ORIGINAL ARTICLE

"Drug Release Kinetic Modeling and Gamma Scintigraphic Studies of Dual Ca^{2+} and SO_4^{2-} Cross-linked Microbeads for Colon Specific Targeting"

Sandeep Kumar Sonkar, S. K. Lanjhiyana

Pharmaceutics Research Division, Institute of Pharmaceutical Sciences, Guru Ghasidas Vishwavidyalaya (Central University), Bilaspur, Chhattisgarh, India

Abstract

Objectives: Ionic gelation-based polyelectrolyte complexes of blend chitosan-sodium alginate polysaccharides based microbeads of methotrexate were prepared by dual cross-linkages with divalent Ca^{2+} and SO_4^{-2} ions for colon targeting. **Materials and Methods:** Those developed dual cross-linked formulations were characterized for particle size, entrapment studies, drug content, swelling degree, etc. **Results:** The surface morphology results showed that the optimized formulations were semi-spherical and rough-surfaced. The *in vitro* drug release carried out in various simulated fluids at various pH had shown lesser release profiles in acidic media as compared to alkaline media at the end of 24 h studies. A significant drug release ($P > 0.05$) was observed in colonic fluids containing 2 and 4% w/v rat cecal as compared with control. *In vivo* targeting ability for the colon-specific region was established through gamma scintigraphic imaging technique. Differential scanning calorimetry and X-ray diffraction analysis further confirms for semi-crystalline and complete cross-linking state. The release profile and mathematical kinetic modeling revealed for anomalous non-Fickian type formulations were best fitted with Higuchi and Hixson-Crowell model, respectively. **Conclusion:** It can be concluded that the optimized formulations may be effective for colon targeting and promising to achieve drug targeting for colorectal cancer.

Key words: Dual cross-linked beads, gamma scintigraphy, *in-vitro* studies, kinetic modeling, matrix polysaccharides, methotrexate, rat caecal content

INTRODUCTION

Development of polyelectrolyte and ionic gelation-based multiparticulate systems (MPs) had gained much fame over the single unit systems for oral drug delivery as per reports of the last few decades. Polyelectrolyte complexes (PEC) or "egg-box-junction" is a gel formation process which takes place when oppositely charged cationic amino groups of chitosan and anionic carboxylic acid groups of sodium alginate polysaccharides interact electrostatically in an aqueous media resulting into three-dimensional structures.[1] Beads or microparticles of these complexes were widely used for easy manipulation of mechanical properties as well as drug-releasing properties.[2]

There has been considerable research for designing of colonic drug delivery system by numerous researchers. Colon targeting has been achieved by several approaches, including prodrugs, microbial dependent, pH, and time-dependent systems. Among several polymers, those pH-sensitive sodium alginate and chitosan were also have been widely used by various researchers and proved to be promising matrix carriers in the pharmaceutical dosage forms. Chitosan is a linear cationic polyelectrolyte consisting of β-1,4-linked glucosamine (deacetylated units) and N-acetyl-D-glucosamine (acetylated units) residues. Sodium alginate contains (1,4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) monomers

Address for correspondence:
S. K. Lanjhiyana, Institute of Pharmaceutical Sciences, Guru Ghasidas Vishwavidyalaya (Central University), Bilaspur - 495 009, Chhattisgarh, India.
Tel.: 07752 260027, Fax: 07752 260148,
Cell: +91 9826252991. E-mail: sklanjh1975@gmail.com

Received: 10-09-2020
Revised: 28-102020
Accepted: 04-10-2020

having a significant affinity toward divalent or polyvalent cations.[3-5]

The alginate-chitosan-based polymeric beads prepared by either single cross-linking or dual cross-linking beads had been widely investigated by those researchers, but their potential stability, kinetic modeling, or *in-vivo* performances have not been adequately researched.[6] The objective of the present investigation was to design and evaluate stable methotrexate-loaded dual cross-linked beads using natural polymers for site-specific delivery to the colon for possible management of colon cancer. This may be achieved by targeting drug directly into the vicinity of the desired colonic region while bypassing the assaults of the upper gastrointestinal (GI) tract. The influences of numerous process and formulation variables were also examined on physicochemical properties, modified mechanical potential, *in-vitro* sustained release characteristics, kinetic model fittings, and *in-vivo* targeting abilities.

MATERIALS AND METHODS

Methotrexate was obtained from Unimed Technologies Ltd., Gujarat, as a gift sample. Chitosan (MW ~ 3.0×10^5) was received from Central Institute of Fisheries Technology (Cochin, India). Sodium alginate (viscosity of 2% solution at 25°C ~ 250 cps), calcium chloride, and sodium sulfate were purchased from Hi-Media Laboratories (Mumbai, India). All of the other chemicals and reagents were of analytical grade and were used without additional purification.

