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Wheat germ agglutinin-conjugated chitosan–Ca–alginate microparticles for local colon delivery of 5-FU: Development and *in vitro* characterization

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ABSTRACT

The aim of this work was to prepare lectin-conjugated chitosan–Ca–alginate microparticles (MPs) loaded with acid-resistant particles of 5-fluorouracil (5-FU) for efficient local treatment of colon cancer. MPs were prepared by a novel one-step spray-drying technique and after wheat germ agglutinin (WGA) conjugation, they were characterized for size, swelling behavior, surface charge, entrapment efficiency and *in vitro* drug release. Prepared particles were spherical, with 6.73 μg/mg of WGA conjugated onto their surface. The size and zeta potential increased after conjugation, from 6.6 to 14.7 μm and from 9.6 to 15.3 mV, while drug encapsulation was 75.6 and 72.8%, respectively after conjugation. The swelling behavior of beads was mainly determined by properties of the cross-linked chitosan–alginate network. *In vitro* drug release studies carried out in simulated *in vivo* conditions with respect to pH, confirmed the potential of the particles to release the drug in a controlled manner. Also, the drug release was not significantly affected by WGA conjugation. The retention of biorecognitive activity of WGA after covalent coupling to MPs was confirmed by haemagglutination test. Functionalized MPs showed excessive mucoadhesiveness *in vitro*, due to the positive surface charge, pH-dependent swelling of the matrix and lectin–sugar recognition.

1. Introduction

In the last 15 years, selective drug delivery to the colon has been the focus of increasing interest in research. The site specific drug delivery to the colon would be beneficial not only for the oral delivery of proteins and peptide drugs, but also for the delivery of low molecular weight compounds used in the treatment of diseases associated with colon or large intestine, such as amoebiasis, inflammatory bowel diseases and colorectal cancer (Minko, 2004; Jain et al., 2006; Mladenovska et al., 2007a,b; Anande et al., 2008; Jain and Jain, 2008; Simonoska Crcarevska et al., 2008).

Among chemotherapeutic compounds, 5-fluorouracil (5-FU) is one of the most widely used agents for the treatment of colorectal cancer (Lai et al., 2006; Sastre et al., 2007). However, the cytotoxicity of 5-FU is not limited to tumor tissue. Haematopoietic cells and normal epithelial cells of the gastrointestinal (GI) tract are susceptible to 5-FU-induced cytotoxicity, which produces severe leucopenia and intestinal toxicity, leading to lethal translocation of intestinal microflora (Kucuk et al., 2005). Until now, there is no dosage form of 5-FU for oral delivery, although this would be particularly useful in colon cancer therapy.

To improve these disadvantages, pharmaceutical technologists have been working on ways to deliver the drug more effectively to the colon *via* the GI tract, where it can target the tumor tissues. Colon-targeted drug delivery is most often associated with particulate carriers such as microparticles (MPs) and nanoparticles, which are designed to effectively protect drugs against premature degradation, to localize drug molecules at the targeted site of action and to control the time and rate of drug release (Yin et al., 2006; Sastre et al., 2007).

Polyanion/polycation cross-linked hydrogel MPs based on alginate (ALG) and chitosan (CTS) have gained much attention in recent years as carriers for local colon drug delivery due to their favorable properties including biocompatibility, biodegradability, pH sensitivity and excessive mucoadhesiveness (Mladenovska et al., 2007a,b; Simonoska Crcarevska et al., 2008; Xu et al., 2007). Physicochemical changes in the hydrogel environment induces relaxation of the polymer network which initiates mucus layer interaction and drug-targeted delivery at the site of action as a result of pH, ion exchange and microbiologically induced swelling of the polymer network. This useful mechanism for increase of the residence time as well as controlled drug release regulated by swelling and cross-linking matrix density may provide a relatively high local drug concentration at the site of action and increased therapeutic activity (He et al., 2008).

The main disadvantage of these MPs is adherence to the substrate by non-specific interactions, i.e. mucoadhesive polymers

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cannot distinguish between the adherence to intestinal mucus or to the surfaces of other gut parts or contents. To overcome these limitation, functionalized MPs could be prepared by conjugation of ligands such as lectins to particles surface and could be potentially used to deliver anti-cancer agents directly to the site of the disease (Montisci et al., 2001; Kim et al., 2005; Anande et al., 2008).

Because of their selective carbohydrate specificity and their resistance to protease degradation, several lectins have been explored for pharmaceutical applications. The wheat germ agglutinin (WGA, 36 kDa) from *Triticum vulgaris* is being used widely in drug delivery research because it is well characterized and one of the least immunogenic lectins and putative nontoxic. WGA binds to *N*-acetyl-glucosamine and sialic acid. As compared to other plant lectins with different carbohydrate specificity, WGA binding has been demonstrated as somewhat more specific to intestinal cell lines of human origin, human colonocytes and prostate cancer cells (Zhang et al., 2006). Also WGA acts as an excellent mucoadhesive agent and binds to the glycoproteins of mucus layer of the colon (Peppas and Huan, 2004).

In that course, the aim of the present study was to formulate lectin-conjugated 5-FU loaded CTS–calcium–ALG MPs by combining different principles of targeting and controlled release with muco/bioadhesivity of the system and to evaluate the ability of functionalized MPs to improve oral delivery of 5-FU to colon region. To achieve this goal, spray-dried acid-resistant particles of 5-FU were encapsulated into CTS–calcium–ALG matrix using a novel one-step spray-drying procedure and were further functionalized with WGA. The physicochemical and biopharmaceutical characteristics of the prepared MPs were investigated. The retention of biorecognitive activity of WGA after covalent coupling to MPs surface was confirmed by haemagglutination test. *In vitro* adsorption studies were conducted to estimate the binding activities of lectin-conjugated MPs with crude pig mucin (PM).

2. Materials and methods

2.1. Materials

5-FU was supplied as a gift sample from EBEWE Pharma, Germany. Chitosan (CTS), low viscous, was obtained from Fluka, Switzerland and sodium alginate (Protanal® LF 10/60) (ALG) was kindly donated by FMC BioPolymer, Norway. Hydroxypropyl methylcellulose phthalate (HP-55) was purchased from Shin Etsu Chemical Co. Ltd., Japan. Calcium chloride ($CaCl_2$) was obtained from Alkaloid, Macedonia. Lectin from *T. vulgaris* (WGA), glycine and crude mucin from pig (mucin from porcine stomach, Type II, partially purified; PM) were purchased from Sigma–Aldrich, Germany. 1,1′-Carbonyldiimidazole (CDI) was obtained from Fluka, Canada and *N*-acetyl-D-glucosamine from Fluka BioChemica, China.

All other chemicals were of analytical reagent grade and were used as received.

2.2. Methods

2.2.1. Preparation of acid-resistant particles of 5-FU

Acid-resistant particles of 5-FU (*samples HP-5FU*) were prepared using one-step spray-drying procedure. For this purpose, HP-55 was used, polymer widely applied in oral pharmaceutical formulations as an enteric coating material for tablets and granules. This polymer was selected to avoid release of 5-FU during the transit of the particles through the upper region of the GI tract because it could cause deleterious effects and lack of effectiveness. HP-55 is insoluble in gastric fluids (soluble at pH >5.5) and 5-FU is water-soluble cytotoxic drug with *Mw* 130.08 (Wong et al., 2007; He et al., 2008). In briefs, equal volumes of polymer solutions (coat and core) were prepared by dissolving HP-55 into phosphate buffer pH 7.4 (Ph.Eur.4) at room temperature. 5-FU was than dissolved in the core-polymer solution. The resulting solutions were spray-dried through two-fluid pressurized nozzle of a mini spray-dryer (Buchi 290, Mini Spray Dryer, Swiss). The core/coat polymer ratio was 1:1 and final polymer/drug ratio was 5:1.

