5-FLUOROURACIL ENCAPSULATED CHITOSAN MICROSPHERES

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ABSTRACT

5-Fluorouracil (5-FU) is a chemotherapeutic agent used in therapies for both systematically and topical treatment of different types of cancers. Depending on the period of application, its administration may lead to different side effects such as nausea, headache, pain or even photosensitivity. To avoid them, the drug may be encapsulated into polymeric particles. In the present study, bio polymeric spheres based on chitosan, a linear polysaccharide, are presented. The spheres are formulated by an emulsion technique with solvent evaporation. Three types of particles are synthesized: without crosslinker, with sodium tripolyphosphate and glutaraldehyde crosslinker. The resultant structures are evaluated regarding their size, morphology, and encapsulation efficiency. The crosslinking process and the drug presence in the particles is confirmed by FT-IR. A drug release study is conducted to examine the release kinetics and understand the release behaviour depending on the presence and the type of the crosslinker.

Keywords: microparticles, chitosan, 5-fluorouracil, sustained release, crosslinker, sodium trypolyphosphate, glutaraldehyde.

INTRODUCTION

5-Fluorouracil (5-FU) is one of the most frequently chosen chemotherapeutic drugs due to its inhibition of tumor cells growth and antimetabolite activity regarding the RNA replication [1]. It has been exploited in treatment of various types of cancer such as: breast cancer [2], brain cancer [3], head and neck cancer [4], skin cancer [5] etc. This drug shows promising effects for treatment both solid tumors and cancer cells [6], also it can be single or additional therapy as it is first line anti-neoplastic agent [7]. As it is classified as Class III compound according to the biopharmaceutical classification system, it means that it has high solubility, but low permeability. Having an extremely short half-life, when orally applied it is 6 - 20 min and IV-application even shorter - 5 - 10 min, this leads to poor absorption and fluctuating bioavailability [8, 9]. To prevent frequent administration and dose dumping and at the same time provide stable plasma concentration within the optimal therapeutic window, a suitable tailored delivery matrix may be employed for prolonged delivery. Natural polymers and especially polysaccharides have shown great potential to play the role of cargo system for various types of drugs. One of the most promising candidates for this purpose is chitosan - linear polysaccharide, obtained after

deacytilation of chitin. Chitosan is also a weak polyelectrolyte, and its structure is abundant in NH_2 groups which can be protonated. As the internal pH microenvironment of the tumor tissue is around 5.5 - 5.8, pH-responsive delivery of the drug may be enabled due to the positive charge of chitosan [9]. The presence of amino active groups gives the opportunity to chitosan to interact by both physical and chemical cross linkers. Crosslinking by Schiff-base reaction with glutaraldehyde or by electrostatic interaction with sodium trypoplyphosphate are the most common approaches to obtain chitosan-based structures [10, 11]. Ellagic acid, compound with low absorption and great anti-cancer activity, loaded into physically crosslinked chitosan particles were developed by Arulmozhi et al. [12]. They found that the particles obtained sustained drug release and in all examined concentration loaded particles had better anti-cancer activity than the native ellagic acid, due to the improved absorption as a result of the immobilization process. Shakeran et al. have proved that methotrexate loaded into chemically crosslinked chitosan particles had higher cytotoxicity against breast cancer cell lines due to the complex formation with the cell membranes [13]. In addition, a reduced side toxic effect is also observed. With that being said, it is worth to investigate the effect of loading of 5-FU into polymeric particles in order to improve its bioavailability and reduce toxicity. Due to their biocompatibility and biodegradability, along with the sensitivity for external stimuli, chitosan-based particles loaded with 5-FU are on the focus of the present study. A comparison between non-crosslinked, chemically crosslinked and physically crosslinked chitosan spheres obtained by emulsion technique is done. Depending on the crosslinking mechanism the final structure may have different sizes, morphology, swelling properties and even release rate of the encapsulated drug and therefore it can affect the treatment outcome.

EXPERIMENTAL

Materials

Low molecular mass chitosan (degree of deacetylation ≥ 85%) was purchased from Glentham Life Sciences. Sodium tripolyphosphate (NaTPP), Glutaraldehyde (GlutAl), Sorbitan trioleate $C_{60}H_{108}O_8$ (Span 85) were bought from Sigma Aldrich. All other used chemicals were with analytical grade.

