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Biopolymeric Micro- and Nanoparticles as a Delivery System for Benzydamine

Abstract

On a dissertation
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Scientific supervisors: Prof. Maria Marudova-Zsivanovits, PhD
and Assoc. Bisera Pilicheva, PhD

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This dissertation contains 159 pages, in which 46 figures and 13 tables are presented. 162 literary sources were used.

The PhD student works as an assistant professor at the Department of Physics at the Faculty of Physics and Technology of “Paisii Hilendarski” University of Plovdiv.

The experimental research related to the dissertation work was carried out at the Department of "Physics" at the Faculty of Physics and Technology of Plovdiv University "Paisii Hilendarski", in the laboratories of the Technology Center at the "Paisii Hilendarski" PU, at the Department of Pharmaceutical Sciences at the Faculty of Pharmacy of MU-Plovdiv and in the laboratories of the Department of Pharmaceutical Technologies at the University of Szeged, Hungary.

In relation with this dissertation work, 3 scientific articles have been published in indexed journals. The results have been presented at 5 international scientific forums.

The dissertation work was discussed and proposed for defense to the departmental council of the Department of Physics at the Faculty of Physics and Technology of the PU "P. Hilendarski", held on 28.06.2024. The open final meeting of the scientific jury will take place on 27.09.2024 in “Compass” hall of University of Plovdiv before a jury composed of:

Assoc. prof. Asya Petrova Viraneva, PhD
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Reserve members:
Assoc. prof. Ivanka Yordanova Vlaeva, PhD - external
Assoc. prof. Ginka Kalcheva Exner, PhD – internal

The defense materials are available for free access to those interested in the library of "Paisii Hilendarski" Plovdiv University.

CONTENTS

Abbreviations and designations used:
AFM – atomic force microscopy
BAS – biologically active substance
BHCl – Benzydamine Hydrochloride
Cas – Casein
Chit – Chitosan
DLS - dynamic light scattering
DSC – differential scanning calorimetry
EE – encapsulation efficiency
NaTPP – sodium tripolyphosphate
PE – polyelectrolytes
PEC - polyelectrolyte complexes
SEM – scanning electron microscopy
TEM – transmission electron microscopy
FTIR – Fourier Transform Infrared Spectroscopy

I. INTRODUCTION

Polymers are proving as increasingly successful basis for developing a new generation of materials and composites, thanks to their high functionality and diversity of their structure and properties. Underlying reason for this increased attention is the fact that they can form structures with different architectures and morphologies through a wide range of preparation methods. Bipolymers, distinguished by biocompatibility, biodegradability, non-immunogenicity and lack of toxicity, attract special attention.

The influence of polymer micro- and nano-sized particles in the field of pharmacy in the preparation of drug delivery systems is significant. This type of structure allows for an individual approach tailored to the needs of the respective therapy and the selected active substance. Due to their significantly smaller size and high biotolerance, these structures can pass through different biological membranes (e.g. blood brain) or deliver the drug inside the cells themselves. Their surface functionalization allows a specific interaction with receptors or a certain type of cells, which ensures the delivery of an active substance in a certain target (the so-called targeted delivery). In the case of drug delivery through a polymer matrix, the possible routes of administration are significantly increased, and the local application of the therapeutic system is essential. In addition, these structures allow modulation of the release profile, providing higher therapeutic efficacy and prolonged circulation of the active substance, while potentially shortening the required number of administrations and treatment time.

Despite the availability of already existing drug delivery systems, the development of a polymer structure with a high encapsulation efficiencies and yield as well as a suitable release profile for water-soluble drugs is quite a challenge. Due to their high solubility, the active substances quickly migrate back into the aqueous medium or release within a few minutes, not allowing prolonged release. The creation of particles based on biopolymers of different biological origins (eg, polysaccharide and polypeptide), as well as the wide variety of methods for obtaining structures, enable diverse combinations and approaches to this problem. In addition, the structures themselves must have an appropriate size, conformation to the site of potential application, morphology, and physicochemical properties and characteristics.

II. GOAL AND OBJECTIVES

After a literature review and systematization of publications dealing with chitosan and/or casein-based structures, no sufficiently detailed studies were found regarding their potential as a benzydamine hydrochloride delivery system with controlled release.

Therefore, the main objective of the present work is to develop micro- and nano-sized structures based on biodegradable and biocompatible polymers to serve as a drug delivery matrix for the controlled release of the substance benzydamine hydrochloride.

In order to achieve this goal, the following implementation tasks have been formulated:

1) Development and characterization of benzydamine hydrochloride-loaded chitosan nanoparticles that were prepared by ionotropic gelation while varying the concentration of polymer and crosslinker.
2) Development and characterization of benzydamine hydrochloride-loaded casein microparticles that were prepared by ionotropic gelation in acidic pH while varying the concentration of polymer and crosslinker.
3) Preparation and characterization of casein/chitosan polyelectrolyte complex loaded with benzydamine hydrochloride at different ratios of the complex-forming partners.
4) Preparation and study of chitosan particles (empty and loaded with benzydamine hydrochloride) by spray drying in the presence of crosslinker and excipient.
5) Preparation and study of benzydamine hydrochloride-loaded casein nanoparticles prepared by spray drying while varying the polymer/drug ratio.
6) In vitro study of the release profile of the medicinal substance benzydamine hydrochloride from the developed micro- and nano-sized structures.

III. MATERIALS AND METHODS

3.1. Materials

The following reagents were used in the present work: low molecular weight chitosan (Glentham life sciences, Great Britain), sodium salt of casein obtained from cow's milk (Sigma Aldrich, Germany), calcium dichloride dihydrate (Valerus, Bulgaria), sodium tripolyphosphate (Sigma Aldrich, Germany), benzydamine hydrochloride (Sigma Aldrich, Germany).

3.2. Methods for obtaining micro- and nano-sized particles

3.2.1. Ionotropically cross-linked chitosan particles with sodium tripolyphosphate

A solution of chitosan with concentrations of 0.1%, 0.2% and 0.3% in 2% acetic acid was used. A 5% solution of sodium tripolyphosphate (NaTPP) is added dropwise into the volume of the polymer solution, under constant stirring, until polymer:crosslinker mass ratios of 3:1, 5:1 and 7:1 are reached, respectively. This mixture was stirred at 1500 rpm for 2 h at a temperature of 25 ºC. The gel particles were separated from the reaction mixture by centrifugation for 30 min and for 15 min after washing with water at 15,000 rpm. The obtained structures were lyophilized for 72 h at 10 Pa pressure and a temperature of –50 ºC, after which they were stored in a desiccator at RH 20%. For the synthesis of drug-loaded particles, the drug was added to the chitosan solution and homogenized for 30 min on a magnetic stirrer.

3.2.2. Ionotropically cross-linked casein particles with sodium tripolyphosphate

A solution of casein (1% or 2%) in highly acidified water (pH = 3) was used to form these particles. To a certain volume of the polymer solution, under constant stirring, sodium tripolyphosphate is dripped in a mass ratio of 1:3, 1:5 and 1:10 in favor of the polymer. The solution was stirred for 2 h at 1500 rpm and temperature of 25 °C. Colloidal particles were separated by centrifugation at 14,000 rpm for 15 min, rinsing with distilled water and centrifugation again at the same rpm for 10 min. The resulting structures were lyophilized for 72 h and stored in a desiccator at RH 20%. For the drug-loaded systems, the drug was added to the casein solution 30 min before cross-linking.

3.2.3. Ionotropically prepared polyelectrolyte complex between chitosan and casein

0.5% polymer solutions were used. Chitosan was dissolved in 3% acetic acid and casein in distilled water. Immediately before their mixing, the pH of the chitosan solution increased to 5, while that of the casein remained unchanged (pH ≈ 7). 5 types of particles were obtained based on different molar ratios between the active groups of the complexing polyelectrolytes - 5:1, 3:1, 1:1, 1:3 and 1:5 (chitosan:casein) at pH = 6. The thus obtained colloid the system was allowed to stir for 15 min at 1500 rpm on a magnetic stirrer, then sonicated in an ice bath (UP100H Hielscher Ultrasonics GmbH, Teltow, Germany) for another 20 min at maximum amplitude and 80% fill factor. The gel particles were centrifuged for

15 min at 14,000 rpm and lyophilized at a pressure of 10 Pa and a temperature of −50 °C for 72 h. Stored in a desiccator at RH 20% When obtaining particles with loaded BHCl, the drug was added to the chitosan solution 30 min before mixing with casein.

3.2.4. Chitosan particles obtained with Büchi Mini Spray dryer (B-191)

A solution of chitosan with different concentrations (0.1%, 0.2% and 0.3%) in 2% acetic acid was prepared. Three types of particles were obtained: chitosan particles (uncrosslinked), chitosan particles crosslinked with sodium tripolyphosphate and chitosan particles with mannitol added as an excipient. The first type of particles are sprayed from solutions of different concentrations. For the development of cross-linked particles, one chitosan concentration of 0.2% was fixed and 3 different concentrations of sodium tripolyphosphate (0.05%, 0.1% and 0.5%) were used. Particle systems containing mannitol were developed with 0.2% chitosan solution, achieving three mass ratios between them – 1:5, 1:7.5 and 1:10 in favor of mannitol. The drug is added 30 min before the crosslinking process or sputtering in mass ratio with chitosan 1:2 in excess of the polymer.

3.2.5. Casein particles obtained with Büchi Nano Spray dryer (B-90 HP)

A 1% solution of casein in strongly acidified water to pH=2 was used for the preparation of these particles. The cross-linking was carried out with a 1 M solution of calcium dichloride, with a concentration of 2 μL/mL. The thus obtained solution is homogenized for 15 min at 25,000 rpm, stirred on a magnetic stirrer for 30 min at 500 rpm. The solutions were then sprayed under the following conditions: mesh size – 4 μm, inlet temperature – 40 °C, solution pumping speed – 50%, spray intensity – 70% and drying gas flow rate – 120 L/min. Polymer:drug mass ratios of 1:1, 2:1, 4:1, 6:1 were achieved.

3.3. Methods for characterizing the resulting polymer structures

3.3.1. Dynamic light scattering

Sizes of the obtained hydrogel particles were determined by a Nanotrac particle size analyzer (Microtrac, York, PA, USA) using a helium/neon laser with a power of 3 mW and a wavelength of 780 nm.

3.3.2. Scanning Electron Microscopy (SEM)

To characterize the morphology of the structures, small amount of samples in the dry state are used, which are metallized with a 5 nm gold coating in a vacuum environment with a QUORUM Q 150T ES Plus. Images were obtained by accelerating electrons with energies between 10 and 15 kV (Prisma E SEM, Thermo Scientific, Waltham, MA, USA).

3.3.3. Atomic force microscopy (AFM)

A volume of 150 μl of particle solution was dropped onto a microscope slide and allowed to dry. The samples thus obtained are examined in tipping mode with a Nanosurf FlexAFM atomic force microscope (Nanosurf AG, Liestal, Switzerland) with a cantilever with a 10 nm tip radius of the Tap190Al-G type.

3.3.4. Yield

For each developed system, the yield was calculated as a quantitative characteristic of the efficiency of the process of obtaining them. The following expression was used for the calculation:

$$\text{Yield (\%)} = \frac{\text{Mass of the obtained sample}}{\text{Sum of the masses of the materials}} \text{ x } 100 \qquad \text{(III.1.)}$$

3.3.5. Amount of drug included

In order to determine the encapsulation efficiency (EE) of the drug, two methods were used depending on the type of system developed – direct and indirect.