2.2.2. Preparation of CTS–Ca–ALG MPs loaded with acid-resistant particles of 5-FU

Spray-dried acid-resistant particles of 5-FU (*sample HP-5FU*) were further incorporated into CTS–Ca–ALG matrix using a novel one-step spray-drying procedure published recently by our research group (Simonoska Crcarevska et al., 2008). The goal was to prepare MPs with sufficient mechanical strength, defined swelling properties, controlled release of encapsulated drug substance and certain surface properties as factors important for the efficacy of the designed system and its prolonged residence time at the site of action due to mucoadhesivity of the cross-linked matrix (Glavas-Dodov et al., 2007; Simonoska Crcarevska et al., 2008).

Therefore, polymers used in the formulation were chosen according to their physicochemical properties. Selected ALG (Protanal® LF 10/60), consists of 65–75% of guluronic acid (G) and 25–35% of manuronic acid (M). Having in mind that MG types compared with MM and GG types of ALG have best flexibility, and that polymer gels formed from ALG with high percentage of guluronic acid (>70%) have highest mechanical strength and stability towards monovalent ions, LF 10/60 was chosen for preparation of MPs.

CTS with low viscosity, highly deacetylated was chosen for polyelectrolyte complexation with ALG. The fact that the deacetylated chains are fully stretched by the electrostatic repulsion among the $-NH^{3+}$ groups (and the acetylated blocks are micelle-like agglomerates because of the hydrophobic forces), leads to a conclusion that higher degree of deacetylation might contribute to the efficient process of coating (NATO SfP: 978023, 2006).

In a typical procedure; homogenous 1% *w*/*v* aqueous solution of CTS in 1% *w*/*v* acetic acid was prepared and after 6 h of gentle stirring at ambient temperature cross-linking agent $CaCl_2$ was added. The acid-resistant particles of 5-FU were suspended in previously prepared 2% *w*/*v* aqueous solution of ALG. Thus prepared mixtures were stirred for 15 min before use (300 rpm; Variomag, Multipoint HP15, Germany) and were spray-dried through the double-fluid nozzle of a mini spray-dryer (Buchi 290, Mini Spray Dryer, Swiss). The spray-dried MPs (*samples MPH-5FU*) were harvested from the apparatus collector and kept under vacuum at room temperature. The volume of feed-solutions sprayed for the preparation of the samples was in range of 100–300 ml. Drug empty MPs (*samples MPH*) were also prepared as a comparison.

Schematic presentation of the preparation procedure for 5-FU loaded MPs and the composition of the prepared formulations are presented in Fig. 1 and Table 1, respectively. At least six different samples were prepared for each batch formulation.

Production yields (expressed as the weight percentage of the beads obtained with respect to the initial amount of polymers and drug used for the preparation) (Beck et al., 2007) were around 50% (Table 1), which can be explained by the relatively low volumes of feed-solutions sprayed for the preparations of each batch of MPs, the structure of the apparatus that is not equipped with a trap to

Table 1
Composition and production yield of spray-dried MPs (data shown are the mean ± SD; $n = 6$).

Sample	CTS:ALG:5-FU mass ratio	Conc. $CaCl_2$ (% *w*/*v*)	Production yield (%) ± SD
HP-5FU	–	–	58.7 ± 0.8
MPH	1:2:–	2.5	53.1 ± 0.5
MPH-5FU	1:2:0.75	2.5	52.3 ± 1.9

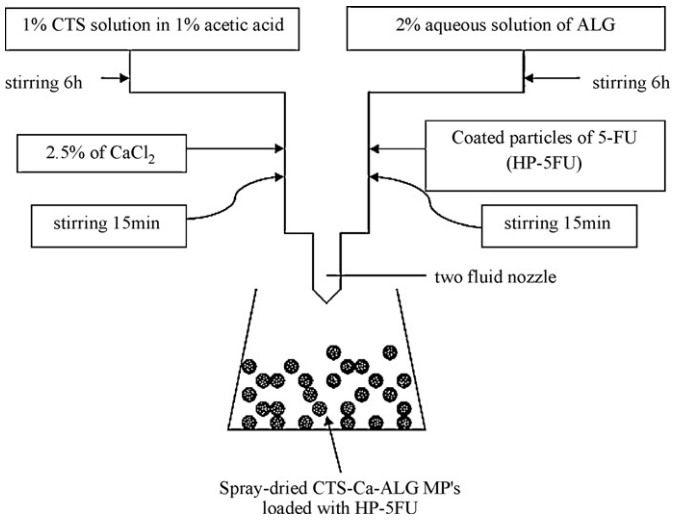

Fig. 1. Schematic presentation of the preparation process of cross-linked CTS–Ca–ALG MPs loaded with acid-resistant particles of 5-FU.

recover the smaller and lighter particles exhausted by the aspirator and the loss of material mostly due to powder adhering to the cyclone walls.

2.2.3. Covalent binding of WGA to CTS–Ca–ALG MPs

To conjugate the surface of MPs with WGA, a modified two-step carbodiimide method was performed (Kim et al., 2005). The process involved activation of the surface hydroxyl groups of CTS with CDI in aprotic solvent, followed by binding to the amino groups in WGA. At the beginning of our experiments the stability of MPs during the coupling procedure as well as after the conjugation with WGA and lyophilization was examined. From the obtained results (presented in addition in Section 3) the following coupling procedure was applied. The MPs were incubated in two repeatable cycles with 1 h duration each, with anhydrous acetone containing CDI (first cycle: mass ratio MP:CDI = 1:1.25 *w*/*w*, second cycle: mass ratio MP:CDI = 1:0.65 *w*/*w*) at room temperature with continuous shaking. Afterwards, the CDI solution was discarded and the MPs were washed several times with anhydrous acetone to remove the unbound CDI. Than, the activated beads were suspended in borate buffer, pH 9.0, to which 0.4 mg of WGA was added under vortexing. The reaction was allowed to proceed for 1 h. MPs were washed several times with the borate buffer to remove the unbound lectin (3 × 5000 rpm, 10 min; Tehtnika Centric 322B, Slovenia) and were incubated in glycine solution (200 mg/ml) to quench activated sites to which WGA was not bound. After several washing steps, MPs were dispersed in water, freeze-dried (−40 °C, 0.75 mBa, period 24 h; Labconco, FreeZone 2.5 l Freeze Dry System, USA) and stored at 4 °C. Functionalized, blank MPs (*samples WGA–MPH*) were also prepared.

To estimate the amount of WGA-conjugated onto the surface of MPs, the amount of WGA in the supernatant and the washings before lyophilization was subtracted from the amount of WGA taken for conjugation (400 μg/30 mg MP).

2.3. Characterization of MPs

2.3.1. Study of morphology

The shape and surface morphology of MPs were investigated using scanning electron microscopy (SEM). The samples for SEM study were prepared by lightly sprinkling the formulation on a double adhesive tape stuck to an aluminum stub. The stubs were then coated with gold to a thickness of about 300 Å under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. The coated samples were then randomly scanned and photomicrographs were taken with a scanning electron microscope (Jeol-SEM 6400, Japan).