Preparation of chitosan-alginate blended MPs by dual ionic gelation method

Methotrexate-loaded chitosan-alginate microbeads were prepared using the ionotropic gelation technique (PEC). The blend mixture solution (100 ml) of polyelectrolytes containing 2% (w/v) chitosan-sodium alginate was prepared with mass proportions of 6:6, 4:8, and 2:10, respectively. An accurately weighed amount of alginate powder was dissolved in deionized water to prepare a sodium alginate solution at ambient temperature. Model drug (5% w/w of dry polymer weight) was suspended completely into the alginate dispersion using a homogenizer for 25 min. Separately, the chitosan solution was prepared while dissolving chitosan powder in deionized water containing acetic acid (1.0%, w/v) at room temperature. Then, the chitosan solution was added into a sodium alginate solution with different mass proportions and homogenized again for another 30 min at 45°C to get a uniform blend mixture. The blend solution containing drugs were ultrasonicated for another 10 min for complete debubbling and were adjusted to pH 5 using NaOH (0.1 mol/l). Thereafter, the bubble-free dispersion was extruded through 0.45 mm inner diameter needle using hypodermic glass syringe at a dropping rate of 1 ml/min and falling distance of 6 cm into Ca^{2+} ions containing calcium chloride (2% w/v) solution. This resulted into the formation of smooth and spherical calcium single cross-linked microbeads but mechanically weak. The obtained composite gel beads were cured into the same calcium chloride solution for 60 min for hardening and then subsequently decanted and washed thrice using deionized water. Immediately after that, those beads were double cross-linked by exposing into different concentrations of SO_4^{-2} ions containing sodium sulfate solution (2, 4, and 6% w/v) to form sulfate cross-linkages which were again cured for another 60 min period. The resultant calcium-sulfate double cross-linked microbeads with improved mechanical properties were decorated and washed thrice with deionized water. The beads were then finally dried at room temperature for 48 h, followed then oven drying at 45°C for 12–15 h. The obtained stable dried products were stored at room temperature into a suitable container till further characterizations. The optimization, core compositions, and independent variables that were used for preparation were shown in Table 1. All batches were prepared in triplicate.

Characterization of prepared microbead systems

Surface morphology by scanning electron microscopy (SEM)

The surface morphological characterizations for those selected microbeads were studied with the help of a scanning

Table 1: Different independent variables used in the preparation of methotrexate-loaded microbead system

Formulations	Drug (% w/w of total polymer weight)	Polymer ratio (Chitosan: sodium alginate)	Concentration of calcium chloride: Sodium sulfate (% w/v) solution	Curing time (min)
M_XCA_{B1}	5	6:6	2:2	60
M_XCA_{B2}	5	6:6	2:4	60
M_XCA_{B3}	5	6:6	2:6	60
M_XCA_{B4}	5	4:8	2:2	60
M_XCA_{B5}	5	4:8	2:4	60
M_XCA_{B6}	5	4:8	2:6	60
M_XCA_{B7}	5	2:10	2:2	60
M_XCA_{B8}	5	2:10	2:4	60
M_XCA_{B9}	5	2:10	2:6	60

electron microscope (JSM-5800, JEOL, Japan) facility. That specimen was kept in an ion sputter with a thin layer of gold (~300Å) for 150 s and at 20 kV to make them electrically conductive and then examined with accuracy and precision.[7]

Particle size determination

After the drying process, the mean particle diameter of selected 100 dried beads from batch formulations was calculated using an optical microscope (Olympus, Germany). The Feret's diameters were determined for those randomly selected beads from each batch by placing on the clean glass slide (sterilized). All readings were noted under 4 × magnifications with accuracy as average of three trials ± standard deviation (S.D) [Table 2].

Determination of drug content, percent yield, and drug entrapment efficiency

Accurately weighed 100 mg beads were taken and then placed in 100 ml of phosphate buffer saline (pH 7.4) for 24 h with occasionally shaking at 37 ± 0.5°C in a graduated flask. These samples were ultrasonicated and continued for 30 min until it gets completely dissolved. Thereafter, the polymeric dispersion was centrifuging at 3000 rpm for 30 min to remove the polymeric debris. Then, the solution was filtered through Whatman filter paper (# 41) and aliquot was used to assay for actual drug content spectrophotometrically (Shimadzu UV 1800) against an appropriate blank at 258 nm, respectively [Table 2]. The determinations were made in triplicate, and results were averaged properly. The entrapment efficiency and percent yields were measured using the following formula:

$$\text{DEE}(\%) = \frac{\text{Acutal content}}{\text{Theoretical content}} \times 100$$

$$\text{Percent yield} = \frac{\text{Total weight of beads}}{\text{Total weight of drug and polymer}} \times 100$$

Swelling degree studies

The equilibrium swelling degree of the prepared bead formulations was determined in three different mediums, namely, simulated gastric fluid (SGF) (pH 1.2), simulated intestinal fluid (SIF) (pH 6.8), and simulated colonic fluid (SCF) (pH 7.4). Accurately weighed dried beads (50 mg) of each batch were immersed in separate USP dissolution apparatus II (basket type) containing 500 ml of swelling solutions. The beads were allowed to swell in apparatus for up to constant equilibrium weight at a constant temperature of 37 ± 2°C and maintained under 50 rpm speed for 4 h period. Thereafter, those swollen beads from various mediums were removed, blotted with filter paper for moisture removal and instantly followed by weighing on an electronic balance.[8] All experiments were performed in triplicate and represented as a mean value [Table 2]. The percentage swelling ratio of each sample beads was determined with respect to time using the below equation:

$$\text{S.D. } (\%) = \frac{(W_e - W_i)}{W_i}$$

Whereas, W_e is equilibrium weight of swollen beads in the medium at a given time, and W_o is absolutely initial weight of dried beads.

Differential scanning calorimetry (DSC)

DSC thermograms of pure drug, physical mixture of drug polymers, and bead formulation were recorded using differential scanning calorimeter (DSC 4000, Perkin Elmer, Germany). About 2-2.5 mg of dried powdered samples were accurately weighed out and then hermetically sealed inside 40-µl aluminum pan. It was then heated at the scan speed of 10°C/min over the temperature range from 30°C to 350°C. The temperature inside the system was maintained with a continuous nitrogen gas supply at the flow rate of 100 ml/ min.