3.3.5.1. Direct method

In this method, 10 or 20 mg of dry particles are placed in a dialysis membrane bag immersed in a volume of 10 mL of the desired medium (distilled water, acetic acid solution or buffer) for 72 hours. According to a previously prepared calibration curve, the amount of drug released at a wavelength of 306 nm in 1 mL of the medium was calculated and recalculated for the mass of the sample. The results are calculated using the expression:

$$\text{EE (\%)} = \frac{\text{mass of the drug included}}{\text{mass of the tested sample}} \text{ x } 100 \qquad \text{(III.2.)}$$

3.3.5.2. Indirect method

The indirect method for the determination of EE is based on measuring the extinction of the obtained supernatant after the first step of centrifugation of the samples. The amount of loaded drug is calculated by the following formula:

$$\text{EE (\%)} = \frac{(\text{initial amount} - \text{amount in supernatant})}{\text{initial amount}} \text{ x } 100 \qquad \text{(III.3.)}$$

3.3.6. Fourier Transform Infrared Spectroscopy (FTIR)

A Thermo Fisher Scientific, Pittsburgh, PA, USA, attenuated total reflection operating mode with a Nicolet iS 10 FTIR diamond head scanning in the range 600 to 4000 cm−1 with a scan resolution of 4 nm and 64 scan lines was used. .

3.3.7. Differential Scanning Calorimetry (DSC)

The thermal stability and phase state of the substances included in the composition of the final structures were investigated using a differential scanning calorimeter DSC 204F1 Phoenix (Netzsch Gerätebau GmbH, Selb, Germany). The investigated samples were tested after encapsulation in aluminum crucibles in the temperature range from 20 °C to 300 °C with a heating rate of 10 K/min.

3.3.8. In vitro release of benzydamine hydrochloride

In order to establish the release profile of the active substance from the polymer structures, a couple of model particles were selected for each type of system, with which an in vitro release test was conducted in an artificial saliva medium. For this purpose, a phosphate buffer with a pH of 6.8 is prepared with a

composition of: 1.44 g of disodium hydrogen phosphate, 0.245 g of potassium dihydrogen phosphate, 8.00 g of sodium chloride and 0.200 g of potassium chloride. A precalculated amount of particles equivalent to 5 mg or 10 mg of incorporated drug is placed in a dialysis membrane bag with 1 mL of buffer and this bag is immersed in 20 or 30 mL of the buffer. The release was carried out at a temperature of 37 ± 0.5 °C and constant stirring at a speed of 50 rpm. Samples are taken from the acceptor medium at predetermined time intervals. Same volume of pure buffer was added back to the medium, and the samples were analyzed spectrophotometrically at a wavelength of 307 nm according to a previously developed calibration curve. The dependence of the amount of drug released in percent of time is plotted.

3.3.9. Mathematical models used to describe the release process

Mathematical models are a suitable auxiliary tool for a better description and understanding of the drug release process and transport mechanism from the polymer matrix. The models applied for the present analyzes were: first-order model, Higuchi model, Korsmeyer-Peppas model, and Weibull model.

- A first-order model

$$\log C = \log C_0 - Kt/2{,}303, \qquad \text{(III.4)}$$

where: C_0 - initial drug concentration; K - first-order release rate constant expressed in s-1; t – release time.

- Korsmeyer-Peppas model

$$\frac{M_t}{M_\infty} = Kt^n, \qquad \text{(III.5)}$$

where: M_t/M_∞ - the part of the drug released during time t; K - specific release rate constant; n – the exponent index, indicating the release mechanism

- Higuchi model

$$Q = K_H t^{1/2}, \qquad \text{(III.6.)}$$

where: Q – accumulated released drug at time t; K_H – Higuchi's constant, which determines the release mechanism; t – release time

- Weibull model:

$$M = M_0 \left[1 - e^{-\frac{(t-T)^b}{a}}\right], \qquad \text{(III.7.)}$$

where: M – amount of drug released from the matrix at a given time t; M_0 - the total amount of the drug that is released from the system; T- parameter, which is responsible for the delay in the measured time as a result of the dissolution process, for our systems always T = 0; a - describes the dependence on time; b - describes the shape of the dissolution curve, takes values from 0 to 1.

IV. RESULTS AND DISCUSSION

IV.1. Formation of chitosan particles by ionotropic gelation in the presence of sodium tripolyphosphate

Ionotropic gelation occurs as a result of electrostatic interaction between the protonated NH3+ groups in the chitosan molecules and the negatively charged phosphate groups in the crosslinker (Antoniou et al. 2015).

5 types of chitosan particles loaded with benzydamine hydrochloride were developed by varying the polymer concentration (0.1% w/v; 0.2% w/v and 0.3% w/v) and the polymer:crosslinker ratio (Chit:NaTPP = 3:1; 5;1 and 7:1). The obtained hydrogel particles were characterized according to their size and size distribution, yield and drug incorporation efficiency. The parameters and characteristics of the obtained gel particles are presented in Table IV.1.1.

Table IV.1.1.: Sizes, yield and enapsulation efficiency of the developed particles when varying the concentration of the polymer or crosslinker (n=3).

Sample	Chit. Conc., %	Chit:NaTPP ratio	Yield ± SD, %	Sizes ± SD, nm	EE ± SD, %
F1	0,1	3:1	56 ± 4	81 ± 3	14,7 ± 0,2
F2	0,1	5:1	47 ± 2	63 ± 4	10,5 ± 0,3
F3	0,1	7:1	23 ± 3	25 ± 1	12,5 ± 0,4
F4	0,2	7:1	61 ± 5	460 ± 15	9,9 ± 0,1
F5	0,3	7:1	69 ±4	588 ± 23	24,3 ± 0,4

With increase in the polymer concentration, a significant rise in mean particle diameter was observed, which is consistent with previous research by another team (Rampino, et al. 2013). It is observed that the yield of the gelation process also increases significantly for the systems with higher polymer concentration, ranging from 23% to 69%. When higher concentrated polymer solutions are used, significantly larger particles are obtained, which are more easily separated from the production medium. The effect of the crosslinker is similar. When chitosan interacts with tripolyphosphate, both intra- and intermolecular bonds are formed through physical cross-linking (Thandapani, et al. 2017). When using a higher concentration of the oppositely charged ions, a greater number of macromolecules cross-link or participate in the formation of the gel network, due to the reduced distance between the individual chains. Therefore, an increase in the mean hydrodynamic diameter of the formed particles is observed (Kalam, et al. 2016). By using a larger amount of crosslinker, the gel network is denser and this leads to an improvement in the incorporation efficiency. The drug encapsulation efficiency values are relatively low – between 10% and 25%.

When examining the morphology of the obtained structures by atomic force microscopy (Fig. IV.1.1.), separate morphological units with a regular oval shape and sizes close to those determined by means of dynamic light scattering are observed. At the same time, a strong aggregation of individual particles observed.

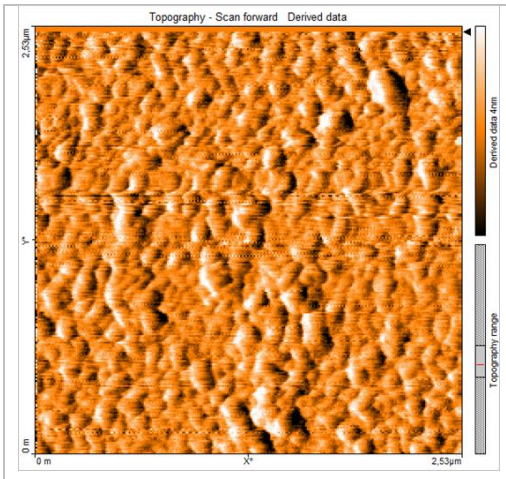


Fig. IV.1.1.: AFM image of chitosan (0,1%): NaTPP = 3:1, image scale 2,5 x 2,5 µm

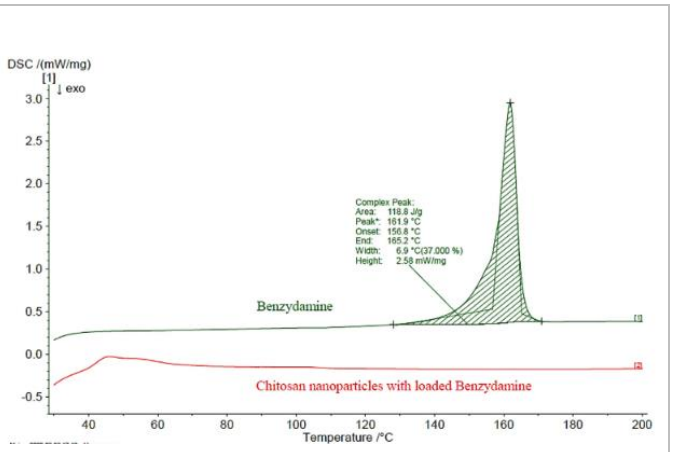


Fig. IV.1.2.: Thermogram of: pure BHCl (green line); BHCl-loaded particles (red line)

Using the DSC method, the phase state and thermal stability of the active substance before and after its inclusion in chitosan particles were investigated - fig. IV.1.2. Benzydamine hydrochloride is a water-soluble salt of the main substance benzydamine and is characterized by a highly manifested crystalline phase state (Alves, et al. 2020). When examining the pure substance, it was found that it melts at a temperature of 161.9 °C and the enthalpy of this transition is 118.85 J/g. In the thermogram of the drug incorporated in the particles, it is clearly seen that there is no peak in the range around the melting temperature of benzydamine, which means that it is in the amorphous phase state.

The physical cross-linking of the chitosan particles and the successful incorporation of the drug into the polymer matrix were confirmed by FTIR (Fig. IV.1.3. and Fig. IV.1.4). When benzydamine hydrochloride was included in the particles, no interaction with the carrier was observed.