2.3.2. Particle size analysis

Particle size of the prepared beads (*samples HP-5FU*, *MPH-5FU*, *MPH*, *WGA–MPH* and *WGA–MPH-5FU*) was measured by laser diffractometry using Mastersizer 2000, Malvern Instruments Ltd., UK equipped with Scirocco 2000, Malvern Instruments Ltd., UK, for dry powder measurements. Six measurements for each sample were performed. The conditions were: vacuum −2 bar and feed rate −50%, while obscuration was set between 0.2 and 4%.

The particle size distribution was also expressed in terms of *SPAN* factor determined as:

$$SPAN = \frac{d_{90} - d_{10}}{d_{50}}$$

where d_{10}, d_{50} and d_{90} are the diameters in μm at 10, 50 and 90% cumulative volumes, respectively. Thus, the *SPAN* factor gives a measure of the range of the volume distribution relative to the median diameter (Raffin et al., 2006). A high *SPAN* value indicates a wide size distribution (Gavini et al., 2007).

2.3.3. Swelling studies

Dry ionically cross-linked beads increase their volume after minutes in water or in buffers at different pH and composition, due to matrix rehydration and in accordance to the degree of cross-linking. Maintains of the polyelectrolyte complexes depends considerably of the ion exchange in the tested buffer solution. Therefore, the swelling properties of the WGA-conjugated CTS–Ca–ALG MPs (WGA–*MPH-5FU*) were determined by measuring the particle volume increase (expressed as volume increase ratio-*VIR*) of prepared formulations in buffer solutions pH 2.0, 4.5, 6.8 and 7.4 (Eur.Ph.4), by laser diffractometry using Mastersizer 2000, Malvern Instruments Ltd., UK equipped with dispersion unit Hydro 2000S; Malvern Instruments Ltd., UK.

Samples of beads of known weight (30–40 mg) were suspended into 5 ml of swelling solutions and allowed to swell on magnetic stirrer (300 rpm; Variomag, Multipoint HP15, Germany) at room temperature. At predetermined time intervals (after 1, 3, 5 and 24 h, respectively), the homogenous dispersions were examined to determine the particle size. At least six measurements were performed while the sample was in the cell under stirring (2520 rpm/stirrer rate) and ultrasound (50%), previously applied for 1 min. The obscuration was set between 10 and 12%.

The volume increase ratio (*VIR*), in predetermined time interval, was calculated according to the equation:

$$VIR\,\text{ratio} = \frac{A\,d_{50}\,(\mu\text{m})}{B\,d_{50}\,(\mu\text{m})}$$

where *A* is particle diameter d_{50} (μm) of the swollen beads and *B*, particle diameter d_{50} (μm) of the beads measured as dry powder.

2.3.4. Zeta potential measurements

The zeta potential (ζ) of WGA-conjugated and unconjugated MPs was measured using Zetasizer Nano Series, Nano-ZS, Malvern Instruments Ltd., UK. Particles were suspended in distilled water (pH 5.1) and sonified for 2 min. Six different measurements for each sample were performed.

2.3.5. Drug entrapment efficiency

The encapsulation efficiency (*EE*%) of 5-FU in the prepared formulations was determined by extracting and quantifying the encapsulated drug using an HPLC assay described elsewhere in detail (Glavas-Dodov et al., 2005). For this purpose, blank MPs were

also analyzed, in the same manner and same time as MPs loaded with 5-FU.

Analyses were performed on Agilent 1100 HPLC system, equipped with 1100 Quaternary Pump and Agilent 1100 diode array detector. The column used was LiChrospher® 60, Rp Selected B, 125 mm × 4 mm i.d., 5 μm. The mobile phase was 100% 0.02 M phosphate buffer pH 7.4. Chromatographic conditions set for this method were: flow rate 1 ml/min, column temperature 20 °C, UV detection at 266 nm, injection volume 20 μm.

A predetermined amount of the MPs (10 mg) were added to 10 ml of phosphate buffer at pH 7.4 (Ph.Eur.4) and were subjected to shaking at 37 °C, to ensure complete extraction of the drug substance. Samples were centrifuged (1500 × *g*, 3 × 15 min; Tehtnika Centric 322B, Slovenia) and 2 ml of the clear centrifugate was filtered through 0.45 μm membrane filter (Ministrar RC 25; Sartorius, Goettingen, Germany) and injected onto the HPLC column. Results were calculated from linear regression of the external standard of 5-FU.

The drug encapsulation efficiency (*EE*%) was calculated from the following equation (Sairam et al., 2006):

$$EE\,(\%) = \frac{\text{Actual drug loading}}{\text{Theoretical drug loading}} \times 100$$

2.3.6. Determination of the amount of bound WGA to MPs

The amount of bound lectin to the surface of the MPs was determined by SEC-HPLC analysis. The HPLC system (Waters 600E, USA) was equipped with Rheodyne 7725I, photodiode array detector 996, column FRACTOGEL BioSEC, Merck; 600–16 mm. The mobile phase was H_3PO_4 0.1 M, pH 2.5; 100 mM NaCl. Chromatographic conditions set for this method were: flow rate 2 ml/min, column temperature 25 °C, injection volume 200 μl, UV detection at 215 nm. The method was validated and reported to be linear in the range of 12.5–100 μg WGA/ml ($r^2 = 0.9992$). The RSD for repeatability was 0.50% ($n = 6$) and for intermediate precision was 0.54% ($n = 18$). Accuracy for 25, 50 and 75 μg WGA/ml was 101.70, 100.34 and 99.12%, respectively.

Aliquots of the clear supernatants obtained from centrifugations during the removal of the unbound lectin before lyophilization of the particles were taken for analysis. The quantity of bound lectin was calculated as the difference between the initially added WGA (400 μg/30 mg MP) and WGA which was recovered by centrifugation. Clear supernatants obtained from MPs without added WGA were used as a blank (Arangoa et al., 2000; Surti et al., 2008).

2.3.7. In vitro release studies

In vitro drug release studies were carried out in closed glass tubes at 37 °C and 50 horizontal strikes/min (horizontal shaker; Shaker Unitronik OR, Selecta, Spain) where the suspension of WGA-conjugated or non-conjugated MPs (equivalent to 2 mg of 5-FU/10 ml) was placed. To compare the 5-FU release under different pH conditions, the experiments were performed in acidic buffer (pH 2.0, for 2 h) to simulate fasted stomach, in phosphate buffer (pH 4.5) to simulate duodenum, in phosphate buffer (pH 6.8) to simulate mid jejunum and in phosphate buffer (pH 7.4) to simulate ileo-colon conditions for 24 h (Kalantzi et al., 2006; Mladenovska et al., 2007a; Sonaje et al., 2009). Samples (5 ml) were withdrawn at specified time intervals (every 30 min in the first 2 h, afterwards every hour till the end of the experiment) and were centrifuged at 1500 × *g* for 10 min. The supernatants were membrane filtered and assayed for drug release, UV spectrophotometrically (266 nm; Lambda 16, PerkinElmer, USA). All dissolution tests were run in triplicate and the release results were plotted as the cumulative and percentage of the content into dissolution medium versus time.