Methods

Synthesis of chitosan particles by emulsion technique

Water-in-oil emulsion technique with addition of crosslinking agent was used for preparation of blank and loaded chitosan particles. 10 mL of 1 % chitosan solution (pH >> 4) was dropped to 100 mL liquid paraffin containing 0.5 % w/v surfactant agent Span 85 at a temperature of 65°C. 15 min after the addition of the chitosan, 0.5 mL crosslinking agent (NaTPP or GlutAl) in different molar ratios with respect to the chitosan's protonated amino groups, was added dropwise. This mixture was stirring for 2.5 h at 1100 rpm with mechanical stirrer (Velp Scientifica, Usmate, Italy) and the final solution was filtrated with 0.45 μm cellulose nitrate membrane filter and washed 2 times with petroleum ether. The same procedure was used to prepare loaded samples, adding 50 mg of 5-FU to the chitosan solution.

FT-IR spectroscopy

The spectra of the blank and 5-FU loaded chitosan particles that are not cross-linked and cross-linked with NaTPP or GlutAl, were collected with a Nicolet iS 10 FT-IR spectrometer (Thermo Fisher Scientific, Pittsburgh, PA, USA) in ATR mode. The spectra were taken at wavelengths in the range of 600 to 4000 cm^{-1} with a resolution of 4 nm and 64 scans. The obtained experimental data were analyzed with the OMNIC® software package (Version 7.3, Thermo Electron Corporation, Madison, WI, USA).

Laser Diffraction Technique

The mean diameters of the blank chitosan particles were analysed by laser diffraction technique using LS 13320 analyser (Beckman Coulter, USA). Dried particles with weight of about 100 mg were examined with the Tornado system, which is applied for powdered samples. The presented results are an average value from three repetitions [14].

SEM

The morphology of blank and loaded particles was examined using Scanning electron microscopy (SEM) (Prisma E SEM, Thermo Scientific, Waltham, MA, USA). The samples were loaded on a copper sample holder and sputter-coated with carbon followed by gold

using a vacuum evaporator (BH30, Prisma E SEM, Thermo Scientific, Waltham, MA, USA).

The images were recorded at a 15 kV acceleration voltage at various magnifications using a DBS (back-scattered electrons) detector (Prisma E SEM, Thermo Scientific, Waltham, MA, USA) [15].

Yield of the synthesis process

In order to estimate the amount of the transformed into particles chitosan during the synthesis, after the filtration and washing steps, the particles were dried in air at room temperature in a Petri dish until constant mass was achieved. Then the yield was calculated by the Eq. 1:

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

This presented data were the average values from three repetitions.

Swelling

To investigate the swelling behaviour of the chitosan particles, the following procedure was performed: 10 mg of dry particles were immersed in 2 mL saliva buffer solution (pH = 6.8).

In a period of 8 h, the change in paticles' mass was measured after each passed hour. The experiment was done in triplicates. The swelling degree was calculated by the Eq. 2:

$$\text{Swelling}\ (\%) = \frac{m_t - m_0}{m_0} \times 100 \quad (2)$$

where m_t is sample mass at a given time, and m_0 is the initial mass of the sample.

Loading efficiency

The 5-FU content in the synthesized chitosan particles was determined spectrophotometrically using a preliminary determined calibration curve. 10 mg of drug loaded particles were closed in dialysis membrane and submerged in 10 mL of distilled water. Afterwards they were sonicated and left for 1 week to ensure full release of 5-FU. A couple milliliters of the acceptor media was filtrated (Chromafil Xtra RC (Macherey-Nagel, Dueren, Germany), 0.45 μm and the 5-FU concentration was determined by the UV-VIS spectrophotometer, Metertech SP-8001(Metertech Inc., Nangang, Taipei, Taiwan), at a wavelength of 266 nm. The loading efficiency (LE) was calculated based on the equation, keeping in mind the dilution steps (Eq. 3):

$$LE\ (\%) = \frac{mass\ of\ the\ loaded\ 5-FU}{initial\ mass\ of\ 5-FU} \times 100 \quad (3)$$

Differential Scanning Calorimetry

Thermal stability of blank and loaded particles, as well as the phase state of loaded 5-FU were examined with differential scanning calorimetry equipment DSC 204F1 Phoenix (Netzsch Gerätebau GmbH, Selb, Germany) [16]. The instrument was calibrated for both heat flow and temperature with an indium standard (T_m = 156.6°C, ΔH_m = 28.5 J g^{-1}). All of the examined samples (about 5 mg) were sealed in aluminium pans and an empty pan was used as a reference. The samples were heated from 20°C till 300°C with a rate of 10°C min^{-1} under argon atmosphere.