Table 2: Particle size, percentage yield, entrapment efficiency, and degree of swelling of different beads formulation as dependent variables

Batches	Particle size (µm) (mean±S.D.)	Percentage yield (mean±S.D.)	DEE (% w/w) (mean±S.D.)	Drug content (% w/w) (mean±S.D.)	Degree of swelling in pH 1.2 (4 h)	Degree of swelling in pH 6.8 (4 h)	Degree of swelling in pH 7.4 (4 h)
M_XCA_{B1}	991.14±0.35	75.46±0.18	66.73±0.39	82.05±0.62	0.12±0.005	0.14±0.006	0.15±0.010
M_XCA_{B2}	986.27±0.72	74.17±0.24	64.29±0.43	83.13±0.59	0.10±0.003	0.14±0.008	0.15±0.009
M_XCA_{B3}	988.63±0.58	76.43±0.19	69.37±0.58	82.41±0.39	0.10±0.004	0.18±0.005	0.17±0.011
M_XCA_{B4}	976.38±0.37	78.59±0.62	65.17±0.72	84.29±0.78	0.08±0.006	0.19±0.007	0.20±0.016
M_XCA_{B5}	975.73±0.68	80.39±0.73	70.59±0.45	85.48±0.45	0.09±0.005	0.22±0.009	0.21±0.006
M_XCA_{B6}	968.25±0.48	82.73±0.29	74.63±0.53	87.57±0.68	0.07±0.008	0.24±0.009	0.25±0.005
M_XCA_{B7}	966.11±0.45	83.19±0.46	74.14±0.64	88.21±0.54	0.07±0.002	0.25±0.002	0.25±0.008
M_XCA_{B8}	964.43±0.17	84.27±0.73	75.48±0.49	89.92±0.36	0.06±0.001	0.27±0.004	0.26±0.008
M_XCA_{B9}	963.53±0.19	85.27±0.25	75.48±0.34	87.92±0.12	0.06±0.002	0.25±0.009	0.28±0.010

Data are the average of values of mean±S.D (*n*=3); whereas, S.D: Standard deviation

X-ray diffraction (XRD)

XRD pattern of pure methotrexate drug, excipients, physical mixture of drug and excipients, and drug-loaded beads were traced using wide-angle X-ray diffractometer (Ultima-III, Rigaku SmartLab, Japan) using a Ni-filtered Cu-K alpha radiation source (1.54056 Å). The system was set up with tube voltage of 30 kV, current of 30 mA, scan speed 5°/min, over the diffraction angle (2θ) range of 20°–80°, and the count range of 3000 cps, respectively.[9]

In vitro drug release studies

The *in vitro* release studies were performed for all those prepared formulations using USP type-II dissolution apparatus (Electrolab TDT-06P, Mumbai, India). Accurately weighed quantities of sample (100) mg from each batch formulations were added into 900 ml of acidic SGF (pH 1.2) that was maintained at a temperature of 37 ± 0.5°C and stirred at the speed of 50 rpm for 2 h intervals. The test was then continued in SIF (pH 6.8) for next 3 h and in SCF (pH 7.4) for up to 12–24 h at similar conditions. The collected 5-ml aliquots each time were filtered, diluted suitably, and analyzed at measured for absorbance at λ_{max} 258 nm against appropriate blank using a UV-vis spectrophotometer (U-1800, Shimadzu, Japan). All experiments were carried out in triplicate; mean values and S.D were evaluated.[10]

Kinetic modeling and mechanism of drug release

The data obtained from *in vitro* release studies was fitted to different mathematical models for the purpose to explore the possible drug transport mechanism through matrix-based system [Table 3]. The various well known classical power-law expressions used for model fittings based on, namely, zero order, first order, Higuchi's model, and Hixson-Crowell models indicative of drug release mechanism. The accuracy and prediction ability of these models were compared with the calculation of squared correlation coefficient (R^2) values.[11] Microsoft Excel software was utilized to perform curve plotting and simulation studies.

Table 3: Results of curve fitting of the *in vitro* methotrexate release data from chitosan-sodium alginate-based bead formulations containing methotrexate

Formulation code	Zero order	First order	Higuchi	Hixson-Crowell
M_XCA_{B1}	0.967	0.884	0.979	0.956
M_XCA_{B2}	0.976	0.911	0.989	0.973
M_XCA_{B3}	0.975	0.922	0.990	0.980
M_XCA_{B4}	0.983	0.937	0.992	0.991
M_XCA_{B5}	0.982	0.935	0.993	0.989
M_XCA_{B6}	0.987	0.936	0.991	0.989
M_XCA_{B7}	0.980	0.939	0.985	0.991
M_XCA_{B8}	0.980	0.934	0.987	0.986
M_XCA_{B9}	0.982	0.931	0.990	0.988

Gamma scintigraphic imaging of beads system

The steps for preparation for the uptake of sodium pertechnetate (99mTc) were carried out as per our previous studies.[12] The *in-vivo* gamma scintigraphic study was carried out which was approved by the Institutional Animal Ethics Committee. During the study, six healthy male rabbits weighing 1.5–2 kg were used to monitor the *in-vivo* transit behavior of optimized formulation. Animals were divided into two groups of 3 animals each (one control group). The animals were fasted for 12 h before the commencement of each experiment for the reason to standardize the condition of GI motility. Thereby, capsules containing optimized formulation were orally administered to the animals of the first group with the help of a feeding tube followed by a sufficient volume of drinking water. All the four legs of the rabbit were tied over a piece of plywood and the location of the formulation in the GI tract was monitored at every 1 h by keeping the subject in front of the gamma camera. The gamma camera was arranged with a field view of 40 cm and was fitted with a medium energy parallel-hole collimator. The 140 keV gamma rays emitted by 99m Tc were imaged. Specific GI tract site (anterior) were imaged by E-cam Single Head Gamma Camera (Siemens, Germany) after a definite time interval. The gamma images were recorded using an online computer system and stored on a magnetic disk and analyzed to determine the distribution of activity in the GI tract.