The *in vitro* release pattern was evaluated to check the goodness of fit to the Higuchi's square root of time equation (Higuchi, 1963) (Eq. (1)), Korsmeyer–Peppas power law equation (Korsmeyer et al., 1983; Peppas, 1985) (Eqs. (2) and (3)) and fitting the data to the heuristic model proposed by Peppas and Sahlin (1989) (Eq. (4)) for quantifying the phenomena controlling the release from swellable matrix, in which the contribution of the relaxation or erosion mechanism and of the diffusive mechanism can be quantified. The goodness of fit was evaluated using the *r* (correlation coefficient) values.

$$M = Kt^{1/2} \tag{1}$$

where *M* is the amount of drug dissolved in time *t*, *K* is the Higuchi dissolution constant, and *t* is the release time.

$$\frac{M_t}{M_\infty} = Kt^n \tag{2}$$

which in logarithmic form is

$$\log \frac{M_t}{M_\infty} = \log K + n \log t \tag{3}$$

where M_t is the amount of drug dissolved in time *t*, M_∞ is the amount of drug dissolved after infinite time (all the drug content in the formulation), M_t/M_∞ is the fractional release of the drug in time *t*, *K* is a constant incorporating the structural and geometric characteristics of the dosage form, *n* is the release (diffusion) exponent, which depends on the release mechanism and the shape of the matrix tested and *t* is the release time. For spheres, when *n* approximates to 0.43, a Fickian/diffusion controlled release is implied. For values of *n* between 0.43 and 0.85 are an indication of both diffusion controlled release and swelling controlled drug release (anomalous transport). Values above 0.85 indicate case-II transport which relate to polymer relaxation during gel swelling (Siepmann and Peppas, 2001; Pasparakis and Bouropoulos, 2006).

$$\frac{M_t}{M_\infty} = K_1 t^{1/2} + K_2 t \tag{4}$$

where M_t is the amount of drug dissolved in time *t*, M_∞ is the amount of drug dissolved after infinite time (all the drug content in the formulation), M_t/M_∞ is the fractional release of the drug in time *t*, K_1 and K_2 are, respectively, the diffusion and erosion terms. According to this equation, if the diffusion to erosion ratio $K_1/K_2 = 1$, then the release mechanism involves diffusion and erosion equally. If $K_1/K_2 > 1$, then diffusion prevails, while erosion predominates when $K_1/K_2 < 1$ (Ferrero et al., 2003; Khan and Craig, 2003; Sankalia et al., 2007).

2.3.8. Assessment of the in vitro activity and specificity (haemagglutination test)

The retention of biorecognitive activity of WGA, after the covalent coupling to MPs, was assessed by WGA-induced agglutination of human erythrocytes (Yin et al., 2006). For this purpose, a serial dilutions of blank WGA–MPs conjugates (*WGA–MPH*), non-conjugated MPs without 5-FU (*MPH*) and WGA solution in phosphate buffer pH 7.4 (0.2 ml), were mixed with the same volume of 2% suspension of fresh human blood group O erythrocytes. Samples were incubated at room temperature for 1 h. The confirmation of bioactivity was performed by comparing the value of the titre dilution (*Td*) of WGA, covalently bound to MPs, which gave the last visible red cell agglutination, against a positive control (WGA solution) as well as negative control (non-conjugated MPs).

In order to confirm the permanence of the specificity after WGA binding to particles surface, different quantities of *N*-acetyl-D-glucosamine (specific inhibitory sugar for WGA) were added to MPs and mixed with 2% suspension of fresh human blood group O erythrocytes. Then, the haemagglutination test was carried out as described above. Similarly, the minimal specific sugar concentration (mg/ml) required to inhibit the agglutination of human blood

group O erythrocytes triggered by WGA–MPs conjugates was determined. The procedures were conducted in triplicate.

2.3.9. In vitro adsorption studies with crude pig mucin (PM)

The *in vitro* interaction of lectin-conjugated MPs with PM was performed according to the method proposed by Kim et al. (2005) and Yin et al. (2006) with little modifications. The *in vitro* activity of blank WGA-conjugated MPs (WGA–*MPH*) was determined by mixing 5 ml of the PM suspension in different buffer systems (pH 2.0, 4.5, 6.8 and 7.4; Ph.Eur.4) with the equal volume of MPs suspension. After incubation period of 1, 3, 5 and 24 h at 37 °C, the samples were centrifuged (10 min at 4000 rpm) and the remaining free PM in the supernatants was measured at 251 nm, UV spectrophotometrically (PerkinElmer, Lambda 16, USA), since interacted PM was sediment together with MPs. The references consisted of the same amount of PM as in the samples. Each test was performed in triplicate.

The PM binding efficiency of MPs was calculated from the following equation:

$$\text{PM binding efficiency}\,(\%) = \frac{Co - Cs}{Co} \times 100$$

where *Co* is the initial concentration of PM used for incubation and *Cs* is the concentration of free PM determined in the supernatant.

2.4. Calculations and statistics

All results were depicted as mean value ± SD. Significance between the mean values was calculated using ANOVA one way analysis. Probability values $p > .05$ were considered significant.

3. Results and discussion

3.1. Preparation of CTS–Ca–ALG MPs

Spray-drying method is a well-known process which is used to produce powders, granules or agglomerates from drug-excipient solutions and suspensions. The particle size of MPs prepared by spray-drying process ranged from a few microns to several tens of microns and usually had a relatively narrow particle size distribution. When a liquid is fed to the nozzle with a peristaltic pump, atomization occurs by the force of compressed air, disrupting the liquid into small droplets, from which solvent evaporates instantaneously leading to formation of free flowing particles.

Recently, a number of papers have been published describing the preparation of MPs by such spray-drying method (Goracinova, 2007). Up to now spray-drying process was reported for production of CTS particles, ALG particles or CTS–ALG MPs with separate cross-linking procedure (Lorenzo-Lamosa et al., 1998; Ganza-Gonzales et al., 1999; Coppi et al., 2002; Huang et al., 2003; Agnihotri et al., 2004; Oliveira et al., 2005; Mladenovska et al., 2007b). The preparation of CTS–Ca–ALG MPs into the one-step spray-drying process is a novel method of MP preparation published recently by our drug delivery group (Simonoska Crcarevska et al., 2008).

In this study we present the one-step spray-drying method for production of CTS–Ca–ALG MPs loaded with acid-resistant particles of 5-FU. The acid-resistant particles of 5-FU were also prepared by the same method. The one-step production process ensured reproducible and simple manufacturing of the micronized systems, i.e. acid-resistant particles of 5-FU in ALG core solidified by cross-linking with $CaCl_2$ and CTS, which occurs spontaneously *via* electrostatic interactions between the negatively charged carboxylate groups on alginate and the positively charged calcium ions and/or protonated amino groups on chitosan (Glavas-Dodov et al., 2006, 2007; Goracinova et al., 2007; Lertsutthiwong et al., 2008).

In our earlier experiments, different concentrations of $CaCl_2$ as a cross-linking agent were evaluated (Glavas-Dodov et al., 2007, 2008). From the obtained results (data not presented), CTS–Ca–ALG MPs containing 2.5% of $CaCl_2$ were selected for conjugation with WGA.

