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Dynamics of controlled release of heparin from swellable crosslinked starch microspheres

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Abstract The microspheres of crosslinked starch have been prepared and characterized by IR spectral analysis and SEM technique. The prepared microspheres were loaded with an anticoagulant drug 'heparin' and the kinetics of in-vitro release of heparin was investigated spectrophotometrically at physiological pH (7.4) and body temperature (37 °C). The influence of percent loading of heparin, chemical architecture of the microspheres and pH of the release medium were examined on the release profiles of the drug. The chemical stability of heparin was tested in phosphate buffer saline (pH 7.4) and the release was also studied in various simulated biological fluids.

Introduction

For many years, the treatment of acute diseases or chronic illness in-patients has been achieved by the delivery of drugs. These drug delivery systems include tablets, injections, cream, ointments, liquids and aerosols. The term drug delivery can be defined as techniques that are used to get the therapeutic agents inside the human body [1]. Conventional drug therapy requires periodic dosing of therapeutic agents. These agents are formulated to produce maximum stability and bioavailability. For most drugs conventional methods of drug administration are effective, but some drugs are unstable or toxic and have narrow therapeutic ranges [2]. Some drugs also possess solubility problems [3]. In such cases, a method of continuous administration of therapeutic agents is desirable [4].

The structural design of polymer carriers intended for use in biochemical and biopharmaceutical delivery applications is based on several critical requirements including biocompatibility, biodegradability, bioresorbability, non-toxicity, stability on storage and appropriate size of the drug. A successful drug delivery system is one, which has a high percentage of drug uptake and a high loading capacity, thereby minimizing the quantity of carriers required for administration. Polymeric microspheres thus are ideal vehicles for many controlled delivery applications due to their ability to encapsulate a variety of drugs, biocompatibility, high bioavailability and sustained drug release characteristics. Moreover, the microspheres protect the medicine from the harsh environment of the stomach until it can be released and absorbed in the intestine. Drug release rates depend strongly on the size of the spheres or capsules containing the drug. Larger microspheres generally release encapsulated compounds more slowly and over longer time period's [5]. For this reason microspheres have been recognized as potential drug carriers for bioactive ingredients such as proteins [6], peptides [7], antigens [8], plasmid DNA [9], oligonucleotides [10], enzymes [11] etc. Their widespread use for oral delivery [12] also aims at improving the bioavailability of drugs with poor absorption characteristics, reducing gastrointestinal (GI) mucosa irritation caused by drugs and assuring stability of drugs in the GI tract [13]. Thus, all these and many more such characteristics of microparticles qualify them as a promising candidate in drug delivery technology.

Heparin is a polysaccharide consisting of a repeating pentasaccharide sequence (three D-glucosamines interspaced with two uronic acid residues) as shown in Fig. 1.

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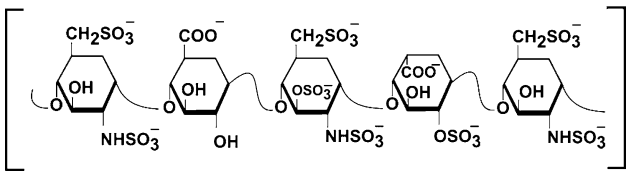

Fig. 1 Structure of the heparin molecule

The abundance of sulphate groups on glucosamine residues adds significant negative charge to the molecule, facilitating heparin's antithrombogenic activity. This activity results when heparin binds itself to antithrombin (AT), neutralizing thrombin and thus preventing cleavage of fibrinogen to fibrin.

The present study focuses on the oral delivery of heparin, since its bioavailability is reduced when the drug is administered by subcutaneous injection, in low or moderate doses. When ingested orally, heparin is degraded to inactive oligomer fragments whereas systemic administration is complicated by the need for continuous infusion and the potential for uncontrolled haemorrhage [14]. It is administered parenterally because heparin does not traverse the gastrointestinal mucosal barrier because of its size and overall negative charge [15]. However, intravenous delivery has substantial disadvantages and the medical implications of any orally delivered heparin solutions are attractive [16]. There has been extensive research on the release mechanism of heparin. There are four methods in which heparin is released from surfaces [17]: (1) negatively charged heparin is ionically bound either directly to a positively charged polymer substrate surface or a positively charged gel that is then coated on the polymer substrate [18], (2) Heparin is dispersed in the bulk polymer and diffuses through the surface into the blood stream [19], (3) Heparin is loaded into a thermosensitive hydrogel-grafted surface at low temperatures [20] and (4) A complex of positively charged polymer and heparin is subjected to an electric current to induce heparin release[21].