Statistical analysis

Experimental data have been represented as mean ± S.D. Mean values of particle size, percent yield, and entrapment efficiency were compared using the Student's *t*-test. Differences are considered statistically significant at the level of $P < 0.05$.

RESULTS AND DISCUSSION

Preparation of methotrexate-loaded chitosan-alginate microbeads and optimization

In this existing study, methotrexate-loaded chitosan-alginate microbeads were prepared by polyelectrolyte complex based ionotropic-gelation technique [Table 1]. When blend polyelectrolytes mixture containing drug-polymer dispersion (5%, w/w of polymer concentration and polymer-to-polymer ratio of 6:6, 4:8, and 2:10) was added drop-wise at fixed flow rate into the cross-linking $CaCl_2$ solutions, then immediately spherical cross-linked networks of gel beads ("egg-box" model) were formed. The formation of spherical surfaced systems was due to the reason of electrostatic ionic cross-linking and hydrogen bonding that had taken place between negatively charged anionic carboxylic acid groups ($-COO^-$) located

on the alginate backbone and positively charged divalent Ca^{2+} ions of the cross-linking solutions. During the further dual cross-linking process, again electrostatic ionic cross-linking taken place between positively charged cationic amino groups ($-NH^{2+}$) on the C-2 position and negatively charged sulfate groups (SO_4^{2-}) of the cross-linking solutions. At the initial stage, those swollen beads were spherical and smooth-surfaced, but after the drying process, it became semi-spherical shaped and rough shaped with reduced sizes. Polymer-blending process of known polymers with desirable physicochemical properties was used to represents a rational approach for designing and obtaining modified pharmaceutical dosage forms. These results were in agreement with earlier reports of those researchers.[13] Such approach improved our carrier beads systems with various functional properties, namely, well-defined swelling at specific pH, excellent drug encapsulation or entrapment, mechanical strength, compact and smaller particle size, higher stability, controlled and sustained drug release rate, etc. Moreover, optimization for two independent variables for the ratio of polymer(s) and cross-linker(s) that were varied at three low, medium, and high levels resulted into overall 9 batch formulations. It is only the optimizations process that positively resulted into matrix systems with modified functional properties and improved mechanically strength suitable for distal colon targeting. Whereas, the concentration of drug and cross-linking times was taken at fixed level throughout the experimentation.

SEM

The SEM was carried out for morphological microanalysis of optimized double cross-linked beads (dried) systems as shown in Figure 1a-f. These figures evident that after drying, the surface of double cross-linked beads is found with compact-sized, semi-spherical, roughness, wrinkled, and minor cracks that might be due to reason of shrinkage and partly collapsing of polymeric gel network during the second steps of dual cross-linking with SO_4^{2-} ions as reported. These results are found in agreement with other findings of researchers Srinatha *et al.*[14]

Particle size

The mean size distributions of semi-spherical beads were investigated using a calibrated optical microscope [Table 2]. It was evident that those average diameters of particles decreased significantly ($P < 0.05$) with decreasing concentration of chitosan polymers, whereas on increments for the mass proportion of sodium alginate resulted into decreased particle sizes. This suggested that the cross-linked networks of sodium alginate were more compact in comparison to chitosan polymers and hence responsible for decreased sizes. Our results are found concord with other investigations of Bera *et al.*[15]

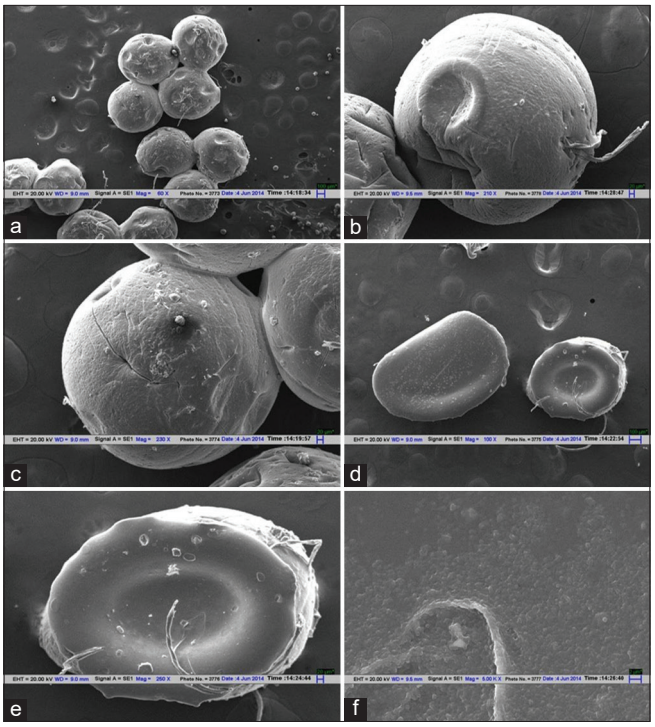

Figure 1: Scanning electron microphotographs of methotrexate-loaded 4:8 ratios containing chitosan-sodium alginate-based double cross-linked microbeads cured with 2 and 6% w/v of calcium chloride (a) surface view, magnified at 60kx, (b) surface view, magnified at ×210, (c) surface view, magnified at ×230, (d) cross-sectional view, less zoom at ×100, (e) cross-sectional view, high zoom at ×250, and (f) surface view, high zoom at 5.00kx