3.2. Conjugation of WGA to CTS–Ca–ALG MPs

Generally, the association of lectins with MPs would be achieved by covalent linkage, which is more stable than simple adsorption. Different strategies can be contrived, depending on the principal functional groups located on the carrier surface. Most conjugates have been prepared from hydroxylated or carboxylated MPs, using glutaraldehyde or CDI as a coupling agent. However, studies concerning the preparations of lectin-conjugated particles loaded with drugs were quite limited (Mo and Lim, 2005; Yin et al., 2006).

The procedure of WGA conjugation to CTS–Ca–ALG MPs using CDI involve formation of an imidazolyl carbamate complex with the hydroxyl group of MPs which may be displaced by binding the free amino group of a ligand, such as a protein. The reaction is an *N*-nucleophilic substitution and results in a stable *N*-alkylcarbamate linkage of the ligand to the polymer. The resulting ligand–polymer complex is stable and resists hydrolysis for extended periods of time (Kim et al., 2005).

From the point of stability of MPs during the conjugation process, in the preliminary studies, the WGA attachment to MPs as well as particle size of functionalized MPs was assessed as a function of CDI concentration and incubation time between the CDI reagent and the MPs (Zhang et al., 2006). The activation of the surface –OH groups was performed with MPs:CDI mass ratios 1:0.5, 1:0.75, 1:1, 1:1.25, 1:1.5 and 1:2 and incubation time of 1, 2 and 3 h. Lectin binding increased with the CDI, but aggregation of the particles was observed when the MPs:CDI mass ratios was higher than 1:1.25 *w*/*w*. Similarly, lectins binding increased with the CDI activation time. Therefore, activation of MPs in two repeatable cycles with 1 h duration, each (first cycle, MP:CDI = 1:1.25 *w*/*w*, second cycle MP:CDI = 1:0.65 *w*/*w*) was considered as optimum for surface modification with WGA.

During the process of covalent immobilization of WGA to the MP surface, special attention has to be paid to the pH and ionic strength of the dispersion (coupling) media. From the point of the stability of the MPs in this solution (borate buffer pH 9.0; 1×10^{-3} M, ionic strength 6×10^{-3}), swelling studies point that no changes appeared during swelling test for period of 24 h in the above-mentioned buffer after the initial slight increase in volume diameter.

Production yield was 36.3% ± 2.3 (*w*/*w*) for functionalized blank MPs and 37.1 ± 2.1 (*w*/*w*) for drug loaded MPs, probably due to the WGA coupling procedure where additional processing steps and repeated washings resulted in low production yields (Mo and Lim, 2005).

3.3. Characteristics of MPs

3.3.1. Physicochemical characterization of the prepared MPs

The scanning electron microscopy pictures of the acid-resistant beads of 5-FU (*HP-5FU*) and CTS–Ca–ALG MPs loaded with acid-resistant beads of 5-FU (*MPH-5FU*) are shown in Fig. 2a and b. Generally, spherical morphology with presence of spherical disks with a collapsed centre or deformations was observed. Surface appearance was smooth with low porosity. Insufficiency of ideal spherical morphology was probably developed during the drying process.

The physicochemical characteristics of different formulations: *HP-5FU* (acid-resistant particles of 5-FU), *MPH* (MPs without 5-FU or WGA), *WGA–MPH* (MPs with WGA conjugation but without 5-FU incorporation), *MPH-5FU* and *WGA–MPH-5FU* are summarized in Table 2.

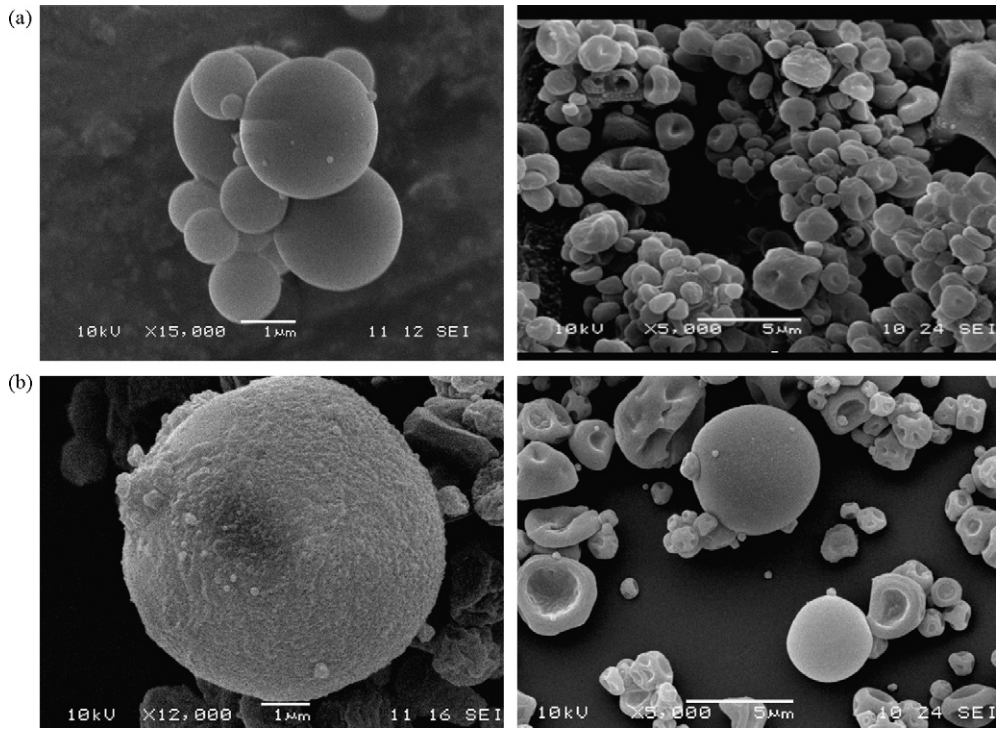

Fig. 2. SEM micrographs of the spray-dried (a) HP-5FU and (b) MPH-5FU MPs.

Mean diameters d_{50} (μm) of these MPs ranged from 3.1 μm for acid-resistant particles of 5-FU to 6.6 μm for non-conjugated and 14.7 μm for WGA-conjugated 5-FU loaded particles, with unimodal narrow size distribution. Also, the *SPAN* factor value slightly increased after the conjugation with WGA. Having in mind that the optimal particle size for localization of MPs and prolonged residence time in the colon is between 4 and 15 μm (in order to achieve a relatively large surface area and excessive adhesion at the site of action) (Lamprecht et al., 2001; Coppi et al., 2002; Goracinova et al., 2005; Glavas-Dodov et al., 2006), prepared WGA conjugates could be promising carriers for local colon delivery of 5-FU.

The zeta potential of MPs ranged from 9.6 mV (*MPH-5FU*) to 15.3 mV (*WGA–MPH-5FU*) (Table 2). The procedure of WGA conjugation was performed at slightly alkaline pH, therefore WGA (p*I* 9.0) would be positive electricity under this condition (Mo and Lim, 2005). This may be the reason for an increase in particles surface charge density as reflected by a higher zeta potential. Considering that CTS–Ca–ALG MPs and WGA conjugates showed a positive value of the zeta potential, a preposition of the presence of CTS on the surface of the particles could be made. Presented surface properties of prepared beads are of great importance, since positive charge originating from CTS is necessary for the interaction with negatively charge mucus and cell membranes, and consequently, muco/bioadhesion (Martinac et al., 2005). These findings are in favor of expected mucoadhesivity of prepared MPs using one-step procedure. In fact, during one-step spray-drying process both CTS molecules and calcium ions are competing which each other at the same time with the negatively charged groups of the ALG molecules and this competition may result in that CTS molecules are only slightly bound and hence keep their flexibility when the particles are suspended in aqueous milieu (Goracinova et al., 2008; Simonoska Crcarevska et al., 2008). As a result of this competition, they are able to interact with the mucus chains and show good mucoadhesiveness.