Starch, being an abundant and inexpensive polysaccharide, is used as a biodegradable polymer. Moreover, the use of starch microspheres has been suggested for embolization [22], parenteral administration [23] and nasal administration [24]. Cross-linking granular starch reinforces hydrogen bonds holding the granule together. This produces considerable changes in the gelatinization and swelling properties of starch granules. This toughening of the granules lead to restriction in the swelling of the granules. A number of multifunctional crosslinking reagents have been suggested of which epichlorohydrin finds wide use [25].

Although various biodegradable microspheres of natural polymers, such as gelatin, chitosan etc. are largely in use as drug carriers in controlled drug delivery; those derived from starch are of prime importance because of their biocompatible, non-toxic, non-carcinogenic and easily degradable nature. These microspheres have been extensively documented in the literature. For example Cortesi and co-workers [26] prepared biodegradable microspheres of starch crosslinked with epichlorohydrin and studied the release of an antiallergic drug Gabexate Mesylate (GM). Microspheres were characterized by SEM, swelling degree and water retention value. The association equilibrium constant for GM binding to microspheres was obtained by UV spectroscopy. The use of starch microspheres for subcutaneous injection and controlled release of protein drugs was reported by Larsson et al [27]. Protein was encapsulated into a starch matrix during emulsification in PEG (polyethylene glycol) solution. The molecular properties of the starch material, presence of additives like protein, PEG polymer, buffer type and pH are the factors which influence the final quality of the spheres. Hamdi and Ponchel [28] prepared starch microspheres, and studied enzymatic degradation by α-amylase. Analysis of the decrease in volume of microspheres by cubic root law, suggested that degradation profiles were dependent on the initial size distribution of the microspheres. No internal rupture of the microspheres were detected suggesting that enzymatic degradation of starch microspheres is surface controlled.

Experimental

Materials

Water-soluble starch was purchased from E. Merck, Mumbai (India) and used without any pretreatment. Epichlorohydrin was employed as a crosslinker for starch and was obtained from Thomas Baker Chemicals, Mumbai, India and used as received. Silicon oil, used as an oil phase, was obtained from Research Lab, Mumbai, India. Heparin sodium injection (I.P.) (1,000 IU/mL) was used as a model drug and purchased from Biological E. Limited, Hyderabad, India. Other chemicals and solvents used for the study were of standard quality (AR) grade and only double distilled water was used throughout the experiment for preparing solutions.

Preparation of microspheres

Various methods for the preparation of microparticles for pharmaceutical use are divided broadly into two categories, those based on physicochemical properties, such as phase separation [29] and solvent evaporation [30], and those based on chemical reactions, such as polymerization and polycondensation. In the present study, the solvent evaporation technique has been used for the preparation of microspheres.

The 'Aqueous phase' was prepared by dissolving a definite amount (1–4 g) of starch in boiling water to prepare a clear solution. Silicon oil was used as the 'oil phase'. The above two solutions were mixed with vigorous shaking (Shaking speed 300 RPM, 0.5 HP Motor Capacity) (Toshniwal, India) for 1 h to form a suspension. To this suspension, 1 mL of epichlorohydrin emulsion prepared in silicon oil (1:5 v/v) was added with constant shaking. The crosslinking reaction was allowed to take place for 4 h at room temperature. Microspheres in silicon oil were extracted using acetone (1:10 volume ratio of the suspension to acetone). Thereafter, the microspheres were resuspended thrice in toluene and twice in acetone and then isolated by filtering the acetone phase. The filtrate was washed with acetone to remove the silicon oil residue. The collected microspheres were vacuum dried and thereafter stored in airtight polyethylene bags. The reaction scheme for crosslinking of starch has been depicted in Fig 2.

Loading of heparin

Loading of a drug onto a polymeric device is done normally in two ways, (i) either by adding the drug into the feed mixture during the preparation of the device, or (ii) by allowing the device to swell in the drug solution until equilibrium is reached. In the present work the later method has been adopted as in the former method purification of the loaded device remains a problem.