Percentage yield, entrapment efficiency, and drug content

Methotrexate containing chitosan-alginate batch formulations were evaluated for micromeritic properties, as provided in Table 2. It was increased significantly ($P < 0.05$) with the increase mass proportions of sodium alginate as compared to chitosan in the core compositions. This suggested that the ionic cross-linked networks of sodium alginate were more strong and compact in comparison to chitosan network and hence responsible for significant increments of yield and entrapment values.[16] The drug content was increased significantly ($P < 0.05$) from 82.05 ± 0.62 to 89.92 ± 0.36% with an increased concentration of alginate polymers and varying ratio of curing agents.

Degree of swelling

The degree of swelling was carried out for beads formulations using simulated GI fluids at various pH of 1.2 (SGF), 6.8 (SIF), and 7.4 (SCF), respectively, as demonstrated in Table 2. The swelling was seem to be almost negligible in SGF at pH 1.2 for most of the batches, except with slight raised values observed with those formulations consisted of increased mass proportions of chitosan polymer. This suggested for overall

acid resistant nature of beads into the acidic environment due to ionic cross-linking of those interpenetrating polymeric networks. Those batch formulations had shown a variable swelling degree in simulated fluids at pH 6.8 and 7.4. Thus, the result suggested that with the increments of percent sodium alginate followed with an increased swelling degree at alkaline medium (pH 6.8 or 7.4). In addition, at the same time, it gets slightly decreased with increased concentration of cross-linkers used during dual cross-linking steps. Whereas with increment of percent chitosan into the mass proportions resulted into slight increased swelling degree at acidic medium (pH 1.2) respectively.

Simultaneously other factors affecting swelling of those pH-sensitive gel bead systems were based on ionization and ion-exchange mechanism theories associated with those charged cationic chitosan and anionic sodium alginates as polyelectrolytes or poly ions. These theories were found equally effective for swelling degrees of those bead systems in addition to cross-linking network theory. These ionizations processes are associated with protonation, deprotonation and chelation that were responsible phenomenon for swelling degree which took place at specific acidic and/or alkaline media. The ion exchange exclusively depends on the magnitude of charges and behavior of those ion exchangers across the gel membrane barriers. Those ion exchangers are, namely, protons (H^+) and hydroxides (OH^-); divalent ions (Ca^{2+}); polyatomic ions (SO_4^{2-} and PO_4^{3-}); carboxylic and amino functional groups ($-COO^-$ and NH^{2+}), etc. As per Gibbs-Donnan effect or Donnan equilibrium, the swelling degree took place due to the concentration gradient of ionic charges (Donnan potential) across the gel phase boundaries or diffusion barriers for those bead systems.[17]

During experimental, the swelling degree of chitosan-alginate beads system at pH 1.2 was found negligible, although chitosan is soluble at acidic media. Chitosan is weak bases due to the presence of amino (NH^{2+}) groups that get protonated upon contact with acidic pH medium as provided with protons (H^+) ions, whereas alginates remain unionized at the same condition. On the other hand, alginates are weak acids due to the presence of anionic carboxylic groups ($-COO^-$) that gets ionized (deprotonated) upon contact with basic pH medium and became soluble, whereas chitosan remains unionized at the same condition. Those protonation and deprotonation processes were the responsible factors for the swelling degree of systems at a specific pH medium. During single and dual cross-linking steps, those anionic carboxylic groups were remained electrostatically cross-linked with cationic Ca^{2+} ions, whereas those cationic amino groups were remained cross-linked with anionic SO_4^{2-} ions. At the same time, few free ions remained unsatisfied or not bonded with any other ions into the same matrix system. The cross-linked poly ions were attributed for the controlled swelling degree of beads at respective acidic and/or basic pH medium. Moreover, these cross-linkages were also contributing to the maintenance of mechanical integrity, controlled, and sustained drug release properties of matrix systems into the respective environments.

Upon contact of beads with an acidic medium at pH 1.2, the protonation of few free amino groups taken place and that was found responsible for ion exchange mechanisms. These ion exchange with outer interstitial fluids across the barrier membrane resulted into the swelling degree of those beads to some lesser extent. Whereas, the rest of the most amino ions were previously bonded with SO_4^{2-} ions and contribute to maintain the mechanical properties of the matrix system. While, those beads upon contact with an alkaline medium at pH 6.8 or 7.4, the deprotonation of carboxylic groups and chelation of PO_4^{3-} (Ca^{2+} sequestrants) ions took place due to which the previous cross-linkages between Ca^{2+} and carboxylic groups gets reversed. The carboxylic groups donated H^+ (proton) to basic proton deficient medium and hence became deprotonated. These isolated carboxylic groups turned into free ions that participated for ion exchanges and then initiated for disruptions of gel matrix networks. Whereas, new cross-linkages might took place between Ca^{2+} and PO_4^{2} ions as due to the reasons for the higher affinity of PO_4^{2} ions toward Ca^{2+} ions than that of carboxylic ions found in agreement with Al-Kassas *et al.*[18] Those disrupted alginates ions were responsible for the swelling degree of beads at alkaline medium and hence act as a triggered mechanism for drug release from matrix systems. However, it was only due to unionized amino ions that were with previously bindings with SO_4^{2-} ions responsible for the maintenance of mechanical integrity and avoids fast disruption into the same system. Hence, these factors leaded for sustained drug release properties for those beads formulation at alkaline medium. These observations are in concordant with the findings of other researchers.[19] Those cross-linkages between various ions and functional groups into the system were confirmed with the help of further DSC and XRD studies.