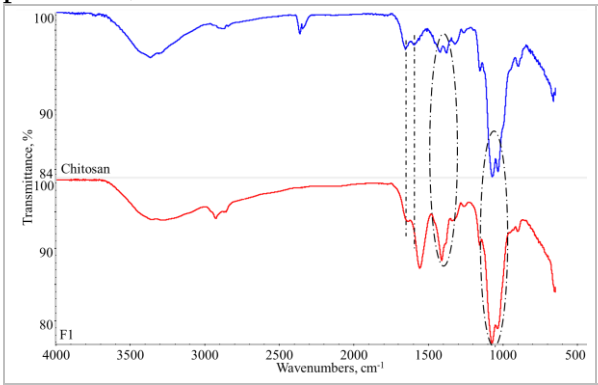


Fig. IV.1.3.: FTIR spectra of: chitosan (blue line) and crosslinked chitosan with NaTPP (red line)

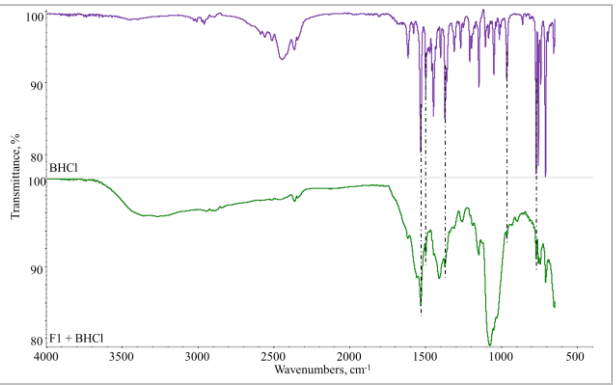


Fig. IV.1.4.: FTIR spectra of: BHCl (purple line) and BHCl-loaded particles (green line)

The results of in vitro simulation of BHCl release at pH = 6.8 are presented in Fig. IV.1.5. and fig. IV.1.6. The drug release profile for all systems is biphasic, characterized by an initial burst effect followed by a delayed and sustained release that is incomplete over a period of 6 hours. It is observed that the particles obtained from the solution with the lowest concentration of the polymer have the fastest release rate. Increasing its value to 0.2% and 0.3% leads to a delay in release. This delay is due to the formation of a layer of the gel as a result of the swelling of the chitosan, which hinders the diffusion of the drug to the acceptor medium (Katsarov, et al. 2017). Particles with the least amount of crosslinker (Chit:NaTPP = 7:1) released with highest rate, followed by those with a Chit:NaTPP = 5:1 ratio in favor of the polymer, and those with the lowest polymer ratio released the slowest (Chit:NaTPP = 3:1). This is due to the reduced space between individual macromolecules and the presence of entanglement in them (Ferreira Tomaz, et al. 2018). As a result, diffusion is hindered, and hence the amount of drug released is lower. In addition, particles with a smaller amount of crosslinker are also smaller in size, resulting in a larger contact surface between the particles and the external environment and easier diffusion of the drug substance (Gokce, et al. 2014).

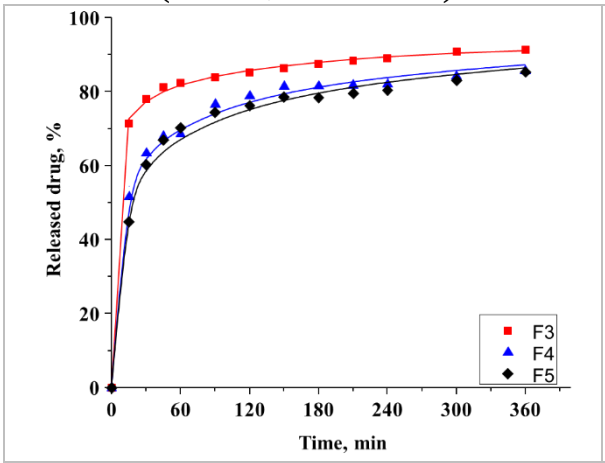


Fig. IV.1.5.: Release profiles of benzydamine hydrochloride from models with different polymer concentration

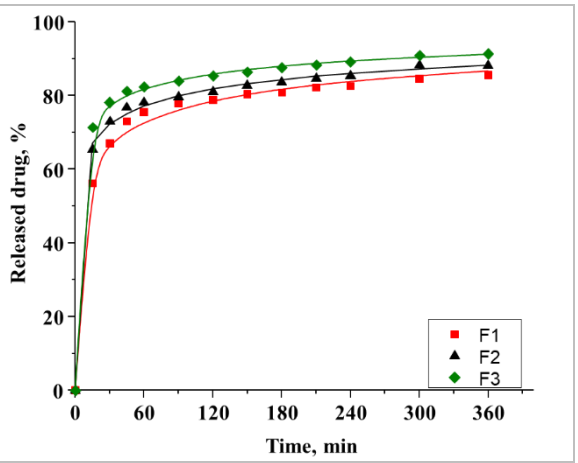


Fig. IV.1.6.: Release profiles of benzydamine hydrochloride from models with different degrees of cross-linking

After obtaining and processing the release results, they were analyzed and modeled by regression analysis according to the selected mathematical models. As an optimal model, based on the value of the coefficient of determination R^2 and the residues derived from the software used, the Weibull model was chosen as best fitting. According to the values obtained for the parameter *b*, which determines the shape of the curve, the type of mechanism responsible for the release process can also be determined. Corsaro et al. (2021) published that for values of *b* less than 0.75, the release mechanism is Fickian diffusion. For all presented types of particles, the value of this parameter is even less than 0.3,

which means that the release process is completely subject to Fick diffusion, i.e. in these cases, there is no dissolution of the drug-delivery system upon its contact with the acceptor medium, but only swelling.

IV.2. Formation of casein particles by ionotropic gelation in acidic media

In the present study, sodium tripolyphosphate cross-linked casein particles were developed by the ionotropic gelation method at highly acidic pH conditions, far from the isoelectric point of the protein. Systems were obtained based on solutions with different polymer concentration (1% w/v, 2% w/v), different crosslinker ratios (3:1, 5:1, 10:1) and presence (5% v/v) or lack of ethanol.

Based on the dynamic light scattering results, it is observed that all the obtained samples have sizes in the range of about (0.9 ± 0.1) μm to (4.3 ± 0.1) μm (Table IV.2.1.). It is noteworthy that these sizes are significantly larger compared to the same particles obtained at pH above neutral, where casein is negatively charged (Głąb, et al., 2017). When the protein is dissolved at pH < 4.6, the enveloping layer of κ-casein is degraded, which leads to the reduction of the charge on the surface of the micelles, and from there to their aggregation (Vasbinder, et al., 2003).

Table IV.2.1.: Sizes, yield and encapsulation efficiency of the different types of particles (n=3)

Sample	Conc. of Cas, % w/v	Cas:NaTPP ratio	Ethanol conc., % v/v	Sizes ± SD, μm	Yield ± SD, %	EE ± SD, %
S1	2	3:1	5	3,9 ± 0,1	64,4 ± 3,4	14,7 ± 0,8
S2	2	5:1	5	3,2 ± 0,1	75,7 ± 4,8	22,2 ± 1,2
S3	2	10:1	5	2,2 ± 0,1	68,9 ± 4,0	9,9 ± 0,5
S4	2	3:1	0	2,0 ± 0,1	66,6 ± 4,9	19,2 ± 1,0
S5	2	5:1	0	0,9 ± 0,0	73,8 ± 4,3	22,4 ± 1,3
S6	2	10:1	0	2,4 ± 0,1	55,3 ± 3,6	5,4 ± 0,3
S7	1	3:1	5	2,5 ± 0,1	65,2 ± 4,6	8,0 ± 0,5
S8	1	5:1	5	1,6 ± 0,0	76,1 ± 4,3	13,7 ± 0,8
S9	1	10:1	5	4,3 ± 0,1	72,5 ± 4,2	4,6 ± 0,3
S10	1	3:1	0	1,2 ± 0,0	63,9 ± 4,6	18,2 ± 0,9
S11	1	5:1	0	2,1 ± 0,1	71,4 ± 4,4	12,5 ± 0,7
S12	1	10:1	0	2,2 ± 0,1	78,3 ± 4,9	10,6 ± 0,6

As a result, significantly larger particles with a high tendency to form aggregates are observed. The tendency to form aggregates with larger sizes increases with decreasing polymer concentration and presence of ethanol in the polymer solution. This is a result of both the impaired stability of the micelle,absence of κ-casein layer, and due to the presence of alcohol. This effect is known as ethanol induced micelle collapse. It is expressed in two main effects:

destabilization due to disruption of the surface roughness layer and changes in the equilibrium of calcium phosphate, reported and observed as early as 1985. The presence of a crosslinker in a higher concentration generally leads to a reduction in dimensions due to the formation of denser packing of the micelles. Probably the surface charge of the particles is increased, which leads to their mutual repulsion and stabilization in the colloidal system. The lower concentration of the crosslinker is a prerequisite for the formation of a looser network between the individual chains and micelles, which makes them more susceptible to swelling in solution, hence they have bigger sizes. In the systems obtained from solutions with the higher concentration of casein and the presence of ethanol, an inverse trend for the sizes was observed when compared of the particles formed without ethanol. All types of particles obtained are having relatively high yield – between 55% and 78%, which is not affected by the presence of ethanol. Despite the significantly better yield compared to chitosan particles obtained by the same method, the benzydamine encapsulation efficiency was similar to theirs and ranged from 4.6% to 22.4%. Increasing the polymer concentration resulted in an increase in the efficiency of benzydamine incorporation into the particles, possibly due to an enhanced hydrophobic effect favoring the entrapment of the drug in the casein micelles (Elzoghby, et al. 2013a). As the crosslinker concentration decreases, the drug incorporation efficiency is also lower. Probably, the lower degree of crosslinking results in a too loose and porous polymer network.

The morphology of the obtained particles was investigated by both AFM and SEM techniques (Fig. IV.2.1. and Fig. IV.2.2.).

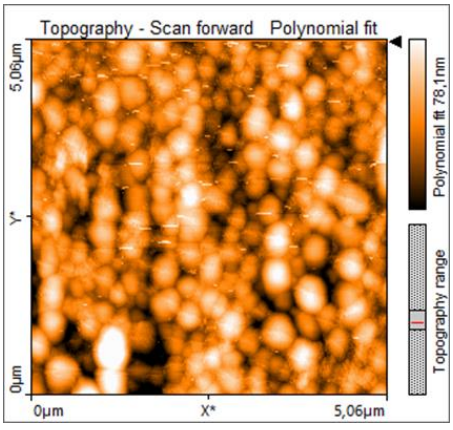
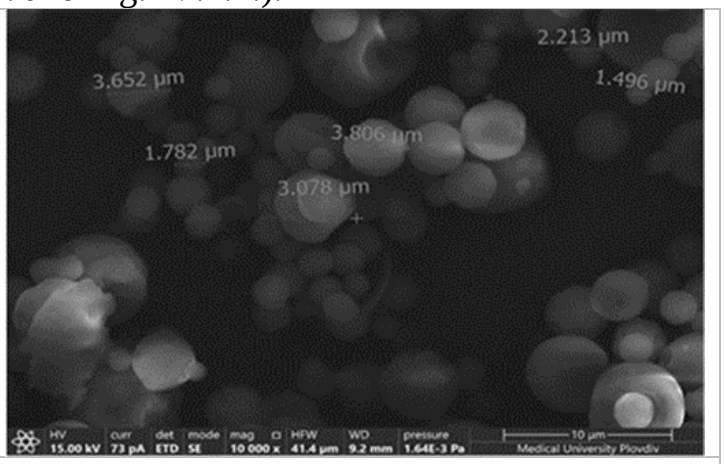


Fig. IV.2.1.: AFM image of cross-linked casein particles, image scale 5x5 µm



Фиг. IV.2.2.: SEM image of cross linked casein particles, scale bar dimension10 µm

Based on the images obtained by both techniques, the shape of the particles is predominantly oval-spherical, but defects are also observed. According to Elzoghby et al. (2013b), in acidic conditions, casein micelles can be sterically stabilized, similar to polyelectrolyte brushes, even at high crosslinker

concentrations, correspondingly lower surface charge. Because of this, distinct and clearly separated casein particles are observed.

Cross-linking of casein by sodium tripolyphosphate at pH below its isoelectric point was confirmed by FTIR (Fig. IV.2.3. and Fig. IV.2.4.).