A varying degree of heparin loaded microspheres were prepared by allowing 0.5 g of microspheres to swell until equilibrium was reached in freshly prepared drug solutions (10 mL).

The percent loading of drug was calculated by the following equation:

$$\%\text{Loading} = \frac{W_d - W_o}{W_o} \times 100$$

where W_d and W_o are the weights of loaded and unloaded microspheres, respectively.

In vitro release experiments

The release of the encapsulated heparin was carried out by placing the dried and loaded microspheres (0.05 g) in a test tube containing a definite volume (5 mL) of phosphate buffer saline (PBS) as the release medium (pH 7.4) (1.2 mM KH_2PO_4, 1.15 mM Na_2HPO_4, 2.7 mM KCl, 1.38 mM NaCl). The resulting suspension was gently shaken for definite time period. Thereafter, the sample was centrifuged for 30 min and the supernatant was removed and assayed for heparin spectrophotometrically by recording absorbance at 256 nm (Systronics, Double beam uv–vis spectrophotometer-2201, Ahmedabad, India). The amount of heparin released was determined from calibration plot.

Swelling measurements

Swelling of microspheres was studied by a conventional gravimetric procedure. In a typical experiment 0.1 g of microspheres were allowed to swell in a definite volume (10 mL) of PBS taken in a preweighed sintered glass crucible (pore sizes 5–10 μm) and weighed after a definite

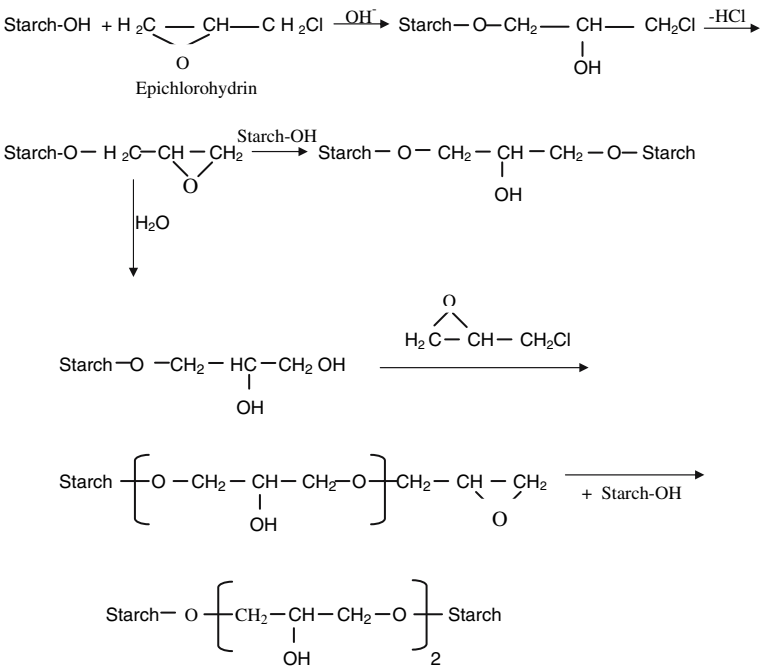

Fig. 2 Scheme of reactions showing crosslinking of starch by epichlorohydrin

period by removing excess PBS by vacuum filtration. The amount of water imbibed by the microspheres was calculated by the following equation:

$$\text{Swelling ratio} = \frac{\text{Weight of swollen microspheres}}{\text{Weight of dry microspheres}}$$

Characterization of microspheres

IR spectral analysis

The IR spectra of crosslinked starch microspheres were recorded on the FTIR spectrophotometer (Perkin-Elmer, 1000 Paragon).

Scanning electron micrograph (SEM) analysis

Morphological studies of crosslinked starch microspheres and heparin loaded starch microspheres were performed on a scanning electron micrograph (JSM-5600 LV, USA).

Particle size analysis

The particle size analysis of the prepared microspheres was performed on a particle size analyzer (Fritsch Particle Sizer, Analysette 22, USA).

Dissolution test

The prepared microspheres were subjected to dissolution test as detailed below:

100 mg of dry microspheres were shaken in 20 mL PBS for 2 h, dried at 40 °C for 72 h and weighed on a digital balance. The extent of dissolution was quantified by the following equation:

$$\% \text{ Dissolution} = \frac{m_d - m_{d'}}{m_d} \times 100$$

where m_d and $m_{d'}$ being the weights of dry microspheres before and after shaking in the PBS, respectively.