***In vitro* drug release**

In vitro release of the loaded into the chitosan particles 5-FU was performed in saliva buffer (pH = 6.8). Amounts of all investigated types of particles, equivalent to 5 mg loaded 5-FU, were suspended in 1 mL of the buffer. These suspensions were placed into a dialysis bags, which were poured into 20 mL of the same buffer at 37°C and continuously stirred at 50 rpm during the experiment. Samples of 3 mL were taken at selected time intervals for spectroscopic analysis at 266 nm, and after taking out each sample, an equivalent amount of buffer was added back to the release medium. The release kinetics was further modelled with known from the literature Weibull model [16]:

$$M = M_0\left[1 - exp\left(-\frac{(t-T)^b}{a}\right)\right] \quad (4)$$

where M is the amount of dissolved drug as a function of time; M_0 - the total amount of released drug; t - time; T - lag time caused by dissolution process; a - scale parameter of the time dependence, and b - shape of the dissolution curve progression.

RESULTS AND DISCUSSION

Formulation and characterisation of blank chitosan particles

The chitosan particles were successfully synthesised by a water-in-oil emulsion technique without and with added cross-linking agent. As both physical and chemical crosslinking was employed to obtain chitosan crosslinking particles, more precise physico-chemical characterization was needed. A suitable approach to confirm the gelation of chitosan by NaTPP and GlutAl and confirm the loading of 5-FU, Fourrier-transformed infrared spectroscopy in ATR mode was applied. The obtained results are presented in Fig. 1.

Blank chitosan particles show characteristic bands in the region of C-N stretching (around 2900 cm^{-1}),