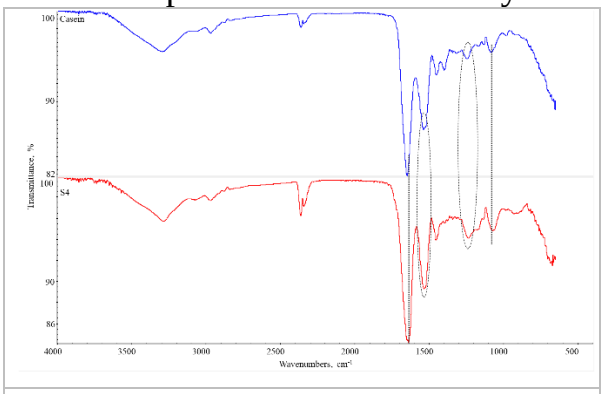


Fig. IV.2.3. : FTIR spectra of: casein (blue line), NaTPP cross-linked casein (red line)

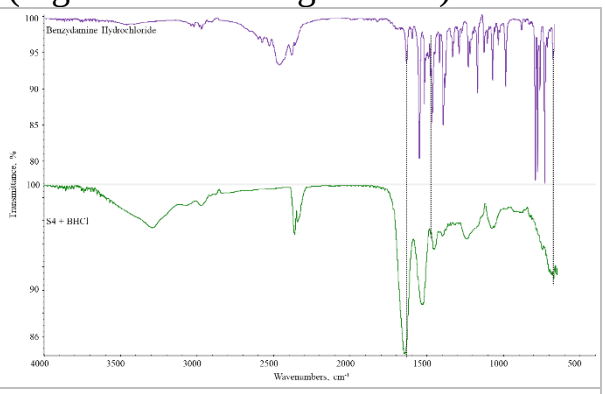


Fig. IV.2.4.: FTIR spectra of: BHCl (purple line), BHCl-loaded particles (green line)

Due to the lack of change or shift in the characteristic peaks of the drug, we consider that it is physically present in the particles, without any structural changes or interactions.

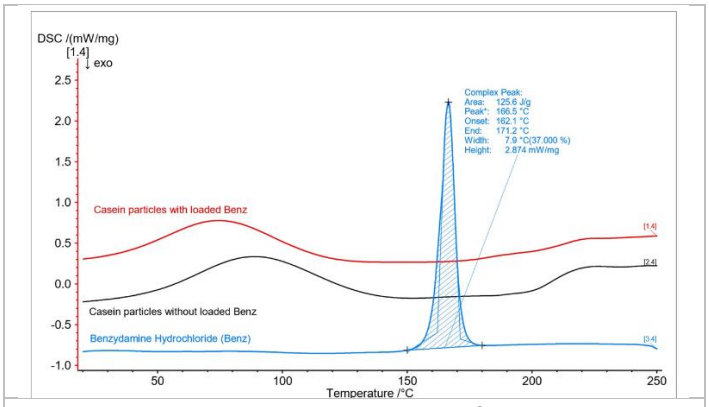


Fig. IV.2.5.: Thermograms of: BHCl (blue line), empty particles (black line), – BHCl loaded particles (red line)

The phase state of the loaded BHCl was established by the DSC method (Fig. IV.2.5.). A broad endothermic peak at around 90 °C is present in the thermogram of blank casein particles, corresponding to the evaporation of bound water in the sample. The other endothermic peak in the same thermogram is observed at a temperature above 200 °C and is due to thermal destruction of the protein chains. No other peaks corresponding to the melting of benzydamine were observed in the drug-loaded particles, leading to the conclusion that it had gone into an amorphous state.

The cumulative release of benzydamine hydrochloride from casein particles at different concentrations of the polymer and the crosslinker was carried out in a buffer at pH 6.8 and a temperature of 37.0 ± 0.5 °C over a period of 8 hours (Fig. V.2.6. and Fig. . V.2.7.). For all investigated systems, an incomplete release between 15% and 32% was observed for this period, without the presence of a "burst effect". The observation is in confirmation with the hypothesis that benzydamine is tightly incorporated into the micelle structures and as a result, the

release process will last longer. The delay of the release with the increase of the concentration of the polymer from 1% to 2% is an additional confirmation of this assumption, due to the formation of a dense protein network and difficult migration of the drug out of it. Particles with different degrees of cross-linking have a more interesting behavior. For the first 8 hours, the most strongly cross-linked particle system is released the fastest. But when the experiment was continued for 72 hours, it was found that this trend changed (table IV.2.2.). For this time period, incomplete release is again observed, but the trend for samples with different crosslinker concentration changes. At the end of the 72nd hour, the system with the least crosslinker had released the largest amount and the one with the densest network the least. The formation of a dense polymer network makes swelling difficult, due to the formation of a kind of barrier. Thus, the observed result for the most densely cross-linked system for the 8-h period is possibly due to the accumulation of some amount of drug in the peripheral regions rather than in the core due to the dense network of micelles.

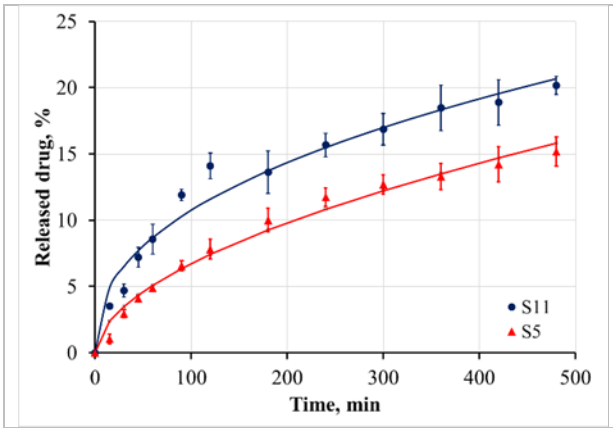


Fig. IV.2.6.: Release profiles of benzydamine hydrochloride from particles with different casein concentration

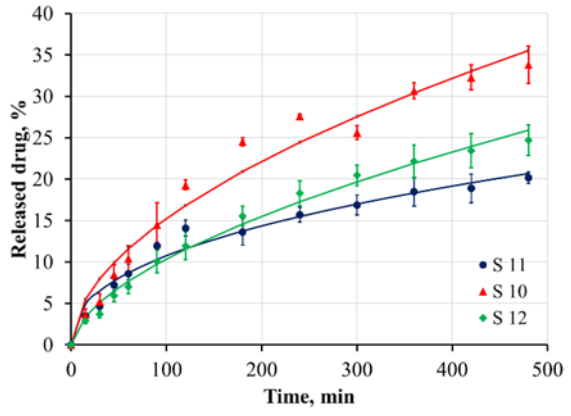


Fig. IV.2.7.: Release profiles of benzydamine hydrochloride from systems with varying degrees of cross-linking

Table IV.2.2.: Amount of released BHCl after 8 h, 24 h and 72 h (n=3)

Sample	Amount of drug released in mg ± SD after:		
	8h	24h	72h
S10	33,8 ± 2,2	51,2 ± 3,9	85,9 ± 6,7
S11	20,2 ± 0,7	39,3 ± 4,3	90,1 ± 9,0
S12	24,7 ± 2,5	44,5 ± 2,6	94,6 ± 3,8

In order to explain the mechanism of release from the casein structures, the release data up to the 8th hour were modeled against selected mathematical models. Based on the value of the coefficient of determination R^2 and the residues derived from the software used, the Korsmeyer-Peppas model is the most suitable for the description of the release process. For all samples studied, the parameter n has a value greater than 0.45, indicating anomalous non-Fickian diffusion (Bruschi, 2015). It has both diffusion and swelling release.

IV. 3. Formation of chitosan/casein polyelectrolyte complex at different ratios of the complexing partners

Polyelectrolyte complexes based on casein and chitosan were obtained and characterized in the conditions of slightly acidic pH=6. At the chosen working pH, the two polyelectrolytes are oppositely charged and can interact electrostatically. 5 types of complexes were developed at different stoichiometric ratios, calculated on the basis of their degrees of ionization and the molecular masses of the elementary units - 2 with an excess of casein, 2 with an excess of chitosan and 1 stoichiometric. In order to facilitate the formation of the complexes and avoid their aggregation, they were treated with ultrasound.

Table IV.3.1.: Sizes, yield and efficiency of inclusion of complexes with different stoichiometry (n=3)

Sample	Ratio of charged COO^-/NH_3^+ groups	Sizes ± SD, μm	Yield ± SD, %	EE ± SD, %
5:1	5:1	0,90 ± 0,08	83,5 ± 2,5	30 ± 2
3:1	3:1	0,40 ± 0,07	72,4 ± 4,3	16 ± 3
1:1	1:1	0,39 ± 0,04	47,5 ± 3,7	15 ± 2
1:3	1:3	0,61 ± 0,05	26,7 ± 1,8	20 ± 1
1:5	1:5	1,74 ± 0,11	18,4 ± 0,2	22 ± 2

The sizes of the resulting complexes range from about 400 nm to 1.8 μm, being strongly influenced by the stoichiometry of the given polyelectrolyte complex (Table IV.3.1). The smallest sizes were observed for the stoichiometric complex, due to the formation of a very dense network as a result of the complete mutual compensation of the charges of the complexing partners. At a ratio of 1:5 in favor of chitosan, the largest value for the hydrodynamic radius was observed, due to the high tendency of the complex to swell. Chitosan is a highly hydrophilic macromolecule with a high net charge, and in aqueous media it easily switches to an unfolded conformation, resulting in a looser structure. In contrast, casein has both hydrophilic and hydrophobic domains that help self-assemble the polypeptide chains into tighter micelle packing's. There is a clear tendency to increase the yield with an increase in the amount of casein put into the complexes. The lowest value is in the sample 1:5 in excess of chitosan – 18.4% and the highest in the ratio 5:1 in excess of casein – 83.5%.

The encapsulation efficiency of benzydamine ranged from 15% to 30%, being highest in the casein-excess system. For the developed systems with benzydamine hydrochloride, this behavior is probably the result of two factors: preferential entrapment of the drug inside the complex, more precisely in the casein micelles, and potential electrostatic interaction with it at these pH conditions. This would also explain the fact that the lowest value for the entrapment efficiency is observed for the system in which the ions of the two

polyelectrolytes are in a 1:1 ratio, due to the formation of an excessively dense network that does not allow the inclusion of a large number of drug molecules within it.

The morphology of the 1:1 sample studied by AFM and SEM is presented in Fig. IV.3.1. and fig. IV.3.2. Both individual and aggregated complexes are observed in the AFM photomicrograph. Their morphology is close to spherical, but some irregular ovals are also present. The SEM image shows particles with different shapes: spherical, spherical with a rough surface, and those with an irregular shape and a rough surface. Both individual particles and large agglomerates are seen.