DSC study

In DSC thermogram, the pure methotrexate exhibited a sharp melting endothermic peak (Tm) at 183.22°C [Figure 2]. Whereas, the thermal curves of chitosan and sodium alginate powder were found with broader endothermic peaks (Tg) at 81.31 and 152.63°C and corresponding exothermic peaks (Tc) at 227.8 and 258.72°C, respectively. A small non-pronounced endothermic peak (Tm) at 64.54°C was found with a thermal curve of formulation, which attributed to the peaks for evaporation of adsorbed water and/or volatile compounds. The curves of formulation had been shifted to the lower melting point, which interpreted for the conversion of state drug into amorphous form when complexed with interpenetrating polymeric networks of alginate and chitosan polymers. This behavior may be possible depending on the strength of electrostatic cross-linking reactions likely to take place between those divalent cross-linkers Ca^{2+} and

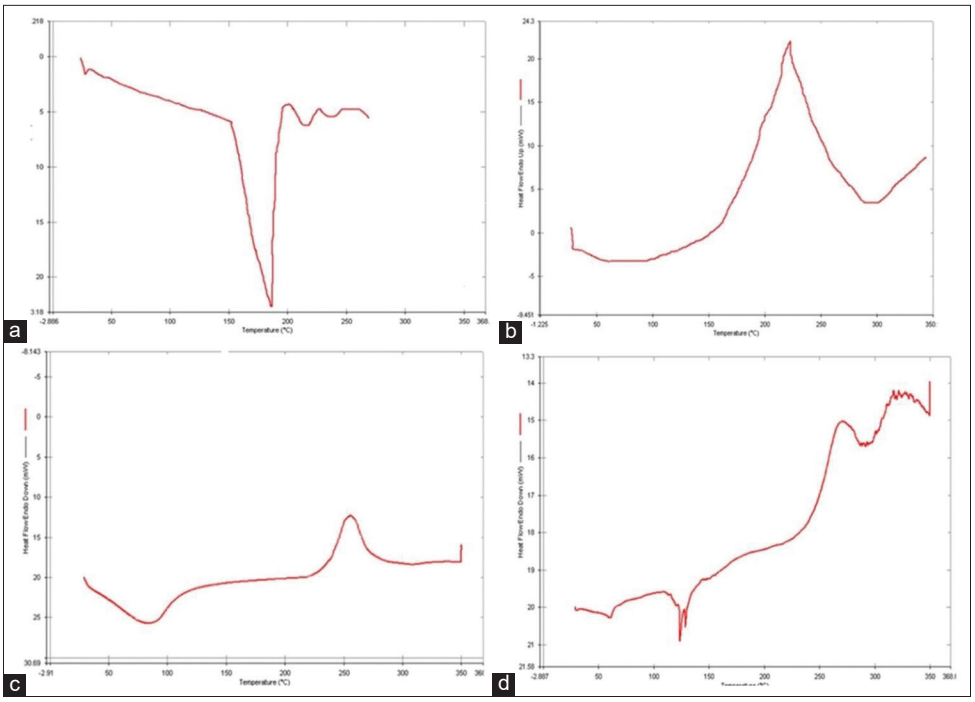

Figure 2: Differential scanning calorimetry thermogram of (a) pure methotrexate, (b) chitosan polymer, (c) sodium alginate polymer, and (d) chitosan-sodium alginate-based methotrexate-loaded dual cross-linked optimized microbead formulation

carboxylic groups of alginate/chitosan. These findings are found in agreement with earlier reports of Aquino *et al.*[20]

XRD study

P-XRD patterns of pure drug, polymer(s), and drug-loaded microbeads were analyzed to further establish for any solid-state transformation of crystalline drugs in the complex matrix systems, as depicted in Figure 3. Methotrexate had shown sharp characteristic peaks that pointed toward its pure crystalline nature. However, those signal intensities for chitosan and sodium alginate were found very weak, as presented in Figure 4b and c. Similarly, the sharp diffraction pattern corresponding to the drug was again diminished in gel beads, as depicted in Figure 4d and e. Thus, the obtained crystallographic data suggested for drug dispersed at the molecular level, transformation of drug from its crystalline to amorphous state, and entrapment of drugs into polymeric matrices possibly due to ionic gelation processes. These results are in concord with the report of Bhattachary *et al.*[21]

In vitro drug release studies

Effect of polymer concentration

An *in vitro* drug release investigation was carried out for the purpose to confirm the potential of methotrexate-loaded batch formulations for resistance against the adverse environment of upper GI tracts, as shown in Figure 4. The loaded methotrexate released into the various simulated medium was found highly depended on mass polymer concentration and cross-linker (independent variables) used into the batch formulations. The drug release profile for those batch formulation in pH 6.8 followed by pH 7.4 showed lesser drug releases from a formulation containing 4:8 and 6:6 mass ratios as compared to formulation batches containing 2:10 mass ratios that were due to an increase in sodium alginate proportion in the polymer concentration.