The swelling behavior of prepared WGA-conjugated MPs in buffer solutions at pH 2.0, 4.5, 6.8 and 7.4, respectively, was observed in a period of 24 h and is presented in Fig. 3. In acidic media only a small degree of swelling (*PVI* ratio of 1.1–1.4) in period of 24 h was observed, which could be attitude to the protonization of the primary amino groups of CTS, and thus creating a repulsive force within the matrix (Lin et al., 2005). However, the presence of hydrogen bounds and the tightly bound ionic cross-links by Ca^{2+} stabilize the matrix and the swelling is minimal (Lin et al., 2005; Pasparakis and Bouropoulos, 2006). In phosphate buffer pH 4.5, initial swelling during the first hour was observed, afterwards there were no significant differences in volume diameters of MPs.

In phosphate buffer at pH 6.8, especially at pH 7.4, prepared formulations exhibited rapid swelling. At pH 6.8, calculated *PVI* ratios were from 2.9 to 9.2 for the period of 24 h. In phosphate buffer with pH 7.4, the *PVI* ratios were from 5.9 to 18.6 for the tested period of 24 h, respectively.

Literature data pointed that at pH >6.0, the CTS shows poor solubility and low binding with ALG due to its lower cationic nature (CTS will lose most of its positive charge and ALG will be decid-

Table 2
Physicochemical characteristics of prepared MP formulations ($n = 6$).

Sample	Production yield (%) ± SD	Mean diameter, d_{50} (μm) ± SD	SPAN factor ± SD	Zeta potential, ζ (mV) in water ± SD	5-FU content into MPs (mg/g MP)	EE (%) ± SD
HP-5FU	58.7 ± 0.8	3.15 ± 0.02	1.83 ± 3 × 10^{-3}	–	165.18 ± 0.8	99.11 ± 0.81
MPH	53.1 ± 0.5	6.39 ± 0.04	1.95 ± 0.03	–	–	–
MPH-5FU	52.3 ± 1.9	6.64 ± 0.03	1.97 ± 0.05	9.6 ± 0.40	58.54 ± 1.1	75.54 ± 0.60
WGA–MPH	36.3 ± 2.3	13.77 ± 0.12	2.13 ± 0.08	–	–	–
WGA–MPH-5FU	37.1 ± 2.7	14.74 ± 0.09	2.28 ± 0.15	15.3 ± 0.30	49.01 ± 1.8	72.78 ± 1.10

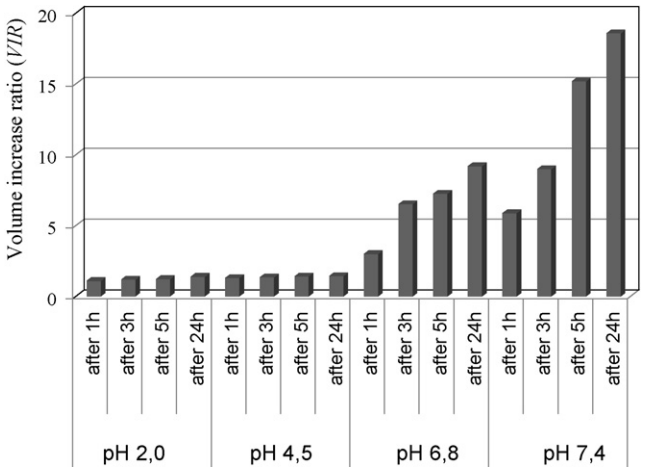

Fig. 3. Swelling behavior of WGA-conjugated MPs loaded with acid-resistant particles of 5-FU (*WGA–MPH-5FU*) in different buffer solutions at pH 2.0, 4.5, 6.8 and 7.4.

edly anionic), leading to increased swelling and dissociation of the complex (Shu and Zhu, 2001; Pasparakis and Bouropoulos, 2006; Sankalia et al., 2007). The swelling behavior of MPs was also related to the exchange of Ca^{2+} ions from the polymer matrix with Na^{+} ions from the phosphate buffers and formation of calcium phosphate (Bajpai and Sharma, 2004). Also, at these pH values (phosphate buffers above a pH 5.5), the affinity of phosphate for calcium is higher that of ALG, due to the chelating action of the phosphate ions (Zheng et al., 2004), so disruption of the matrix occurs faster (Pandey and Khuller, 2004; Anal and Stevens, 2005).

The hydrophilic attributes of WGA, could also be involved in swelling behavior of MPs, making it easier for aqueous solutions to penetrate into the matrix (Yin et al., 2006).

Presented results from the swelling studies are of great importance for the *in vivo* performance of MPs, since physical–chemical changes in the cross-linked hydrogel environment induces relaxation of the polymer network which initiates mucus layer interaction and drug-targeted delivery at the site of action as a result of pH, ion exchange and swelling of the polymer network. Therefore, no interaction and simple passage of the particles through the upper part of GI tract could be expected, while in the colon, adhesion of a carrier system to the mucus may improve residence time and drug contact with the underlying epithelium, thus increasing drug concentration at the site of absorption (He et al., 2008).

5-FU content (mg 5-FU/g MPs) in all prepared formulation, determined by HPLC method, as well as calculated encapsulation efficiency (EE%) is presented in Table 2. EE (%) was found to be quite high, ~100% for acid-resistant particles of 5-FU, 75.6% for CTS–Ca–ALG MPs and 72.8% for WGA conjugates. The slight reduction in the EE% may be due to the diffusion of surface free drug during the conjugation process (Surti et al., 2008). These results clearly presents that during functionalization with WGA no significant ($p > .05$) drug loss occurred and that the preparation method for the incorporation of acid-resistant particles of 5-FU into CTS–Ca–ALG matrices as well as performed conjugation procedure resulted in formation of stable functionalized particles with high EE.

The amount of bound WGA to MPs surface was calculated to be 6.725 μg WGA/mg MP. This value represented a lectin coupling efficiency of ~50% of the initial ligand concentration.

3.3.2. *In vitro drug release studies*

As was previously mentioned, the formulation and the technological approach in the preparation of 5-FU loaded cross-linked hydrogel MPs have to provide a control in the drug release for a relatively long period of time and to delay the drug release in the stomach. These expectations were based on the physicochemical properties of the polysaccharide polymers, the pH sensitive solubility of coated drug particles and the mucoadhesivity of the carrier system.