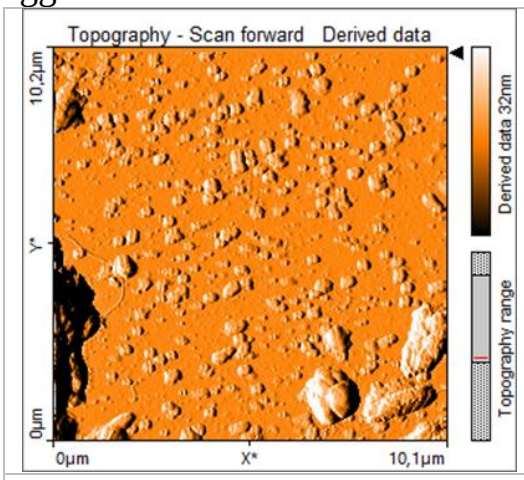


Fig. IV.3.1.: AFM image of complexes, image scale 10x10 µm

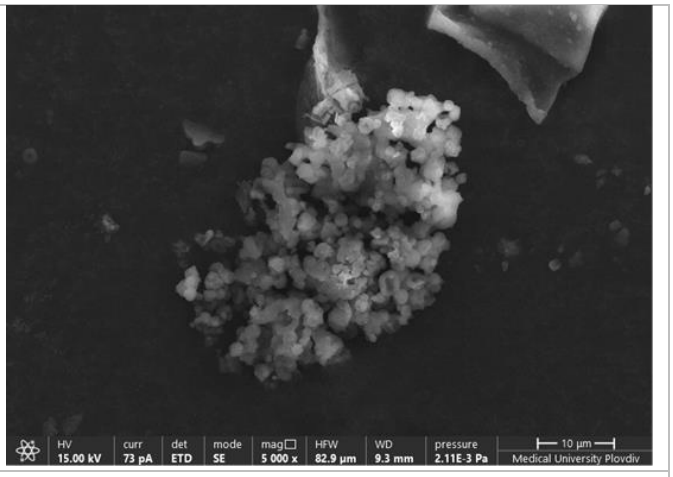


Fig. IV.3.2.: SEM image of complexes, scale bar dimension 10 µm

The polyelectrolyte complex formation between the two polymers is confirmed by a change in their characteristic bands in the general spectrum (Fig. IV. 3.3.). The encapsulation of the drug within the structure formed between the protein and the polysaccharide was confirmed after comparing the two spectra in Fig. IV.3.4.

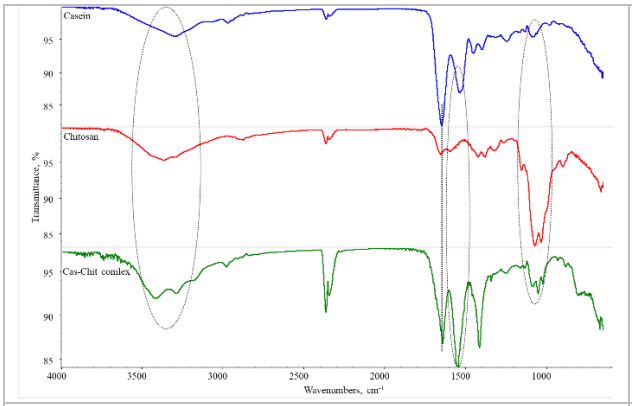


Fig. IV.3.3.: FTIR spectra of: casein (blue line), chitosan (red line), polyelectrolyte complex between them (green line)

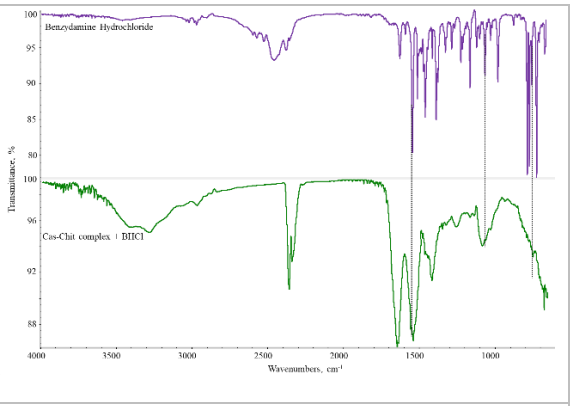


Fig. IV.3.4.: FTIR spectra of: BHCl (purple line), polyelectrolyte complex loaded with BHCl (green line)

The thermal behavior of the stoichiometric complex and the phase state of the drug before and after its incorporation into particles was investigated by DSC and presented in Fig. IV.3.5. A broad endothermic peak is observed in the thermograms of both blank and drug-loaded complexes. It is due to evaporation of bound water from the polymer structure.No other thermal events were observed for the drug-loaded complex, implying that the benzydamine transformed into an amorphous phase state.

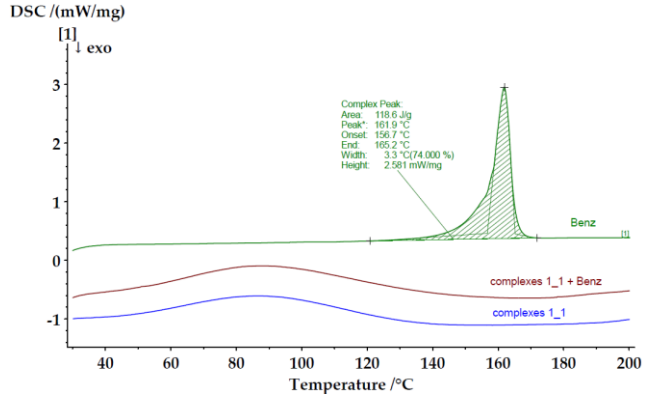


Fig. IV.3.5.: Thermograms of: BHCl (green line); empty particles (blue line); drug-loaded particles (red line)

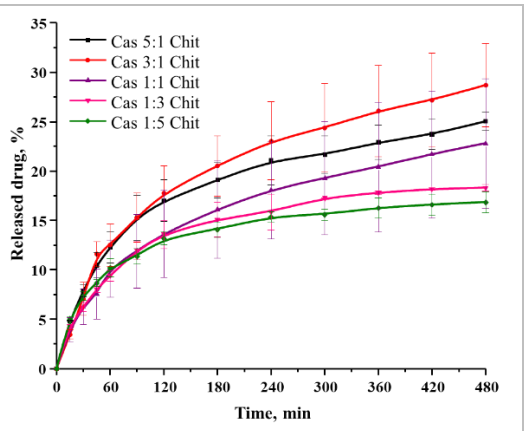


Fig. IV.3.6.: BHCl release profiles from polyelectrolyte complexes with different stoichiometry

The release profile of all developed complexes in artificial saliva buffer is presented in Fig. IV.3.6. As can be seen, the release profile is very similar to that from the casein-based particles, further pointing to the hypothesis that benzydamine is mainly entrapped within the casein micelles. For all samples, incomplete release was observed over a period of 8 h, with values for the amount of drug released ranging between 17% (Cas:Chit = 1:5 system) and 29% (Cas:Chit = 3:1 system). Although the smallest sizes were observed for the Cas:Chit = 1:1 sample, the largest amount was released most rapidly by the 3:1 sample. The two samples have quite similar dimensions, but the non-stoichiometric one has a looser polymer network that is able to swell to a greater extent. At the pH conditions of the release simulation, the casein acquires an even higher charge than that under which the complex was formed. Due to the higher value of the net charge of the protein, the polysaccharide is no longer able to neutralize it, making the network looser. The slowest release of the complex was from the sample with highest excess of chitosan, possibly because of the largest size of the structure observed. Based on the obtained values for the parameters of the applied mathematical models, the Korsmeyer-Peppas model was determined as the optimal model that describes the release profile of benzydamine. The values obtained for the exponent *n* from the equation indicate that the drug release process from the matrix is mainly due to Fick diffusion. Under these pH conditions, the polyelectrolyte complexes are stable and the drug can diffuse from

the core of the complex to its periphery without surface or bulk erosion occurring in the structure.

IV. 4. Formation of chitosan particles by the spray drying method in the presence of a crosslinker or excipient

Spray drying is a single-step method to obtain a dry powder product directly. After initial optimization of the operating parameters of the sputtering process, 9 types of particles were developed: 3 with different polymer concentration, 3 with different polymer:crosslinker ratio and 3 with different polymer:excipient ratio. These variations were chosen in order to evaluate the influence of the crosslinker and the excipient on the properties and behavior of the powdered particles during the release process of incorporated benzydamine hydrochloride. The specific composition of the sprayed polymer particles and the values of the varied parameters, together with the average sizes and the polydispersity index are presented in Table IV.4.1.:

Table IV.4.1.: Particle composition, average size and polydispersity index (n=3)

Sample	Sample composition	Sizes, µm	SD	PDI
C1	0,1% Chitosan	1,827	0,022	0,369
C2	0,2% Chitosan	2,397	0,339	0,264
C3	0,3% Chitosan	2,487	0,107	0,218
CN1	0,2% Chitosan + 0,05% NaTPP	3,002	0,109	0,194
CN2	0,2% Chitosan + 0,1% NaTPP	3,033	0,611	0,261
CN3	0,2% Chitosan + 0,5% NaTPP	3,739	0,474	0,308
CM1	0,2% Chitosan 1 : 5 Mannitol	4,165	0,147	0,177
CM2	0,2% Chitosan 1 : 7.5 Mannitol	6,555	0,202	0,210
CM3	0,2% Chitosan 1 : 10 Mannitol	8,840	0,377	0,508

It is observed that the particles of chitosan concentration of 0,1% have the smallest size and standard deviation. When increasing the concentration of chitosan to 0,2% and 0,3%, due to the increase in the viscosity of the solution, the size of the structures is larger. Such a trend was also reported by Katsarov et al. (2017) in the characterization of chitosan particles by factorial design. The increase in size is based on two factors: an increase in the number of chitosan molecules per unit volume and a subsequent increase in the viscosity of the solution. After addition of crosslinker, even at the lowest weight ratio of 0,05% to polymer, the particle size increased. When the amount of crosslinker increased to 0,5%, the size was almost 50% larger compared to the same uncrosslinked particles. The increase is due to the larger amount of phosphate ions available from the increased crosslinker concentration, which form a network with a larger number of chitosan chains, as reported by Wei et al. (2020). The use of the excipient mannitol results in an increase in particle size between 2 and 4 times depending on its concentration, compared to that without it. The yield and encapsulation efficiency of benzydamine are presented in Table IV.4.2. .

Table IV.4.2.: Yield and encapsulation efficiency of particles with different composition (n=3)

Sample	Sample composition	Yield ± SD, %	EE ± SD, %
BC1	0,1% Chitosan	17,2 ± 0,3	11,84 ± 0,23
BC2	0,2% Chitosan	22,7 ± 1,1	20,98 ± 0,06
BC3	0,3% Chitosan	21,8 ± 2,1	23,13 ± 0,04
BCN1	0,2% Chitosan + 0,05% NaTPP	20,6 ± 0,9	15,67 ± 0,06
BCN2	0,2% Chitosan + 0,1% NaTPP	14,5 ± 0,4	20,49 ± 0,05
BCN3	0,2% Chitosan + 0,5% NaTPP	18,5 ± 1,4	8,73 ± 0,06
BCM1	0,2% Chitosan 1 : 5 Mannitol	21,1 ± 2,4	17,22 ± 0,04
BCM2	0,2% Chitosan 1 : 7.5 Mannitol	38,8 ± 4,1	16,84 ± 0,09
BCM3	0,2% Chitosan 1 : 10 Mannitol	20,1 ± 1,8	14,73 ± 0,05

Yield values ranged from 17% (for particles obtained from a 0,1% chitosan solution without added crosslinker and excipient) to 38% (for particles obtained from a 0,2% chitosan solution with added mannitol in the polymer:excipient ratio 1:7,5). Li et al. (2009) noted that traditionally laboratory spray dryers have lower product yields than industrial ones. In addition to the reasons for the relatively low yield are also the geometry of the spray dryer, as well as the strong adhesion of the chitosan particles, which in the process of sputtering stick everywhere on the volume and its walls, as reported by a number of authors (Sinsuebpol, et al. 2013 ; Li, et al. 2009; Casanova, et al. 2016). The presence of a crosslinker does not significantly affect the amount of dry particles obtained, but the presence of an excipient slightly increases it. Since the mass of the obtained dry particles was used to calculate the encapsulation efficiency of incorporation of BHCl, it follows the same trend as for the yield.