Chemical stability of drug

In order to check if the chemical activity of the entrapped heparin is not lost during release, the UV spectral study (Double Beam UV–Vis spectrophotometric, 2201, Ahmedabad, India) was performed as described elsewhere [31].

Statistical analysis

All experiments were done atleast thrice and a fair reproducibility was observed. The data summarized in tables have been expressed as mean ± SD of atleast three independent determinations. The plots were drawn taking the mean values and each curve has been shown to include error bars.

Results and discussion

IR spectral analysis

The IR spectra of pure heparin and drug loaded crosslinked starch micospheres are shown in Fig. 3a and b, respectively. The spectrum (b) clearly indicates the presence of starch and heparin in the loaded crosslinked starch microspheres. The presence of starch is well evident from the peaks observed at 3,900–3,300 cm^{-1} (O–H Stretching), 2,920 cm^{-1} (C–H Stretching), 1,650 cm^{-1} (δ O–H bending of water), 1,325 cm^{-1} (C–H bending and wagging), 1,250 cm^{-1} (O–H bending) and 950–1,040 cm^{-1} C–O Stretching), respectively. Moreover peaks at 700 and 900 cm^{-1} indicate for low content of coordinated water molecules due to wagging (out of plane bending) and rocking vibrations of the –OH groups in water.

A close examination of IR spectrum (a) of pure heparin implies the presence of a prominent band at 1,038 cm^{-1} that may be assigned to the N-sulphate symmetric stretch ($-SO_3$ vibrations). This band also appears in spectrum (b) thus confirming the loading of heparin onto the starch microspheres. The carboxylate ions present in the anionic heparin molecule are confirmed in the spectrum from an asymmetrical stretching band near 1,650 cm^{-1} and a weaker stretching band near 1,400 cm^{-1}. The bands observed in the region 750–950 cm^{-1} also indicate sulphate half esters in the heparin.

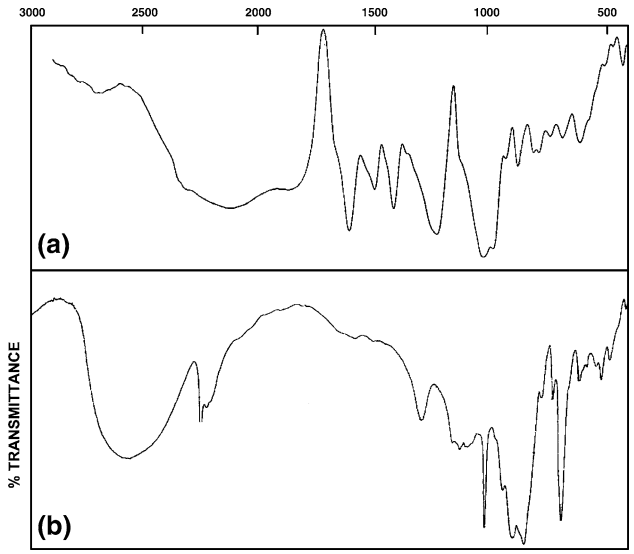

Fig. 3 FTIR spectra of (**a**) heparin, (**b**) heparin loaded starch microspheres

SEM analysis

In order to gain an insight into the morphology of the prepared microspheres the ESEM images of unloaded and heparin-loaded microspheres were recorded as shown in Fig 4a and b, respectively. It is clear from the micrograph (a) that during the preparation of microspheres their internal structures collapsed due to gelatinization as is evident from the heterogeneous nature of the surface containing aggregated starch molecules. Upon loading of the microspheres by heparin a collapsed structure still remains, however, with a reduced extent. The loaded microspheres also shows a few cracks on their surfaces, which could be attributed to result from the operative electrostatic repulsion between the charged drug molecules. A little homogeneity of surface is also visible due to drug–biopolymer interactions.

Particle size analysis

The particle size distribution curve is shown in Fig. 5, which clearly indicates that the size of the microspheres varies between 3 μm and 460 μm, and the largest fraction of the microspheres had a dimension of about 160 μm.