Effect of concentration of cross-linking agents

Drug release profiles were also affected with used variable concentrations of the cross-linking agents and fixed curing time for 60 min during beads preparations. The drug released from batch formulations consisting of variable mass ratios of chitosan:alginate within a group was found decreased with an increase in cross-linking agent concentration in both the dissolution media (acidic and alkaline). Since those Ca^{2+} and SO_4^{2-} ions containing cross-linkers had provided intense cross-linkages and mechanical strengths to the matrix system, due to which the release profile of drug from batch formulations within a group decreases with an increase in cross-linking concentrations. This release behavior was probably for the reason that at higher concentrations of cross-linkers provided the polymeric mesh network with more rigidness due to which the micropores and channels get contracted and saturated. Simultaneously other factors equally affected drug releases into various simulated fluids depends on the swelling phenomenon of those pH-sensitive systems due to the presence of a charged core of cationic and anionic type chitosan and sodium alginate polysaccharides. These phenomena were based on protonation, deprotonation, chelation, ion-exchange mechanism, etc., with those charged

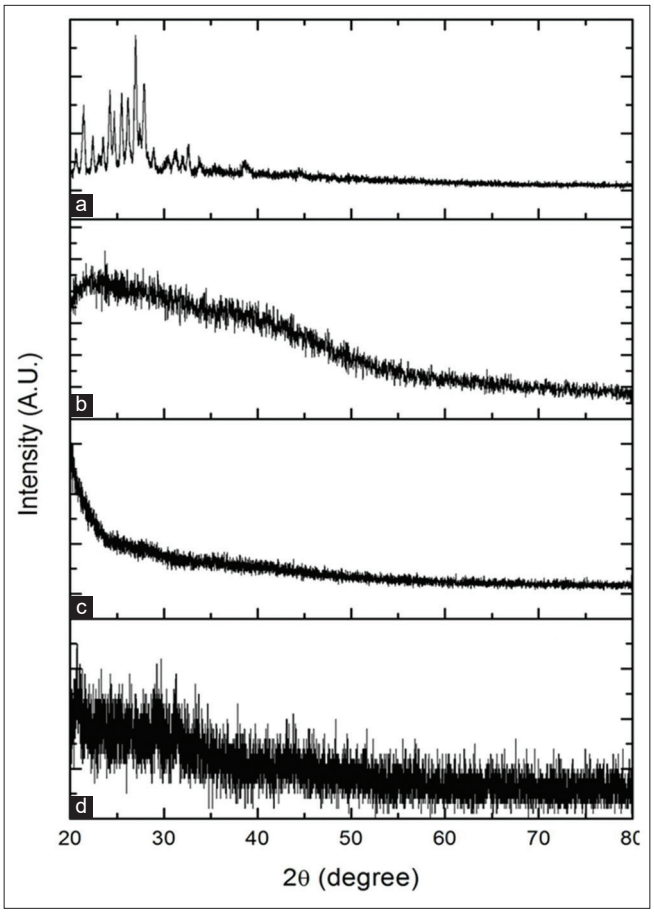

Figure 3: X-ray diffractogram of (a) drug, (b) sodium alginate polymers, (c) chitosan, and (d) chitosan-sodium alginate-based optimized methotrexate-loaded microbead formulation.

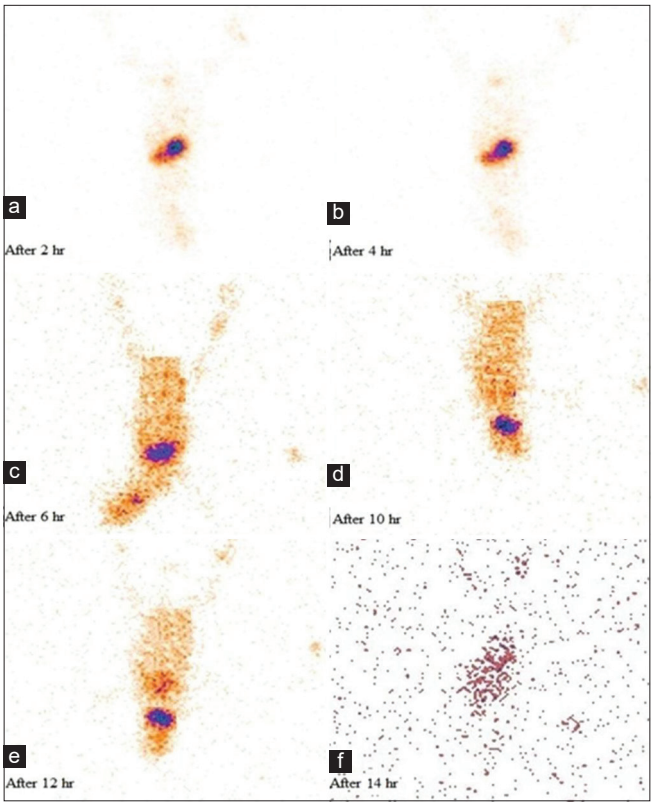

Figure 4: Gamma scintigraphy showing gastrointestinal transit and *in vivo* release of loaded radioactive tracer (^{99m}TC-DTPA) from optimized microbead formulation at different time intervals of 2, 4, 6, 10, 12, and 14 h (n = 3)

poly ions into their respective acidic or alkaline medium. During single and dual cross-linking steps, those anionic carboxylic groups were remained electrostatically cross-linked with cationic Ca^{2+} ions, whereas those cationic amino groups were remained cross-linked with anionic SO_4^{2-} ions. These acidic- and alkaline-based ionizations at the specific medium of alginates and chitosan containing matrix systems were responsible for swelling degree of beads and hence act as a triggered mechanism for drug release from gel bead systems. The optimized mass ratio of chitosan-sodium alginate used during the present study was found able to protect the drug release into those simulated physiological environment of stomach and small intestine, which was confirmed by release studies containing SGF (pH 1.2) for 2 h and further in SIF (pH 6.8) for 3 h, mimicking lag time of 5 h. The batches released fewer amount of methotrexate during 5 h studies, which might be due to the dissolution of surface drug particles being diffused out from systems. The dual cross-linkages with Ca^{2+} and SO_4^{2-} ions provided the beads system for gastro-resistant properties and hence satisfactorily prevented drug dissolution of matrix systems for not less than lag time of 5–6 h. The conventional marketed preparations which were used for colorectal targeting are normally getting dissolved and absorbed in the upper GI tract of stomach and/ or small intestine; thus, a very lesser quantity of dose of the drug reaches up to the colonic region.