The morphology of the selected samples, before and after drug loading, was examined by scanning electron microscopy and the images are presented in Figure IV.4.1. A smooth surface was reported for the chitosan system, confirming the observed size increase for the cross-linked particles. The particles composed of chitosan and mannitol have highly pronounced aggregates and the presence of a rough surface. This is due to two main factors: the fact that mannitol is crystalline and the different Peclet number values for mannitol and chitosan. After loading the same types of particles with the model drug, the morphology of uncrosslinked and crosslinked particles remained unchanged. In the third type of particles with excipient, the presence of needle-like formations on the surface of the spheres is clearly observed, which are probably due to the recrystallization of mannitol.

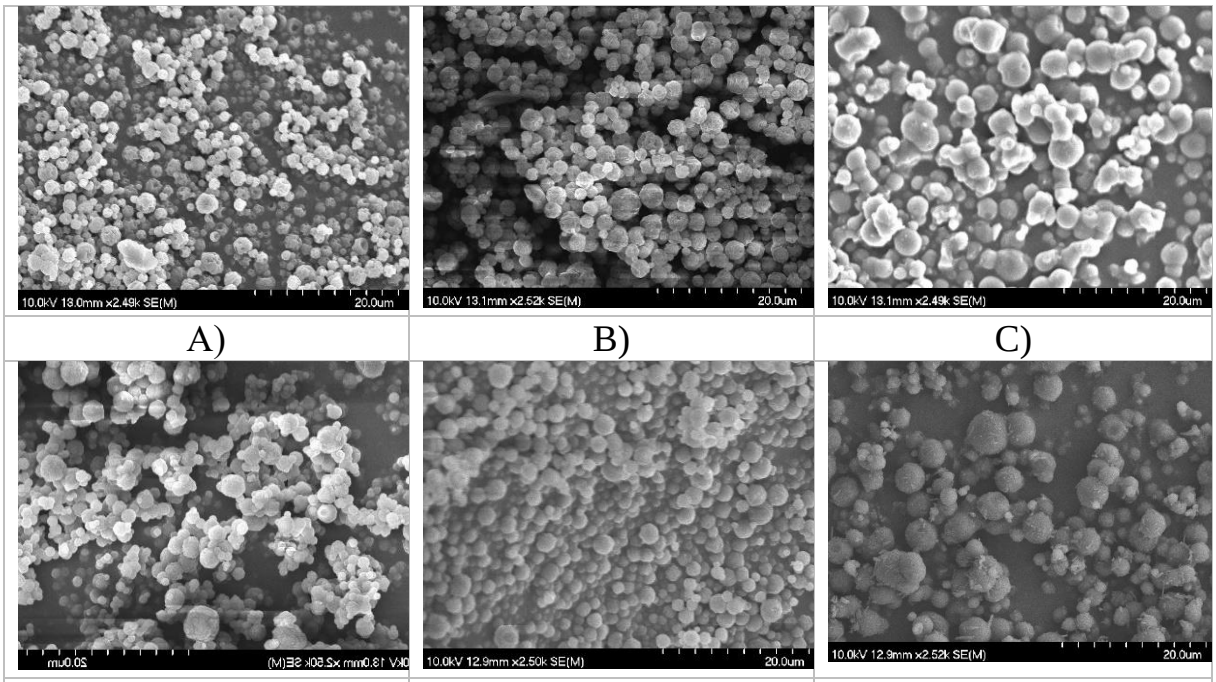


Fig. IV.4.1.: SEM images of empty and loaded. Presented sample codes are: C2 (A), CN2 (B), CM2 (C), BC2 (D), BCN2 (E), BCM2 (F). Scale bar dimension – 20 μm.

The cross-linking process, the physical presence of excipient and the successful incorporation of the drug BHCl into the chitosan spheres were confirmed by the applied FTIR spectroscopy (Fig. IV.4.2. and Fig. IV.4.3.). Due to the lack of shift or change in the type of characteristic bands for the drug, it can be considered that it is physically present in the structures without chemically interacting with the polymer matrices.

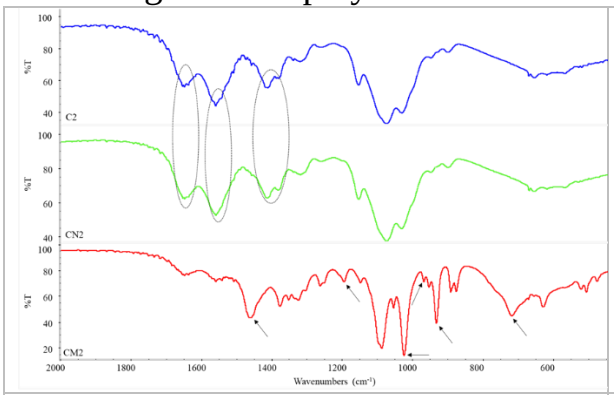


Fig. IV.4.2.: FTIR spectra of: chitosan particles (blue line), chitosan particles cross-linked with sodium polyphosphate (green line), chitosan particles with mannitol (red line)

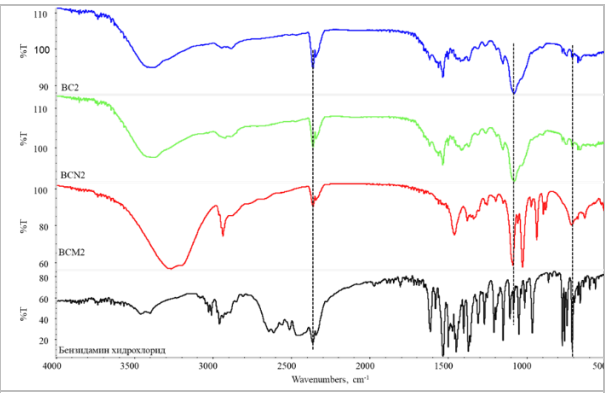


Fig. IV.4.3.: FTIR spectra of: chitosan particles with BHCl (blue line), cross-linked chitosan particles with BHCl (green line), chitosan particles with mannitol and BHCl (red line), pure BHCl (black line)

The thermal stability and phase state of the samples were investigated by the DSC method - fig. IV.4.4. The thermogram of sample CM2 clearly shows the presence of two endothermic and one exothermic transition. The first one is at 157.7 °C with an enthalpy of 44.37 J/g and is attributed to the melting of the δ-polymorph of mannitol. The crystallization transition at 162.1 °C and enthalpy 23.47 J/g is next. The second endothermic transition is associated with melting again, but this time of the α- and β-polymorphs.

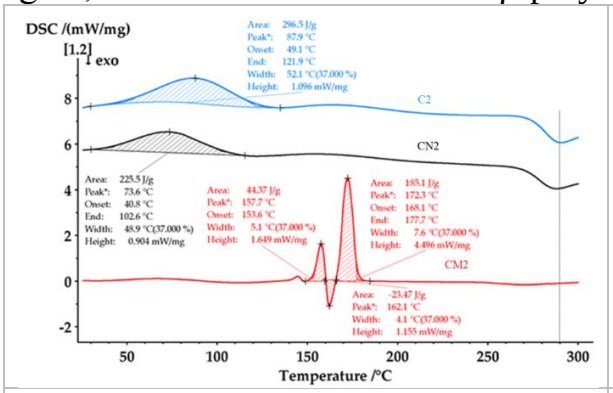


Fig. IV.4.4.: Thermograms of: chitosan particles (blue line); crosslinker chitosan (black line); chitosan with mannitol (red line)

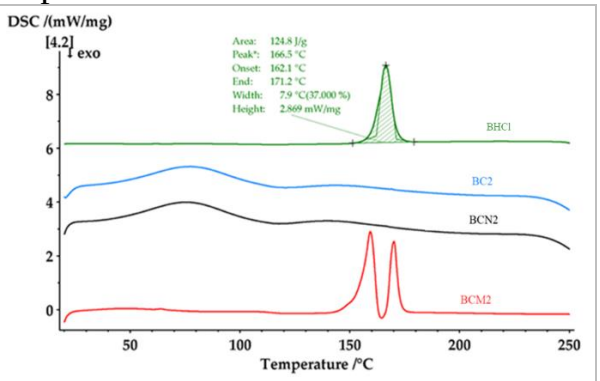


Fig. IV.4.5.: Thermograms of: pure BHCl (green line); chitosan particles with BHCl (blue line);– chitosan with crosslinker and benzydamine (black line); chitosan with mannitol and benzydamine (red line)

The pure drug is characterized by a narrow endothermic peak at 166.5 °C with an enthalpy of 124.8 J/g, due to its highly crystalline structure – Fig. IV.4.5. The absence of peaks corresponding to the drug in samples BC2 and BCN2 led to the conclusion that it had gone into an amorphous state, and the same was true for sample BCM2.

The cumulative amount of drug released from the three types of particles was investigated in buffer at pH 6.8 and temperature 37.0 ± 0.5 °C – Fig. IV.4.6. The release profile of BHCl from all systems was similar and typical of water-soluble drugs – biphasic with an initial rapid release stage, in this case for about 120 minutes, followed by a delayed release of the remaining substance. A possible explanation for this can be found in the process of particle formation. When they are dried, as the water evaporates from the center to their surface, some of the drug dissolved in it migrates along with it, accumulating preferentially at the periphery of the particles. This facilitated migration is due to the high diffusivity of benzydamine and therefore its low Peclet number.

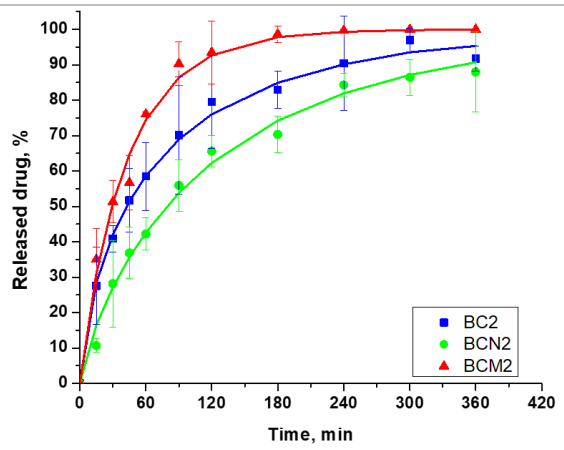


Fig. IV.4.6.: Release profiles of benzydamine hydrochloride from systems with different compositions: chitosan particles (blue line), cross-linked chitosan particles with sodium tripolyphosphate (green line), chitosan particles with excipient mannitol (red line)

The particles with mannitol release fastest, with complete release observed already within 300 minutes. Despite its significantly larger size, during the formation process of the particle, pores and channels are obtained in its interior due to the migration and distribution of mannitol, which greatly facilitates the process of entering the aqueous medium and the migration of the drug. Particles based only on chitosan release 93% in 24 hours, and those with a crosslinker - 89%. As expected, the addition of a cross-linker slows down the release process. As an optimal model for a mathematical description of the behavior of the obtained particles, the Weibull model was chosen. The mechanism of drug release from chitosan-only particles is solely Fickian diffusion. As a result of the cross-linking, a change in the release mechanism in Fickian diffusion, combined with chain swelling and relaxation, is also reported. In addition, cross-linked particles are also larger in size than non-cross-linked, which reduces the contact surface and delays release. A combined mechanism is also reported for the mannitol particles, due to the presence of pores and the accelerated diffusion of water molecules due to the good solubility of the excipient.