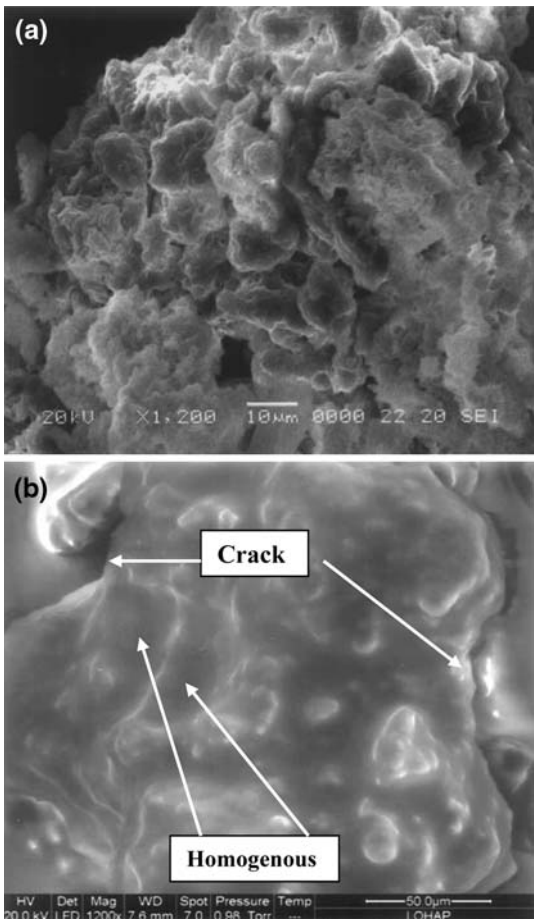

Fig. 4 The SEM images of (**a**) crosslinked starch microspheres and, (**b**) heparin loaded starch microspheres

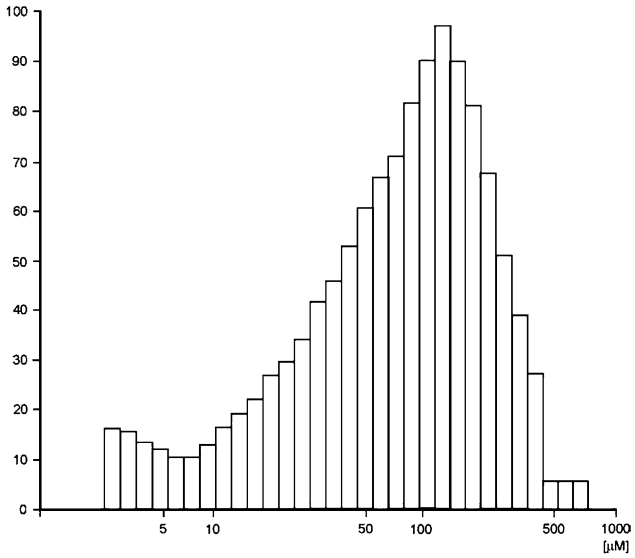

Fig. 5 Particle size distribution curve of crosslinked starch microspheres

Dissolution study

It is known that during the crosslinking reaction of epichlorohydrin with polysaccharide [32] only a minor part of the epichlorohydrin is involved in crosslinkages. Furthermore, it must be noted that epichlorohydrin most likely does not form an ideal tetrafunctional network because of microheterogenities developed due to formation of short free chain ends, loops, entanglements and microaggregation resulting from incomplete crosslinking by epichlorohydrin [33]. Thus, dissolution of starch microspheres as a function of crosslinker content deserves further investigation.

The influence of varying crosslink density on the percent dissolution of microspheres has been investigated by performing dissolution tests for three different microspheres prepared with increasing concentrations of epichlorohydrin (0.006, 0.013, 0.038 mM). The results are shown in Table 1, which clearly indicates that the extent of dissolution gradually decreases with increasing concentrations of crosslinking agent. The results are quite obvious and may be explained by the fact that on increasing the

Table 1 Extent of dissolution of microspheres for varying amounts of crosslinkers content

Crosslinker (mM)	Percent dissolution (%)
0.006	37 ± 1.2
0.013	7 ± 1.8
0.038	6 ± 1.17

concentration of crosslinker the crosslink density of starch microspheres also increases which keeps the microspheres compact and resistant to dissolution.