Xu *et al*.[6] had earlier reported for dual cross-linked bovine serum albumin-loaded alginate/chitosan gel beads with fewer drug releases in SGF (pH 1.0) during 4 h followed with augmented released in SIF and SCF (pH 6.8 and 7.4) during each 3 h studies. Our results are in concord with such findings with fewer drug releases in SGF (2 h) followed with sustained released in SIF (3 h) and SCF (up to 24 h) containing medium. The differences in sustained drug release pattern obtained in our studies in SIF and SCF medium may be due to additional usage of higher mass proportions of 4 and 6% w/v sodium sulfate as cross-linkers (other than lower 2% w/v) during the second cross-linking steps for those batch formulations. Those batch formulations consisted of a higher mass proportion of chitosan (in comparison to sodium alginate) and cross-linked with higher proportions of SO_4^{3-} ions were resulted with enhanced sustained drug releases in SIF and SCF during 24 h studies. The possible reasons of sustaining properties may be that chitosan remains unionized at basic pH media and additionally dense cross-linked with SO_4^{3-} ions on the other hand. In the present study, *in vitro* studies were performed for 24 h period to check performances of the laboratory prepared beads for colon-specific targeting in the intact form. Hence, it may be predicted for the maximum quantity of dose may be delivered into the colonic

region, thereby maximum therapeutic efficacy and minimum drug losses will be meeting up on the other hand.

Mechanism of drug release

In vitro data were treated for best fit into various mathematical kinetic models such as zero-order, first-order, Higuchi, and Hixson-Crowell, respectively. The accuracy and prediction aptitude of these models were determined using regression coefficient (R^2) values and those curve fitting results were represented in Table 3. During curve fittings using several models, the R^2 value of Higuchi matrix model was observed to be the highest and closer to unity ($R^2 = 0.9907–0.9975$) for best-fit and the plot revealed for linearity. The drug release data fit linearly to Higuchi's square root kinetic equation for all those sample formulations. Therefore, it is concluded that drug release from these systems was followed with anomalous non-Fickian transport mechanisms referring to a combination of swelling controlled and diffusion controlled type. These results were found concordant with the reports of Maiti *et al.*[8]

Gamma scintigraphic imaging of MPs

The scintigraphies were performed for the purpose to analyze the colon targeting potential of those radio labeled formulations. After oral administration, the anterior images of abdomen were recorded at different time intervals of 2, 4, 6, 8, 10, 12, and 24 h, respectively, as depicted in Figure 4. The results of scintigrams demonstrated for average gastric emptying time of 2–3 h, small intestinal transit time of 3–5 h, and colonic arrival time of 6–7 h in all groups during the experimental. The release of tracers provided exact information regarding the transit time and location of formulation in GI tract of those experimental groups. Those groups who were orally administered with M_XCA_{B6} formulations were found intact with gamma tracer but showed a very small amount of released ^{99m}Tc-DTPA complex (tracers) in the stomach at 2.0 ± 0.5 h, which may be due to the reason for improper tagging of some gamma tracer within the formulation. Further, again lesser amount of tracers was released after 3 h when particulate reaches small intestine at 5.0 ± 0.5 h, whereas those scintigrams indicated that the systems were began to disintegrate in the colon after 6.0 ± 0.30 h, and then within 12–24 h the formulation got completely disintegrated in the colon. Few amounts of tracers were released into the small intestine may due to same reason for improper tagging of radioactive tracers. On entering the colon, it begins to release with more tracers that confirmed for degradation of core chitosan-sodium alginate of matrix systems that may be due to combined actions of pH-based ionization and microbial degradation properties. The colon arrival time was found to be 6–7 h and found with uniform distribution of the released tracer across the entire colon for 24 h intervals. These results are found similar to the studies of Sharma *et al.*[22]

CONCLUSION

The matrix chitosan-sodium alginate polysaccharides based microbead systems were successfully optimized and prepared using dual cross-linkages by Ca_2^+ and SO_4^{2-} ions showed its potential for oral dose administrations. The *in vivo* gamma scintigraphic studies revealed the targeting potential and sustained release profile of our prepared systems. The *in vivo* results were found to support the *in vitro* release profile data. Therefore, it can be concluded that those pH-sensitive polysaccharides based ionically dual cross-linked microbead system may be suitable carriers for targeting of methotrexate to colon-specific region.

FUNDING

This work was supported by the University Grant Commission of India through its Basic Science Research Fellowship under Grant (No.F-7-345) to complete this research work.

ACKNOWLEDGMENT

The authors are grateful to Scientists Dr. M. Mathur and Dr. N. Ganeshan of Jawaharlal Nehru Cancer Hospital and Research Centre, Bhopal, India, for providing Gamma scintigraphy facility during *in vivo* study. In addition, we are thankful to the Department of Nuclear Medicines, IIT, Delhi, India, for providing sophisticated analytical instrumentation facilities.

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Source of Support: Nil. **Conflicts of Interest:** None declared.