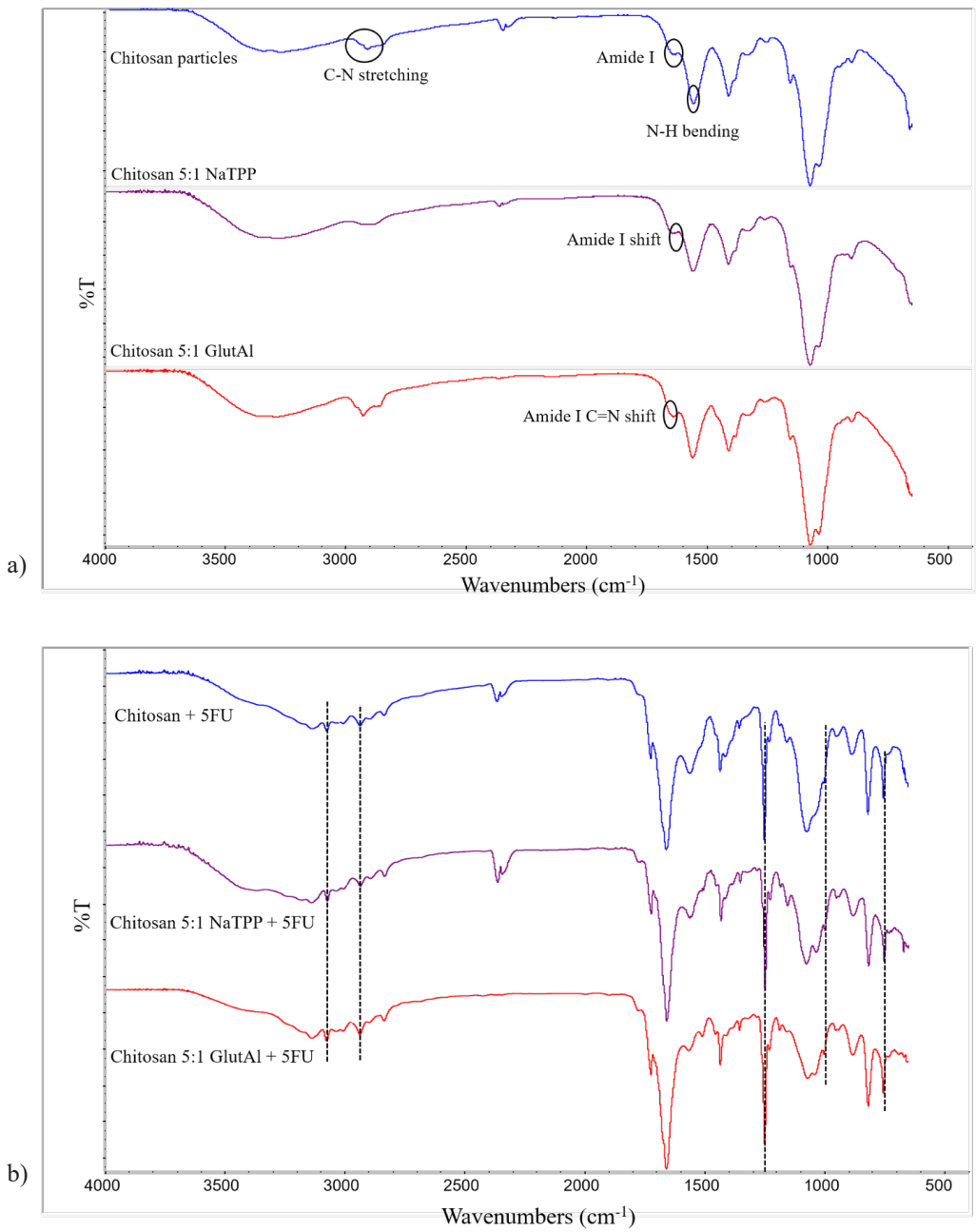


Fig. 1. (a) - ATR FT-IR spectra of non-crosslinked and crosslinked blank chitosan particles; (b) - ATR FT-IR spectra of 5-FU loaded non-crosslinked and crosslinked chitosan particles.

Amide I (1640 cm^{-1}) and N-H bending (1556 cm^{-1}) [17]. The physical crosslinking by NaTPP was confirmed due to the slight shifting of Amide I to 1635 cm^{-1} and N-H bending to 1550 cm^{-1} [18]. As a result of the Schiff-base interaction between the polymer and glutaraldehyde again a shift in Amide I was present, more specifically C=N bond at 1636 cm^{-1} [19].

The mean diameters of the formulated blank chitosan particles, measured by laser diffraction technique, are presented in Table 1. The diameters of all investigated systems are in the micrometer region, varying between 13.8 μm and 79.8 μm. The smallest diameter was found for the non-crosslinked particles. This system is characterized with bimodal size distribution with a small fraction of the particles having dimensions in the nanometric range.

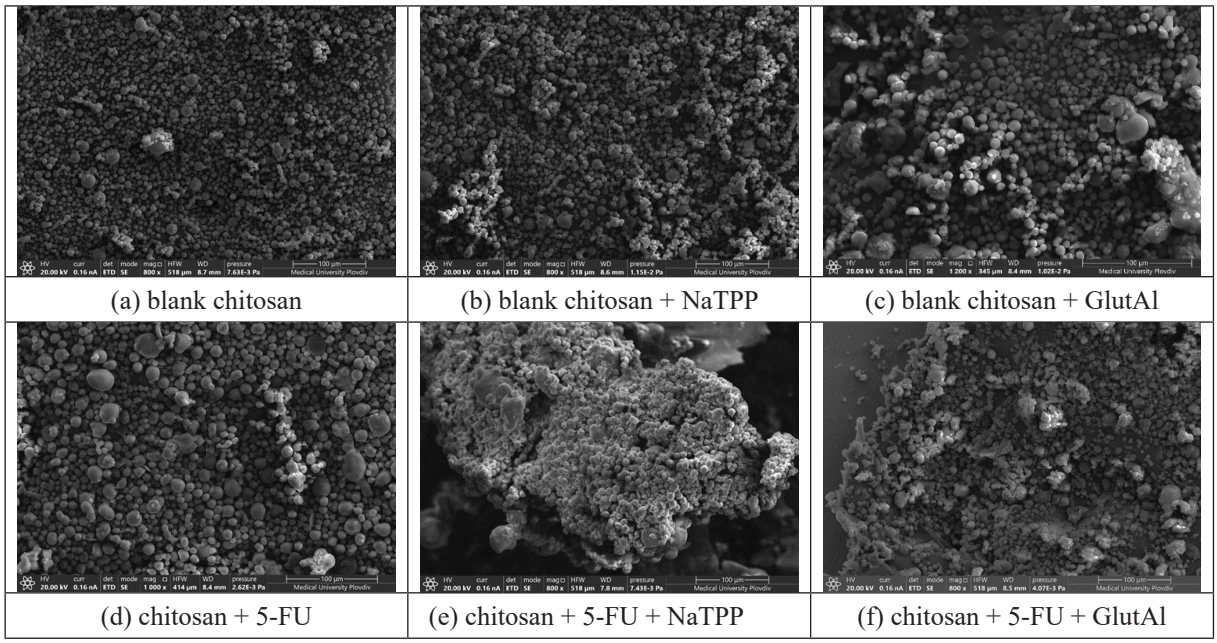
The size distribution of both chemically and physically crosslinked particles are trimodal with diameters up to 200 μm indicating presence of aggregates. It was found earlier, that during cross-linking the viscosity of the polymer solution increases and hence the particle size also increases [20]. The cross-linking reaction generally affects the density of the polymer matrix. The use of GlutAl is assumed to decrease the microsphere size in comparison with NaTPP, which is attributed to the formation of tighter chitosan structure.