Release study of heparin

Effect on percent loading on heparin release

An important parameter influencing the release profile of a drug loaded system depends on the extent of loading, which in the present case has been investigated by allowing the microspheres to swell until equilibrium was reached in the heparin solutions of varying concentrations (50–500 IU/mL). Use of either highly concentrated drug solutions or repeated soaking of microspheres in drug solution and drying them results in higher loading.

In the present study, microspheres of definite composition was loaded with different amounts of heparin in the loading range 40–78%. The release results are shown in Fig. 6, which reveal that the released amount of heparin increases with increasing percentage loading up to 60% while thereafter a decrease is observed. The observed initial increase in the release rate may be attributed to the fact that a larger loading of the microspheres facilitates a faster movement of the invading solvent front, which as a consequence enhances the release of entrapped heparin.

The decrease noticed in released amount of heparin may be attributed to the reason that because of much higher loading of heparin into the microspheres, the drug may get accumulated at their pores, which results in shrinking of the pore sizes. This obviously hampers the expulsion of heparin molecules into the release medium, which brings about a fall in the released amount.

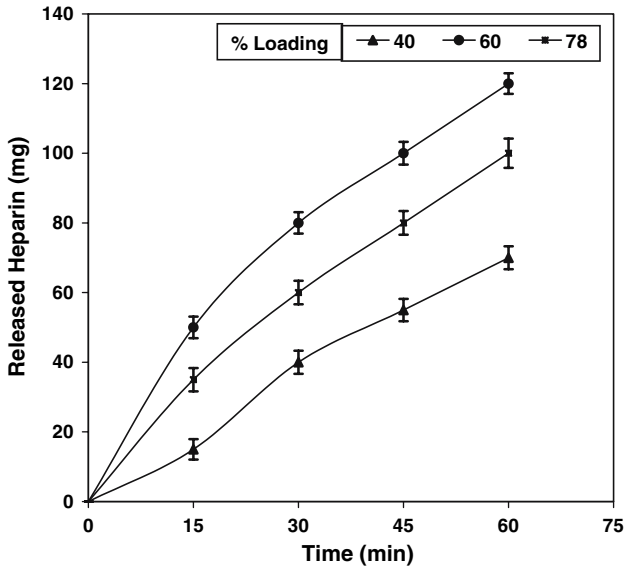

Fig. 6 Effect of percent loading of heparin on its release profiles from loaded microspheres of definite composition [starch] = 3 g, [epichlorohydrin] = 0.013 mM, pH = 7.4, Temp. = 27 °C

Effect of starch on heparin release

Drug release profiles are often sensitive to chemical architecture of the carrier as well as the experimental conditions of preparation of drug carrier. In the present study too, the size and morphology of microspheres are greatly determined by factors such as concentrations of starch and epichlorohydrin in the feed mixture, temperature and shaking time of emulsions. The effect of starch on the release of heparin has been investigated in the range 1.0–4.0 g in the feed mixture. The release profiles are shown in Fig. 7, which clearly indicate that the release of heparin increases in the range 1.0–3.0 g and thereafter a decrease is noticed. The results may be attributed to the fact that starch is hydrophilic in nature and, therefore, its increasing amount may result in an increasing hydrophilicity of microspheres, which obviously brings about an increase in the release of the entrapped heparin. However, the decrease obtained beyond 3 g of starch may be explained by the fact that when the amount of starch becomes much greater, the polymeric microspheres becomes largely crowded of starch molecules and therefore, this consequently reduces the free volume accessible to the penetrant water molecules. This obviously brings about a fall in the released amount of drug.