Obtained results from the *in vitro* dissolution studies were in agreement with these expectations. The release profiles of 5-FU from MPs *in vitro* and the effect of conjugation of WGA on the dissolution behavior of MPs in buffer solutions at pH 2.0, 4.5, 6.8 and 7.4 are presented in Figs. 4–7. Embedment of acid-resistant particles of 5-FU into CTS–Ca–ALG MPs and further WGA conjugation efficiently retained the drug substance inside the particles, maintaining between 82 and 90% of the initial drug loaded after 2 h of incubation at pH 2.0. At increasing pH, phosphate buffer at pH 4.5,

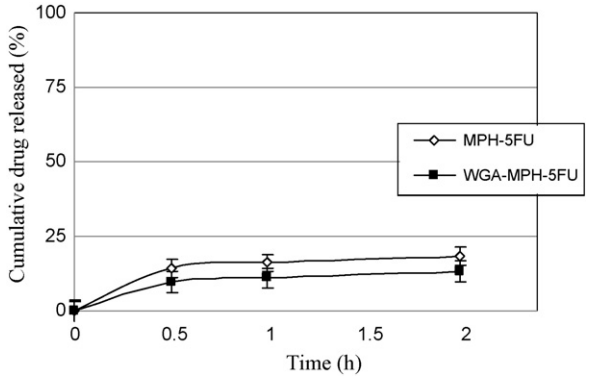

Fig. 4. *In vitro* release profiles of 5-FU from CTS–Ca–ALG MPs and WGA-conjugated CTS–Ca–ALG MPs in buffer solution at pH 2.0 (mean ± SD, $n = 3$).

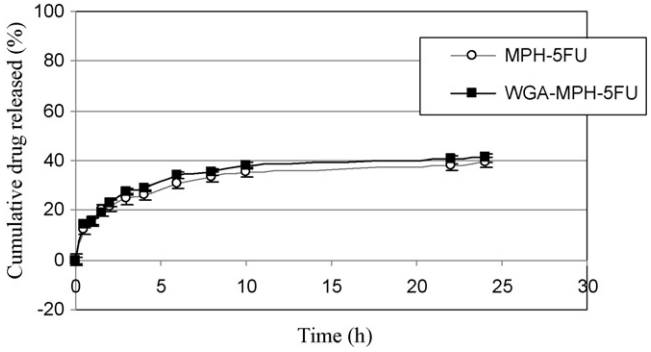

Fig. 5. *In vitro* release profiles of 5-FU from CTS–Ca–ALG MPs and WGA-conjugated CTS–Ca–ALG MPs in buffer solution at pH 4.5 (mean ± SD, $n = 3$).

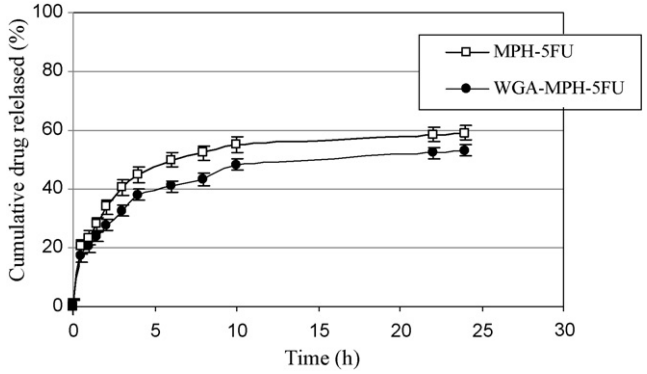

Fig. 6. *In vitro* release profiles of 5-FU from CTS–Ca–ALG MPs and WGA-conjugated CTS–Ca–ALG MPs in buffer solution at pH 6.8 (mean ± SD, $n = 3$).

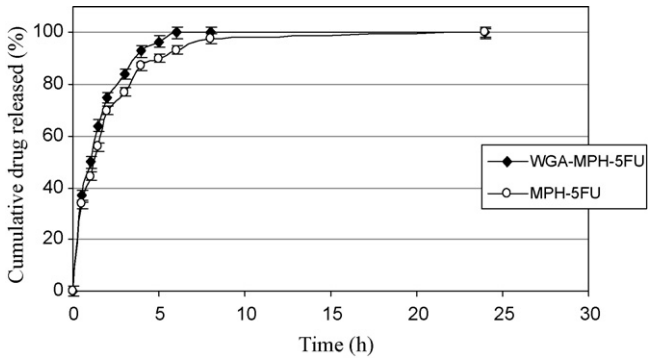

Fig. 7. *In vitro* release profiles of 5-FU from CTS–Ca–ALG MPs and WGA-conjugated CTS–Ca–ALG MPs in buffer solution at pH 7.4 (mean ± SD, $n = 3$).

the increased deprotonation of CTS did not result in faster drug release. In phosphate buffer at pH 6.8, the release of 5-FU was increased governed by dissolution and diffusion of 5-FU through the hydrated paths in the matrix formed by polymers swelling, but still controlled by cross-linked matrix density. A comparatively fast release was observed at pH 7.4 due to the excessive swelling (rapid hydration) of the particles and pH-dependent solubility of the polymers (Motwani et al., 2008). It is important to notice that the release of water-soluble drug, entrapped in a hydrogel matrix, occurs only after water penetrates the network to swell the polymers and dissolve the drug, followed by diffusion along the aqueous pathways to the surface of the device. Therefore, the release of drug is closely related to the swelling characteristics of the hydrogels, which in turn, is a, key function of chemical architecture of the cross-linked hydrogels (Singh and Chauhan, 2009).

Taking into account the poor solubility of CTS at pH 7.4 and low binding with ALG under these conditions (leading to max. swelling of the particles), it was expected that in phosphate buffer with pH 7.4, the 5-FU release would occur through the following steps: (i) water uptake and swelling of the MPs; (ii) dissolution of HP-5FU beads and (iii) diffusion of 5-FU molecules through the swelled and eroded gel matrix. Our results agree well with this release mechanism. Initial swelling and buffer gaining inside the beads leads to easier matrix rehydration and dissolution of the HP-55, afterwards the drug delivery during the time of the dissolution study was controlled from CTS–Ca–ALG matrix.

The comparable 5-FU release profiles from *MPH-5FU* and *WGA–MPH-5FU* MPs suggest that drug release from the MPs was not significantly affected ($p > .05$) by the presence of surface-conjugated WGA. Regarding the drug release in acidic media where swelling was negligible, some differences in the drug release patterns could be related to the effect of acetone as an activation medium for further WGA conjugation, which led to dehydration (contraction of pores and channels) of the beads. At pH 4.5, 6.8 and 7.4, the higher burst of lectin-conjugated MPs as compared to uncoated MPs may be due to the hydrophilic characteristics of WGA, allowing easier penetration of aqueous solution into the matrix thereby dissolving the HP-55 polymer and encapsulated drug substance (Gupta et al., 2006; Yin et al., 2006).

In order to understand the drug release mechanisms, the release data were analyzed using Higuchi's square root of time equation, Korsmeyer–Peppas power law equation and Peppas and Sahlin equation. The main parameter values are listed in Table 3. The goodness of fit was evaluated using the r (correlation coefficient) values.

The drug release data show the best fit to Peppas and Sahlin equation. The prevalence of K_2 over K_1 revealed that the drug release mechanism was controlled mainly by the erosion of the matrix (Toti and Aminabhavi, 2004). It is important to notice the negative values for K_1, calculated from drug release data, suggesting that diffusion process was insignificant compared to the relaxation mechanism (Ferrero et al., 2000). Anyway, a considerable amount of drug might have diffused because of the higher water solubility of 5-FU from the hydrated layer of the matrix (Toti and Aminabhavi, 2004).

This is in accordance with the obtained n values from Korsmeyer–Peppas power law equation, indicating that the release mechanism was somewhat complex and might involve both, the erosion and swelling controlled diffusion (Agnihotri et al., 2004; Kashappa, 2005). This is possibly due to a reduction in the regions of low microviscosity and closure of microcavities in the swollen state (Sairam et al., 2006). The poorer fit to Higuchi model was in an agreement with this observation.