The established by laser diffraction technique particle sizes were confirmed by SEM microphotographs of empty (Fig. 2a-c) and 5-FU loaded particles (Fig. 2d-f).

Particles without cross-linker appear to have narrow size distribution and the smallest sizes. In both crosslinking manners the process led to increase in the size and the polydispersity. did not change their shape, only their size. Addition of crosslinker at high mixing

Table 1. Size, yield and loading efficiency of chitosan particles.

Sample	Size, μm	Yield, %	Loading efficiency, %
Chitosan	13.8 ± 0.2	90.3 ± 4.5	88.5 ± 4.3
Chitosan + GlutAl	54.9 ± 2.8	78.9 ± 5.5	71.8 ± 1.7
Chitosan + NaTPP	79.8 ± 15.9	55.7 ± 1.0	49.4 ± 6.9



(a) blank chitosan | (b) blank chitosan + NaTPP | (c) blank chitosan + GlutAl

(d) chitosan + 5-FU | (e) chitosan + 5-FU + NaTPP | (f) chitosan + 5-FU + GlutAl

Fig. 2. SEM microphotographs of blank and 5-FU non-crosslinked and crosslinked particles.

rates hinders its homogenous distribution, leading to formation of bigger particles and their colliding into aggregates [21, 22]. The addition of 5-FU brings to enlarging the microspheres and making the spheres rougher and more aggregated, probably due to the non-uniform water evaporation during the heating and solidification of the particles in the oil media [23]. In all obtained samples aggregation is partly caused by the filtration of the particle-containing oily media and led to sticking of particles one to another [24].

The yield of the synthesis process is relatively high and varied between 55.7 % and 90.3 %. It is the highest for non-crosslinked chitosan particles and the lowest for ionically crosslinked particles. In the latter case, during the intensive stirring and emulsification on the walls of the beaker in which the synthesis was carried out, large aggregates of macroscopic dimensions were formed, which were removed. Thus, the yield dropped.

The swelling behaviour of drug delivery systems is essential as it largely determines the kinetics of drug release. The swelling of investigated samples during 480 min in saliva buffer is presented in Fig. 3.

When the particles are placed in an aqueous media, processes of polymer network and partial dissolution occur simultaneously. In the case of non-crosslinked chitosan spheres, the dissolution dominates over swelling and the mass of the particles decreases. The swelling is maximal, over 1000 % for physically crosslinked particles, with a decrease in mass due to dissolution after the first hour. The swelling of the chemically crosslinked particles is up to 100 %, again with partial dissolution and mass reduction. In general, the swelling of the last system is the most stable due to the non-reversible chemical binding of the GlutAl and the dense particle structure.

Formulation and characterisation of 5-FU chitosan particles

The loading of 5-FU in the chitosan particles is confirmed by ATR FT-IR analysis (Fig. 1b) and quantitatively characterized spectroscopically by determining the loading efficiency of the polymer structures (Table 1).

According to the literature 5-FU have characteristic peaks at 3070 cm^{-1}, 2932 cm^{-1}, 1242 cm^{-1}, 949 cm^{-1} and 750 cm^{-1} corresponding to F-unbound groups, C-H stretching, C-H in plane stretching, N-H and C-H wagging and vibration of the pyrimidine ring respectively [17, 25, 26]. When a comparison between drug-free and drug-loaded particles is done, it can be seen the presence of 5-FU peaks in the fingerprint region. Also, shift in peaks from 1242 cm^{-1} to 1249 cm^{-1}, 1665 cm^{-1} to 1655 cm^{-1} along with slight shift and change in the peak and intensity in the region 1100 - 1000 cm^{-1} suggest for possible electrostatic interaction between the polymer and the drug [22, 27, 28].

