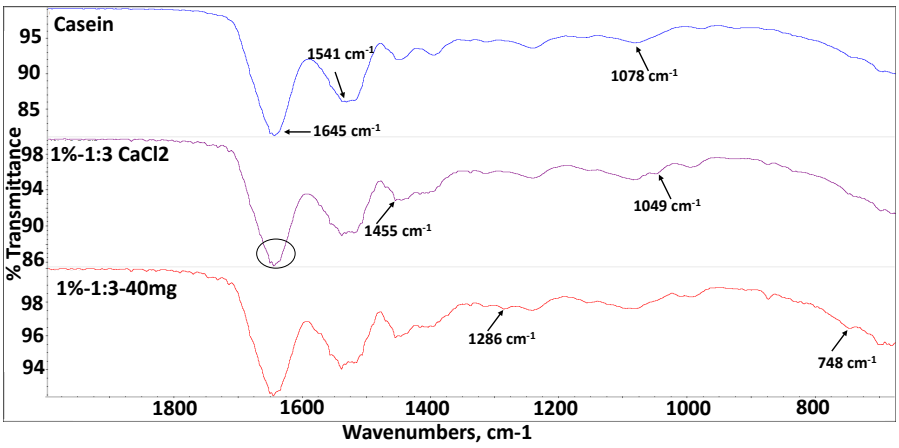

Fig. 4. Characteristic ATR-FTIR spectra of casein (blue line), empty casein particles (purple line) and casein particles, loaded with tolfenamic acid (red line).

Table 1. Particle yields (%) and encapsulation efficiencies (%) of all model samples

Sample name	Yield, %	Loading efficiency, %	Sample name	Yield, %	Loading efficiency, %
0.5%, 3:1, 20 mg	28.39	52.78	1%, 3:1, 20 mg	45.76	71.63
0.5%, 5:1, 20 mg	22.00	52.11	1%, 5:1, 20 mg	44.50	68.55
0.5%, 10:1, 20 mg	6.41	34.22	1%, 10:1, 20 mg	24.54	48.96
0.5%, 3:1, 40 mg	69.92	73.58	1%, 3:1, 40 mg	66.19	86.43
0.5%, 5:1, 40 mg	64.15	83.21	1%, 5:1, 40 mg	59.02	83.41
0.5%, 10:1, 40 mg	8.73	51.48	1%, 10:1, 40 mg	29.20	56.57

The processes of crosslinking of casein micelles by Ca^{2+} ions and the loading of tolfenamic acid into the casein particles are confirmed by ATR-FTIR analysis.

As it is shown in Fig. 4, the characteristic band of amide I at 1645 cm^{-1} (C=O stretching vibration coupled with the bending vibration of NH) [9] changes its shape and transforms from doublet to triplet demonstrating that the carbonyl group might be involved in interaction with calcium. The new band at 1455 cm^{-1} that occurs in the particle's spectrum can be associated with the formation of a complex between the carboxylate ion and calcium [10]. A second new band occurs at 1049 cm^{-1}, suggesting that the phosphate group might involve in the calcium binding [9]. These results therefore demonstrated that Ca^{2+} binding to the casein most likely occurred through interaction with the carbonyl group and the phosphate group, and the presence of calcium might induce the conformational change.

The FTIR spectrum of casein particles formulated in the presence of tolfenamic acid in the reacting solution is characterized with two new bands appearing at 1286 cm^{-1} (C-H stretch deformation) and at 750 cm^{-1} (C-N stretch deformation). They indicate that the tolfenamic acid is loaded to the casein particles without a presence of chemical interactions [11].

To characterize the potential of casein gels loaded with tolfenamic acid as drug delivery systems, both yield and loading efficiency of all variations of the system were examined (Table 1). The models with the highest loading efficiency and yield are those with casein:crosslinker ratio 3:1. This is a result of the densest packed micelle due to the stabilizing properties of $CaCl_2$ towards the casein micelles [12]. These two factors correlate with each other, because the increased casein concentration results in higher micelle concentration, which contributes to more hydrophobic interactions. Thus, they have better loading efficiency and inflated yield [13].

The phase state of the drugs (crystal *versus* amorphous) is one of the most important physical parameters which influence their bioavailability. The thermal behavior of TA in pure and in loaded state was studied by the method of DSC (Fig. 5). The melting peak of crystalline TA was observed at ~214°C, which is close to the values cited in the literature [14]. No peak is observed for the casein particles with loaded TA except for the sample with 0.5% casein concentration, casein:$CaCl_2$ ratio 3:1 and 40 mg loaded TA, suggesting the conversion of crystalline TA into amorphous form. The TA loaded in the sample 0.5%. 3:1, 40 mg is partially crystal with degree of crystallinity 12 %. In this case, as far as the TA concentration is high, it might migrate to the surface of the nanoparticles and form small irregular crystals, which melt at lower temperature than the crystal of neat TA.