3.3.3. *Assessment of the in vitro activity and specificity of lectin conjugates (haemagglutination tests)*

The degree of WGA activity after conjugation depends on the spatial conformation of the molecule and its ability to interact freely with the substrate molecules (Yin et al., 2006). In this study, the activity and specificity of WGA-conjugated MPs was determined by means of an *in vitro* agglutination assay. It is well known that lectins show the ability of erythrocyte agglutination and this phenomenon can be inhibited by adding a specific sugar for the lectin chosen (Arangoa et al., 2000).

The minimal titre of dilution (*Td*) of WGA, conjugated to the surface of MPs, giving the last visible red cell agglutination was determined. For WGA conjugates, *Td* was calculated to be less than 1 mg MPs/ml (corresponding to ~6.8 μg/ml WGA) and 3.2 μg/ml for the free lectin solution. CTS–Ca–ALG MPs (non-conjugated MPs) exhibited negative results at a concentration ≤3 mg MP/ml. These results clearly show that WGA, treated by the covalent coupling procedure still retained the specific carbohydrate binding activity on the surface of the MPs.

With the objective of determining whether or not the interaction of WGA-conjugated MPs with erythrocytes was true lectin binding or simply non-specific adhesion, WGA–MPs were preincubated with different concentrations of specific inhibitory sugar, *N*-acetyl-D-glucosamine, prior to incubation with erythrocytes. In this context, *N*-acetyl-D-glucosamine was tested for its ability to inhibit agglutination of 2% suspension of fresh human blood group O erythrocytes triggered by WGA-conjugated MPs. For 1 mg WGA-conjugated MPs, at least 17 μg of *N*-acetyl-D-glucosamine was necessary to avoid visible agglutination. Similar results were obtained for free lectin. All of these results clearly suggest that WGA-conjugated MPs maintain the same sugar specificity as the WGA and that the selected coupling method could be considered

Table 3
Comparison of different dissolution kinetics models in phosphate buffer at pH 7.4.

Sample	Higuchi[a]		Higuchi		Korsmeyer–Peppas			Peppas–Sahlin			
	K (%h$^{-1/2}$)	r	K (%h$^{-1/2}$)	r	K	n	r	K_1	K_2	K_1/K_2	r
MPH3	28.921	0.969	15.761	0.810	1.696	0.308	0.946	−0.511	3.805	−0.134	0.997
WGA–MPH-5FU	29.752	0.975	16.528	0.799	1.544	0.341	0.982	−1.281	6.145	−0.208	0.998

[a] After the second hour.

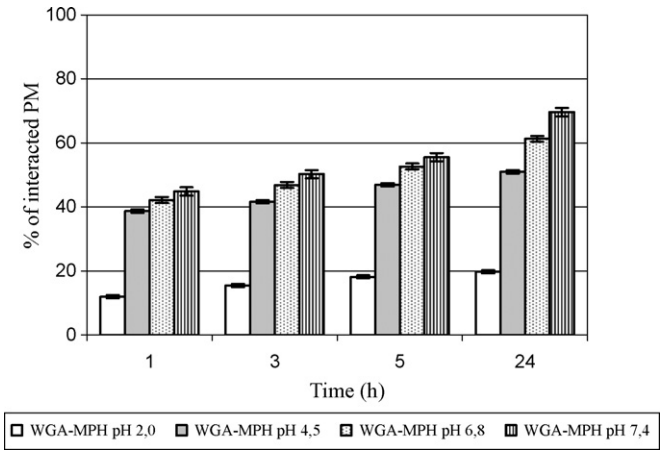

Fig. 8. Binding of PM to WGA-conjugated CTS–Ca–ALG MPs in different buffer solutions (mean ± SD, $n = 3$).

to be suitable for the conjugation of bioactive proteins such as WGA to drug carriers such as CTS–Ca–ALG MPs.

3.3.4. In vitro adsorption studies with PM

It is well known that lectins can specifically recognize and bind to the receptors present on the cell membranes, an lectin-conjugate polymers are regarded as *"the second generation of bioahesives"*, since they are designed to bind directly to the epithelial cells instead to the mucus gel layer. However, they also have strong affinity to mucins in the gel layer (Peppas and Huan, 2004) as a result of specific interaction between complementary structures. Therefore, MPs decorated with lectins of certain carbohydrate specificity can interact with mucin molecules, leading to the enhanced mucoadhesive properties of the carrier system (Gabor et al., 2004).

As known from the literature, native glycoproteins from intestinal mucus contain 77.5% carbohydrate deriving from *N*-acetyl-galactosamine, *N*-acetyl-glucosamine, galactose, fucose and sialic acid at a molar ratio of 1.0:0.6:0.7:0.3:0.5 as referred to the dry weight. Due to the specific interaction of WGA with monomers and oligomers of *N*-acetyl-glucosamine and sialic acid, commercially available PM (pig gastric mucin, Type II), with the comparable carbohydrate composition of human one and the comparable content of native glycoproteins to intestinal mucus (76% carbohydrates at a molar ratio of 1.0:2.8:2.9:1.9:0.2) (Wirth et al., 2002; Güll et al., 2007), was used as a biological model to determine the *in vitro* activity of WGA-conjugated CTS–Ca–ALG MPs (WGA–*MPH)* towards the sugar residues of a glycoprotein.

The *in vitro* mucoadhesive potential of MPs was determined by mixing the PM suspension in different buffer solutions at pH 2.0, 4.5, 6.8 and 7.4, respectively, with the same volume of MP suspension. Fig. 8 shows the amounts of PM (%) interacted with MPs. In acidic environment, low PM binding was observed in a tested period of 24 h. In phosphate buffers at pH 4.5, especially at pH 6.8 and 7.4, excessive adsorption of PM on MPs surface was observed, indicating that MPs–mucin interaction was pH dependent and specific (lectin–sugar recognition) (Yin et al., 2006). Also, these observations were in accordance with the positive surface charge of the particles and their excessive swelling (sufficient chain flexibility) in buffer solution at pH 7.4, leading to enhanced physical entanglement and interpenetration of the polymer chains and those of the mucin and possibility of formation of secondary chemical bonds, hydrogen bonding and van der Waals' forces, thus contributing to the mucoadhesion (Sinha et al., 2004). It is important to notice that this phenomenon has been reported to be advantageous, given that the mucus layer provides an initial yet fully reversible binding site followed by distribution of lectin-mediated drug delivery systems to the cell surface layer (Andrews et al., 2009).

4. Conclusion

Optimized chitosan–Ca–alginate beads loaded with acid-resistant particles of 5-FU were successfully prepared using a novel one-step spray-drying procedure. Prepared particles were further decorated with WGA in an order to design muco/bioadhesive carrier system for efficient local colon delivery of 5-FU. Results from physicochemical and biopharmaceutical characterization of the particles are in favor of their localization and prolonged residence time in colon as well as controlled release of encapsulated drug, thus providing a relatively high local drug concentration at the site of action and enhanced tissue accumulation of 5-FU.

From the data presented, it is expected that these novel ligand-coupled cross-linked hydrogel microparticles would be potential carriers for the oral-targeted delivery of 5-FU to colon region and effective treatment of colon cancer.

The *in vitro* transport and efficacy studies of the lectinized microparticles on Caco-2 cell monolayers and *in vivo* biodistribution of microparticles after peroral administration on Wistar male rats will be reported and discussed in a later paper.

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