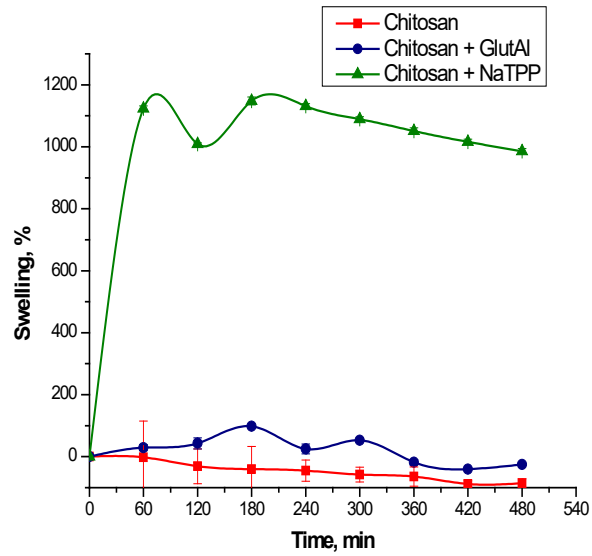


Fig. 3. Swelling behaviour of blank non-crosslinked and crosslinked chitosan particles.

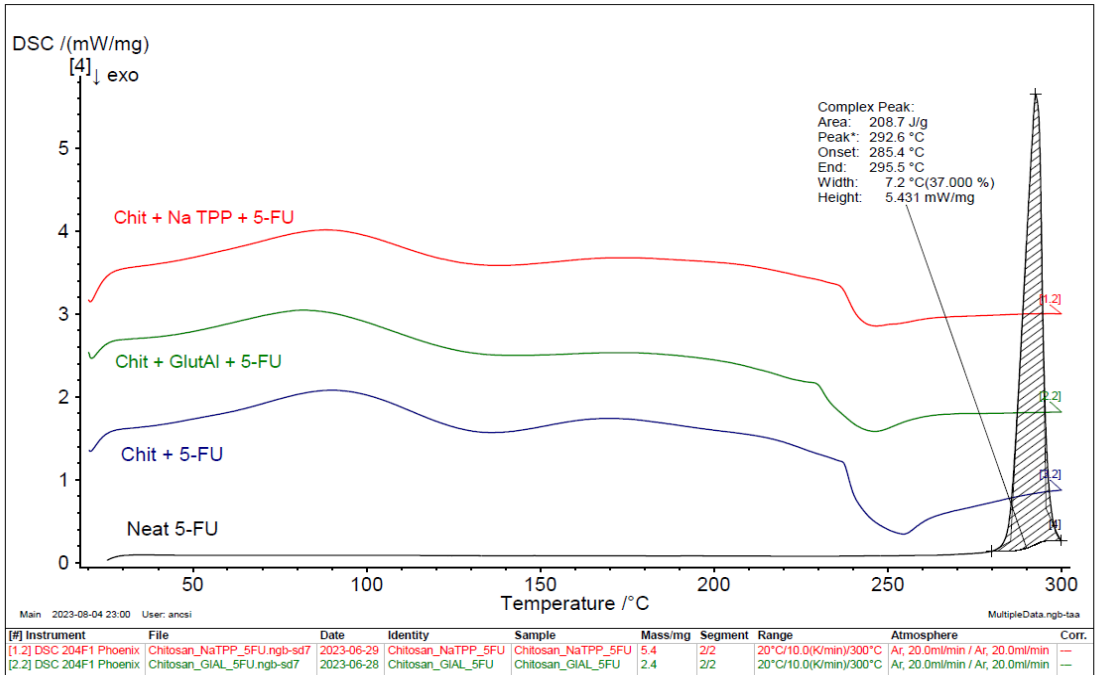


Fig. 4. DSC thermograms of neat 5-FU, and 5-FU loaded in non-crosslinked and crosslinked chitosan particles.

The loading efficiency was relatively high and varied between 49.4 % and 88.5 %. It is the highest for non-crosslinked particles. The reason for this observation could be an interaction between the chitosan and 5-FU. When the system is crosslinked, part of the ionized active groups of the chitosan are involved in interaction with the crosslinker and the interaction with the drug is less pronounced. Therefore, the loading efficiency decreases. Another explanation of the lower lading efficiency of the crosslinked systems, which is reported in the literature, relates to the crosslinking rate [22]. GlutAl and NaTPP cause a fast gelation rate of the chitosan network and a fast solidification of the hydrogel, and this situation prevents the movement of the drug around the polymer chain to entrap 5-FU into the network. As a result, the drug loading capacity decreased with the crosslinked systems [29].