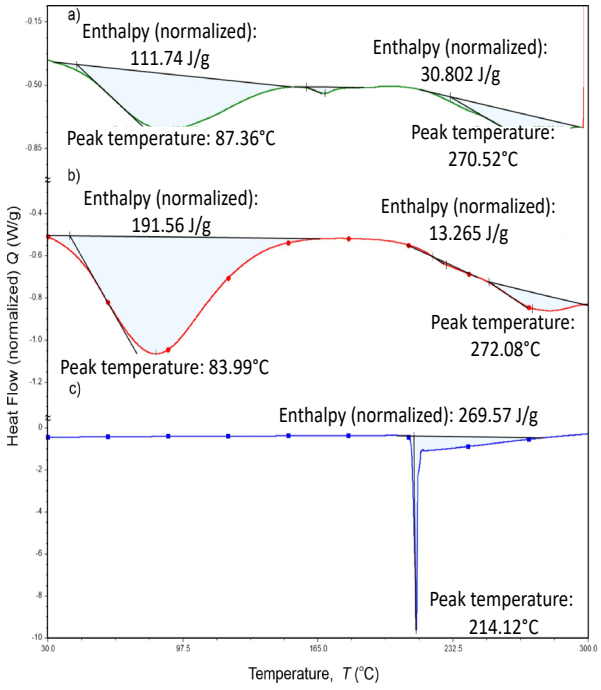

Fig. 5. DSC thermograms of TA (blue line), empty casein particles (red line) and casein particles loaded with TA (green line).

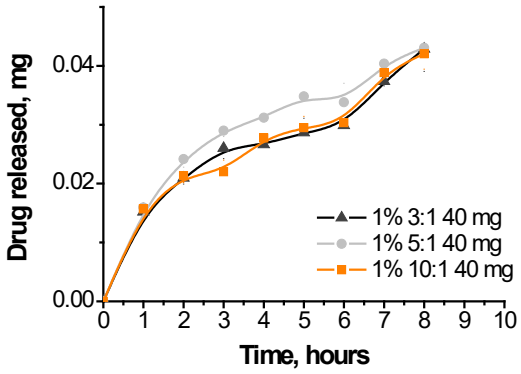

Fig. 6. TA release from casein nanoparticles with different crosslinker concentration

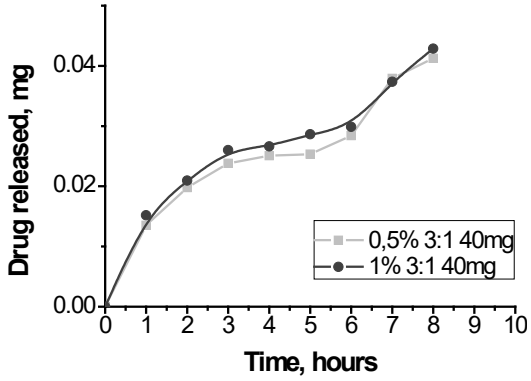

Fig. 7. TA release from casein nanoparticles with different protein concentration

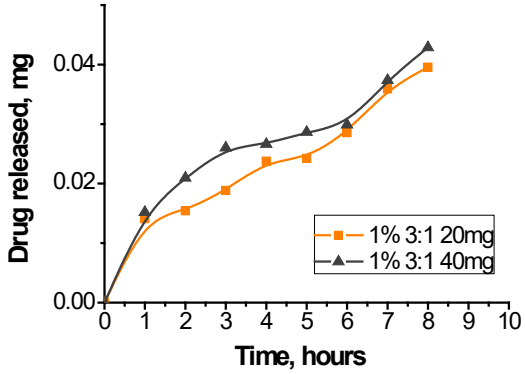

Fig. 8. TA release from casein nanoparticles at different drug concentration

The cumulative release of TA in different media at pH 7.4 and 37 °C for the first 8 hours is shown in Figures 6-8. The cumulative TA release is not more than 0.6 % during this period, demonstrating that no burst effect is realized. Hence, TA is well encapsulated in the casein gels. The slow release of TA could be due to its hydrophobic properties and its difficult dissolution in water media. The slower release is observed between the second and the fourth hour, when the nanoparticles are immersed in acetate buffer at pH 4. This pH is very close to the isoelectric point of casein and in this case the polymer is prone to precipitation [3]. Hence, the matrix is the densest and the drug diffusion is very difficult.

The increase of crosslinker concentration leads to delayed release (Fig. 6). Similar results have been reported by Baimark and Srisuwan [15] who found that the amount of released drug goes down when the concentration of Ca^{2+} crosslinker in alginate gels rose from 5% to 10% due to harder swelling of the alginate network.

Increasing the concentration of the casein solution from which the submicron gel particles were formed from 0.5% to 1% results in an increase in the TA release rate – Fig. 7. These results could be explained with the fact, that at the lower protein concentration the particles are less dense and part of the drug migrates from the bulk to the surface of the particles. This assumption is confirmed by the DSC experiments. Once the drug has come to the surface, its release is faster.

The dependence of the drug concentration on the release rate is presented in Fig. 8. The increase of the drug concentration leads to faster release as a result of the looser structure of the matrix.

The obtained results demonstrated that the TA release profile could be controlled by the concentration of casein and crosslinker.

CONCLUSION

TA-loaded casein gels crosslinked with $CaCl_2$ were investigated in this study. The average size of the gel particles varied from 140 nm to 380 nm, depending on the casein concentration and casein:crosslinker ratio. The loading of TA into the nanospheres did not lead to chemical interactions between the matrix and the drug. The loaded TA was predominantly amorphous, which increased its bioavailability.

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