IV. 5. Formation of spray-dried casein particles when varying the polymer:drug ratio

The present study presents the potential of spray-dried casein particles from highly acidic pH solutions to incorporate and release benzydamine hydrochloride in a controlled manner. Based on preliminary studies, a specific particle system was selected that was optimal according to yield and size values (Zahariev, et al. 2021). The selected drug loading structure was composed of: 1% concentration of casein and 1,5 M concentration of calcium dichloride. This system is characterized by average sizes of (104,1 ± 8,5) nm and yield (64,80 ± 3,02) %. Although at pH ≤ 4.6 casein is positively charged, in Horne's model calcium is reported to have a stabilizing effect on the micelles (Horne, et al. 1998). The parameters of the atomized particles loaded with different mass ratios between the selected polymer and the model drug are presented in Table IV.5.1:

Table IV.5.1.: Sizes, yield and encapsulation efficiency of casein particles (n=3)

Sample	Sizes ± SD, nm	Yield ± SD, %	EE ± SD, %
Cas 1:1 BHCl	994,2 ± 2,2	30,42 ± 4,28	34,61 ± 0,23
Cas 2:1 BHCl	243,6 ± 2,5	74,71 ± 5,41	78,82 ± 0,39
Cas 4:1 BHCl	159,8 ± 2,4	68,76 ± 5,01	76,23 ± 0,28
Cas 6:1 BHCl	135,9 ± 1,7	58,23 ± 5,08	77,44 ± 0,57

The sizes of the resulting structures were investigated by dynamic light scattering and ranged from 136 nm to almost 1 μm. For the casein:drug = 1:1 mass ratio system, a bimodal distribution was observed, probably due to significant aggregation. Due to the high amount of drug in the solution, it is difficult to form homogeneous structures with similar size and morphology, which negatively affects the yield and therefore the encapsulation efficiency, as noted also by Tran, et al. (2014). As the amount of casein in the total solution increases, the average hydrodynamic radius of the structures also decreases. The resulting systems have yields above 74%, except for the first 1:1 mass ratio, where the yield is only about 30%. A probable reason for the behavior of this sample can be two phenomena: disruption of the micelle structure due to the displacement of calcium phosphate from them, which leads to the formation of precipitates even before the solution is atomized; and insufficient supercritical polymer concentration to form a stable structure (García, et al. 2020; Sinaga, et al. 2017). Increasing the amount of polymer in the total solution slightly increased the production yield, probably due to the increase in viscosity and concentration of the solution introduced for sputtering. Encapsulation efficiency is again high for all systems developed except for the 1:1 system, most likely influenced by yield values. For this system, the value for the incorporation efficiency was less than 35%, while for the other particle types it ranged from 76% to almost 79% with a slight increasing trend as the amount of drug in the total solution increased. Higher concentrations of casein are more able to incorporate a large amount of benzydamine, probably because of more intense hydrophobic interactions, which are a prerequisite for better micelle solubility of the drug (Chen, et al. 2020). The hypothesis explaining the low yield and encapsulation efficiency for a 1:1 mass ratio was confirmed after examining the morphology of the four types of particles by means of scanning electron microscopy – fig. IV.5.2. For the 1:1 specimen, there is no distinct morphology approaching even an irregular shape. The presence of a large amount of drug in the total solution makes it difficult to form a dense particle, as well as the stability of the micelles themselves. For the casein:drug ratios of 2:1, 4:1 and 6:1, the formation of particles with a lower degree of aggregation and less cohesion with a distinct morphology was significantly greater compared to the 1:1 ratio.

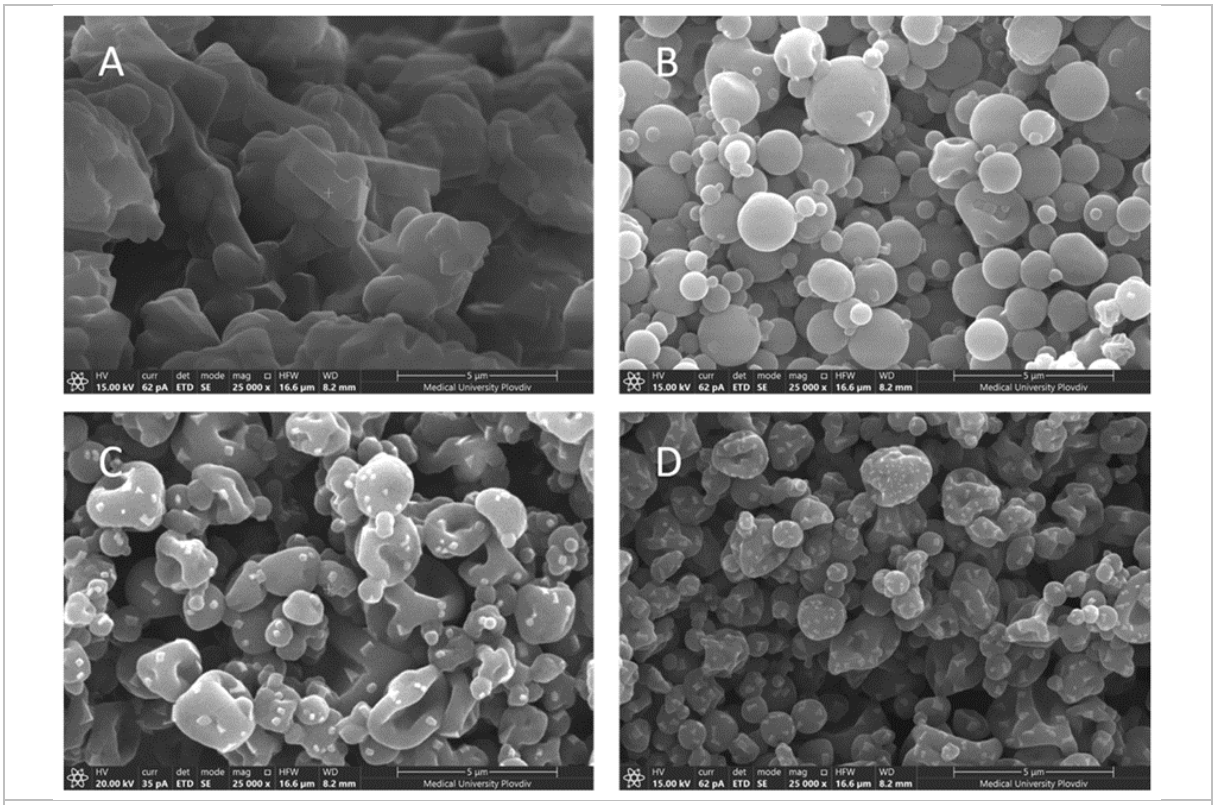


Fig. IV.5.1.: SEM images of particles with different polymer:drug ratios. Shown images are: Cas 1:1 BHCl (A), Cas 2:1 BHCl (B), Cas 4:1 BHCl (C), Cas 6:1 BHCl (D). Scale bar dimension – 5 μm.

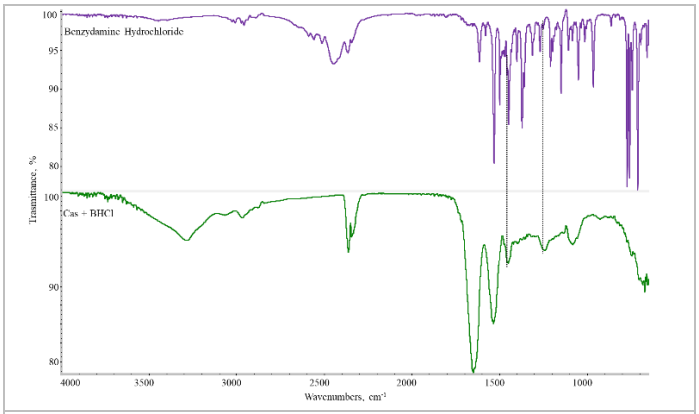


Fig. IV.5.2.: FTIR spectra of: BHCl (purple line), loaded casein particles (green line)

The spectra of the pure substance and loaded particles, studied by FTIR spectroscopy, are presented in Figure IV.5.2. Due to the lack of shift or change in the characteristic bands of benzydamine, we believe that it is only physically entrapped in the particles without interacting with the casein under strongly acidic conditions.

In fig. IV.5.3. the thermograms of blank casein particles are presented, as well as each polymer-drug ratio developed.

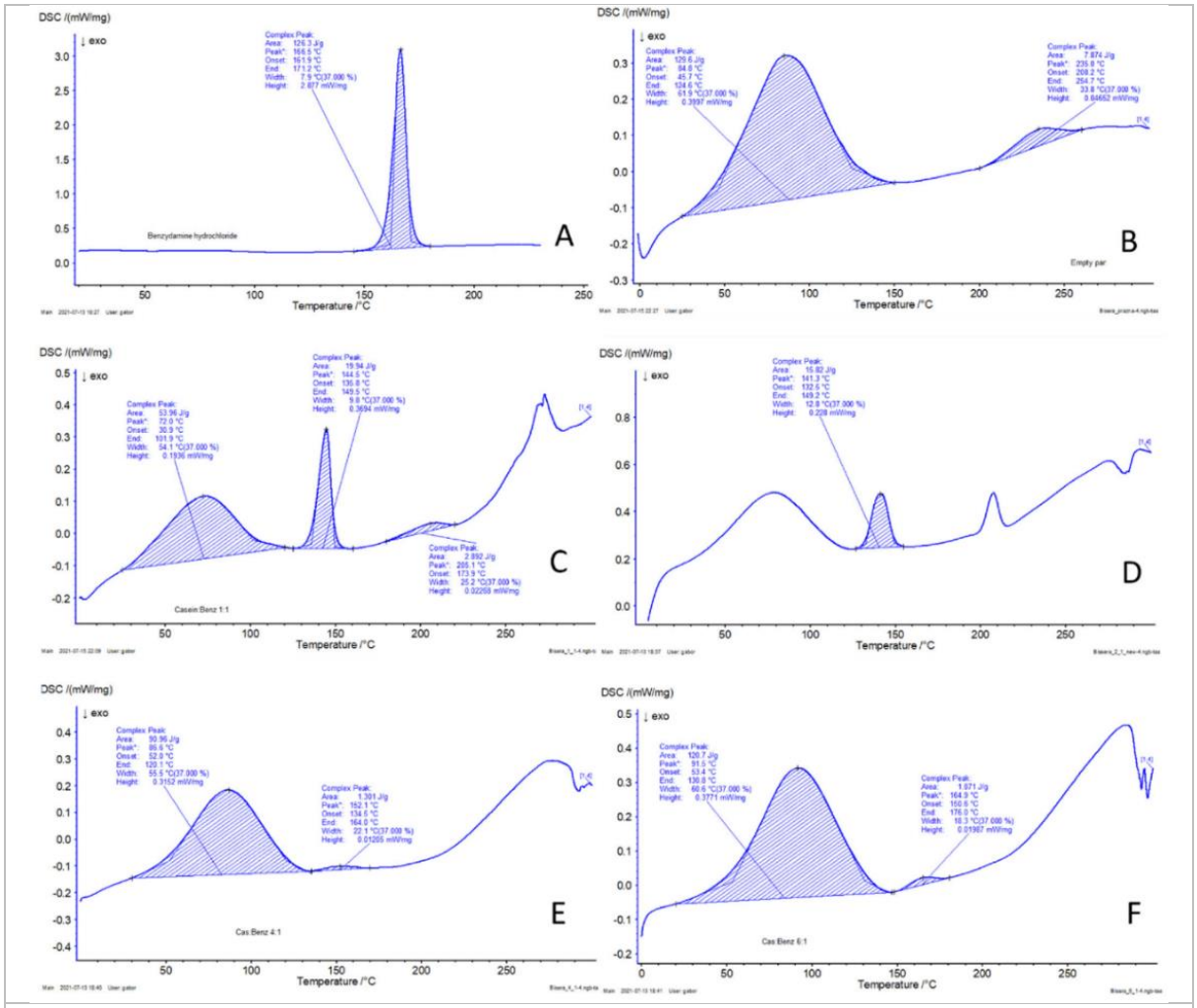


Fig. IV.5.3.: Thermograms of pure drug substance and different particle types: pure benzydamine hydrochloride (A), empty casein particles (B), Cas 1:1 BHCl (C), Cas 2:1 BHCl (D), Cas 4:1 BHCl (E), Cas 6:1 BHCl (F)

For the drug-loaded particles, a second endothermic peak appears for all systems is present. It is related to the melting of benzydamine, due to the presence of a phase transition. Part of the active substance has passed into an amorphous phase. For the particle systems with mass ratios of 1:1 and 2:1 in favor of the polymer, crystallization was observed for nearly 1/3 of the mass of the incorporated drug, while for the other two systems it was about or below 13%. The lowest value is for the 4:1 system, namely 7%.