It is well known that the neat 5-FU is a crystal compound. The differential scanning calorimetry (DSC) technique was applied in order to obtain the phase state of the drug after its loading in the chitosan spheres. The resulting thermograms are shown in Fig. 4.

The chitosan particles' thermogram showed a broad endothermic peak at about 90°C, which is attributed to water evaporation from the samples, and another peak above 250°C, which was due to thermal destruction. The neat 5-FU is a crystal solid with a pronounced narrow endothermic peak corresponding to a melting temperature of 282.80°C [30]. In the drug-loaded samples, no peak corresponding to 5-FU melting was observed. Therefore, an assumption could be made that loaded 5-FU existed in an amorphous state. The transformation from crystal to an amorphous solid most probably occurred when dissolving 5-FU in chitosan solution. These results were confirmed in all tested samples without any noticeable effect of the crosslinking agents.

The cumulative release of 5-FU in saline buffer (pH 6.8) and (37.0 ± 0.5)°C for 480 min is shown in Fig. 5.

The release during the first 480 min was incomplete, varying in the range between 40 % and 55 % depending on the sample composite. It demonstrated burst effect during the first 30 min, after which the released amount remained almost constant. Similar results were reported by other authors [17-19]. The fastest release is realized from physically crosslinked particles probably because of their sharp swelling. The slowest release from the non-crosslinked particles could be explained by the

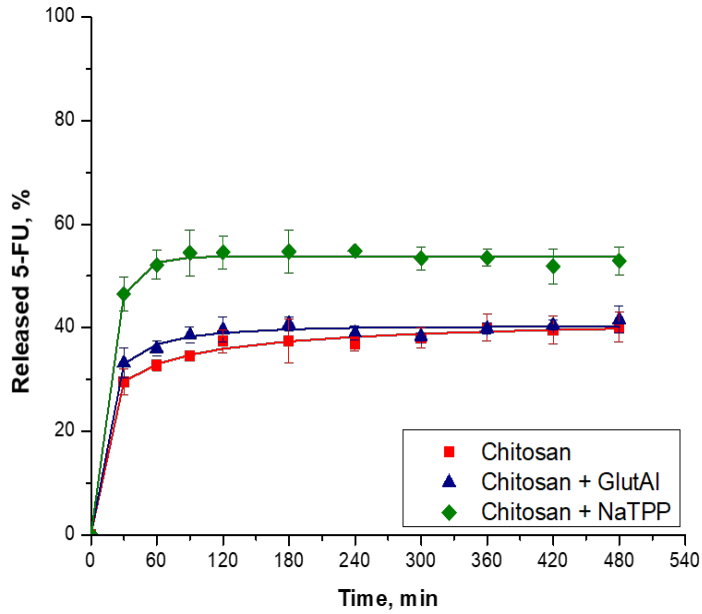


Fig. 5. Cumulative release of 5-FU from non-crosslinked and crosslinked chitosan particles.

Table 2. Weibull model parameters.

Sample	M_0	a	b	R^2
Chitosan	0.41 ± 0.02	15.6 ± 2.9	0.35 ± 0.09	0.996
Chitosan + GlutAl	0.40 ± 0.01	10.4 ± 3.9	0.51 ± 0.14	0.994
Chitosan + NaTPP	0.53 ± 0.03	14.5 ± 4.1	0.94 ± 0.34	0.997

interaction between the polymer and the drug.

For further characterisation of 5-FU release, the experimental data were fitted to Weibull model (Eq. 4). The model parameters are presented in Table 2.

The amount of M0 Is the highest for the physically crosslinked particles, indicating that the maximal release is about 53 %. The slowest release (maximal *a* parameter) is observed from the non-crosslinked particles.

CONCLUSIONS

5-FU was successfully loaded in non-crosslinked and crosslinked by GlutAl and NaTPP chitosan particles. The crosslinking process, as well as the interaction between the drug and the chitosan were established by FT-IR. The loading efficiency of the 5-FU is the highest for non-crosslinked chitosan particles, which possess the smallest size and the narrowest size distribution. The 5-FU release is not full during 480 min. It can be controlled by modifying the crosslinking of the particles.

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