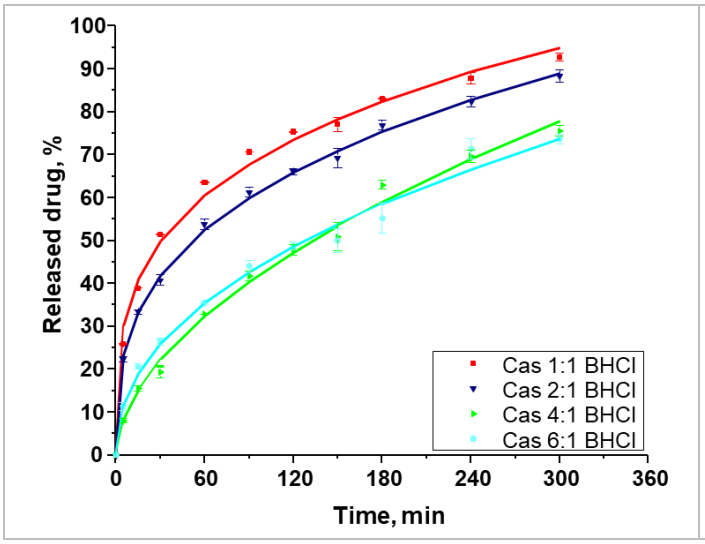


Fig. IV.5.4.: Release profiles of benzydamine hydrochloride from systems with different polymer:drug ratio: 1:1 ratio (red line), 2:1 ratio (dark blue line), – 4:1 ratio (green line), 6:1 ratio (light blue line)

The release profiles of the four developed systems (Fig. IV.5.4.) were studied over a period of 5 hours at pH 6.8 and temperature 37.0 ± 0.5 °C. For this time period, almost complete release was observed for the 1:1 ratio system. With 1:1 and 2:1 systems, a highly pronounced "burst effect" is reported, in which more than 50% of the included drug is released in the first hour. Another factor that significantly affects the release rate may be the degree of recrystallization of the drug. A clear trend is observed between the ratio of drug input in the solution and the amount released in the in vitro test process. Systems with a ratio of 4:1 and 6:1 release at the end of the period almost the same amount, about 75% of the amount of drug put into them. This initial overtaking may be due to the particle sizes and the contact surface between them and the acceptor medium. Probably, the larger amount of polymer put into the solution of the latter two systems forms a denser network that hinders the migration of the drug, despite the larger contact surface compared to the 1:1 and 2:1 systems.

Based on the coefficient of determination R^2 and the distribution of residues, the Korsmeyer-Peppas model was derived as the optimal mathematical model describing the BHCl release profile. For the fastest releasing particles with mass ratios of 1:1 and 2:1, the value of n is less than 0.45, indicating Fickian diffusion as the transport mechanism. In the remaining two systems, the value of $n > 0.45$, indicating non-Fickian diffusion as the main mechanism (Das, et al. 2024).

V. CONCLUSIONS

1. When preparing chitosan particles by the ionotropic gelation method, increasing the concentration of the polymer and the crosslinker increases the size, yield and efficiency of inclusion of the active substance and slows down the release process.

2. When producing casein-based particles by ionotropic gelation, an increase in the incorporation efficiency and yield is reported as the polymer and crosslinker concentrations increase. At the same time, increasing the crosslinker concentration leads to a decrease in the average particle size.

3. The change in the stoichiometry of the casein/chitosan polyelectrolyte complex leads to the optimization of the size of the obtained particles (from 400 nm to 2 μm) and to controlling the release rate of the included benzydamine hydrochloride.

4. The use of a crosslinker or excipient during spray drying of chitosan particles allows modulation of their size in the range of 1-10 μm and of their morphology. Depending on the composition of the particle, a difference in the release mechanism of benzydamine hydrochloride is also reported.

5. Spray drying of an acidified casein solution in the presence of different drug concentrations in it allows the formation of particles with sizes in the submicron range with a relatively high yield (over 58%) and benzydamine hydrochloride incorporation efficiency (about 80%). Based on the values of the model parameters, at an excess of polymer in a mass ratio above 2:1, benzydamine is released by non-Fickian diffusion, while at lower ratios - by Fickian diffusion.

6. The mechanism and release profile of the developed particle systems depends not on the method of preparation, but on the type of polymer. For polysaccharide-based particles, the optimal model for modeling the release process is Weibull, and for those based on protein, as well as their polyelectrolyte complex, Korsmeyer-Peppas.

7. On the basis of yield, dimensions and amount of drug included, the model of powdered casein particles with a mass ratio between polymer and drug of 4:1 can be deduced as optimal.

VI. SCIENTIFIC AND SCIENTIFIC-APPLIED CONTRIBUTIONS

- The potential of chitosan nanoparticles prepared by ionotropic gelation in the presence of sodium tripolyphosphate to incorporate and controllably release benzydamine hydrochloride has been confirmed.
- For the first time, benzydamine hydrochloride-loaded casein particles were developed using the ionotropic gelation method in the presence of alcohol and under acidic pH conditions.
- For the first time, the influence of the stoichiometric ratio of a polyelectrolyte complex based on casein-chitosan on the incorporation and release of benzydamine hydrochloride has been examined in detail.
- For the first time, the influence of a crosslinker or excipient in chitosan-benzidamine hydrochloride structures obtained by the spray drying method was evaluated.
- For the first time, benzydamine hydrochloride-loaded casein nanoparticles obtained by spray drying in acidic conditions in the presence of calcium ions were obtained and characterized, and the influence of the polymer:drug ratio on the main physicochemical parameters of the structures was established.

VII. REFERENCES

The complete literature review, as well as a list of cited literary sources, can be found in the dissertation.

VIII. APPENDICES

LIST OF SCIENTIFIC PUBLICATIONS RELATED TO THE DISSERTATION:

1) **Milenkova, S.**, Pilicheva, B., Uzunova, Y., Yovcheva, T., & Marudova, M. (2022). Casein Microgels as Benzydamine Hydrochloride Carriers for Prolonged Release. Materials, 15(4), 1333.

Indexed in Scopus/Web of Science, Q2 (IF=3.4)

Cited by:

- Dai, C., Li, W., Zhang, C., Shen, X., Wan, Z., Deng, X., & Liu, F. (2024). Microencapsule delivery systems of functional substances for precision nutrition.
- Feitosa, M. L., da Silva Costa, D. V., Teixeira, I. M. M., SAMPAIO, T., Gaspar, D. M., Martins, A. M. C., ... & de Sousa, F. C. F. (2024). Benzydamine induces apoptosis in astrocytes in vitro. *Journal of Health & Biological Sciences*, *12*(1), 1-7.
- Mao, T., Akshit, F. N. U., Matiwalage, I., Sasidharan, S., Alvarez, C. M., Wescombe, P., & Mohan, M. S. (2024). Preferential binding of polyphenols in blackcurrant extracts with milk proteins and the effects on the bioaccessibility and antioxidant activity of polyphenols. *Foods*, *13*(4), 515.

2) Marudova, M., **Milenkova, S.**, Zahariev, N., Yovcheva, T., & Pilicheva, B. (2023). Formulation and characterization of Benzydamine loaded casein/chitosan nanocomplexes. In Journal of Physics: Conference Series (Vol. 2436, No. 1, p. 012028). IOP Publishing.

Indexed in Scopus, SJR=0.18

3) **Milenkova, S.**, Ambrus, R., Mukhtar, M., Pilicheva, B., & Marudova, M. (2024). Spray-Dried Chitosan Hydrogel Particles as a Potential Delivery System for Benzydamine Hydrochloride. Gels, 10(3), 189.

Indexed in Scopus/Web of Science, Q1 (IF=4.6)

PARTICIPATION IN SCIENTIFIC CONFERENCES:

1) „Improved Nutraceutical Based Systems as Benzydamine Hydrochloride Carriers“ - Bucharest Polymer Conference 2nd Edition Held at the University POLITEHNICA of Bucharest, Romania 9-11 June 2021 – дистанционно участие
2) "Formulation and characterization of Benzydamine loaded casein/chitosan nanocomplexes" -22nd International School on Condensed Matter Physics: State of the Art in Functional Materials & Technologies (ISCMP 2022) 28/08/2022 - 02/09/2022 Varna, Bulgaria;
3) “Chitosan-Casein complexes as carriers for bioactive compounds” - Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science 18/01/2023-20/01/2023 Szeged, Hungary.
4) “Chitosan-based spray dried particles as delivery system for Benzydamine Hydrochloride” - 7th International Symposium Frontiers in Polymer Science 29 May- 1 June 2023, Gothenburg, Sweden
5) “Biopolymeric nano- and microsized hydrogel particles as an emerging tool for controlled release of senolytics” - BIOTECHNOLOGIES FOR PERSONALIZED MEDICINE, October 31 -2 November 2023, Plovdiv, Bulgaria

PARTICIPATION IN MOBILITIES:

1) Specialization under the PERIMED project (Competence Center "Personalized Innovative Medicine" (PERIMED) under the Operational Program "Science and Education for Intelligent Growth" under grant procedure BG05M2OP001-1.002-0005-C01) - 13.10.2022. until 11/11/2022 Host University: Faculty of Pharmacy, University of Szeged, Hungary.
2) Short-term doctoral student mobility under Erasmus - 16.01.2023. until 15.02.2023 Host University: Faculty of Pharmacy, University of Szeged, Hungary.

PARTICIPATION IN SCIENTIFIC AND INFRASTRUCTURE PROJECTS:

1) "New micro- and nanostructured drug systems for buccal administration" KP-06-H38/3 of 05.12.2019. funded by the Scientific Research Fund, Ministry of Education and Science - young scientist.

2) Competence Center "Personalized Innovative Medicine" (PERIMED) under the Operational Program "Science and Education for Intelligent Growth" under grant procedure BG05M2OP001-1.002-0005-C01 dated 30.03.2018, financed by the Operational Program "Science and education for smart growth" 2014-2023 and co-financed by the European Union through the European Regional Development Fund - young researcher.