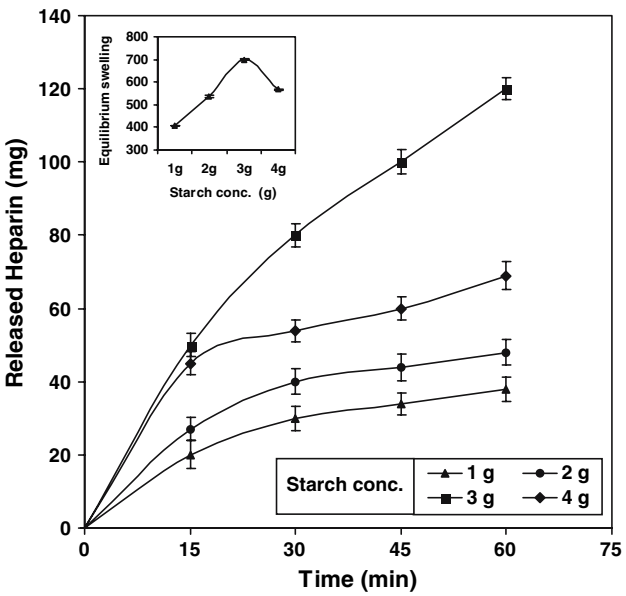

Fig. 7 Effect of starch content of the microspheres on the release profile of heparin from loaded microspheres of definite composition [epichlorohydrin] = 0.013 mM, % Loading = 60%, pH = 7.4, Temp. = 27 °C. Inset picture shows variation in swelling ratio with varying starch concentration

The observed release studies are consistent to the swelling behavior of the microspheres, as shown in the inset of Fig. 7. It is clear from this figure that the swelling ratio increases with increasing starch concentration up to 3.0 g due to increased hydrophilicity of microspheres. However, beyond 3.0 g of starch content the observed decrease in swelling ratio could be attributed to enhanced compactness of the microspheres due to greater interaction between starch molecules.

Effect of crosslinker on heparin release

A large number of crosslinkers are known for starch among which phosphoryl chloride, epichlorohydrin and tri-sodium tri-metaphosphate are the most common. In the present study, epichlorohydrin has been used to crosslink starch in the concentration range 0.006–0.038 mM in the feed mixture. The results are depicted in Fig. 8, which reveal that an initial increase in concentration of epichlorohydrin in the range 0.006–0.013 mM in the feed mixture increases the hydrophilicity. As epichlorohydrin is a low molecular weight crosslinking agent of starch, which at its two terminals react the hydroxyl groups of starch to form crosslinks. Thus, a crosslinked starch network could be imagined as an ultrahigh molecular weight starch molecule that contains wide pore sizes in its structure and, therefore, possesses an abnormal capacity of accommodating water into the network. Thus, capacity to imbibe increasing number of water molecule results in an increased swelling which is clearly shown also in inset of Fig. 8. The observed, increased swelling permits a greater number of heparin molecules to diffuse out of the material and pass into the release medium.

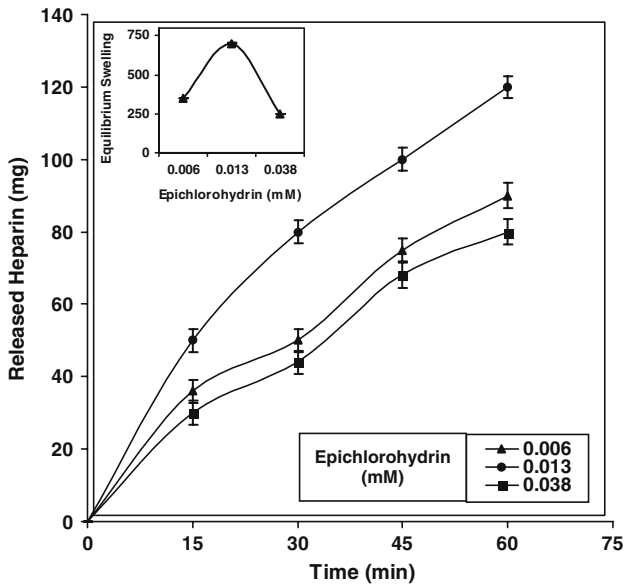

Fig. 8 Influence of crosslinker content of the microspheres on the release profile of heparin from loaded microspheres of definite composition [starch] = 3 g, % Loading = 60%, pH = 7.4, Temp. = 27 °C. Inset picture shows variation in swelling ratio with varying crosslinker concentration

However, beyond 0.013 mM of ECH, the network contains a much greater number of crosslinks, which consequently reduces the free volume accessible to the penetrant water molecules. Similar types of results have also been reported by other workers [34]. Some authors [35] have, however, reported that inclusion of crosslinker into the polymer matrix enhances its glass transition temperature (T_g) which restrains the mobility of network chains at experimental temperature and, therefore, both swelling and release of heparin decrease.

Effect of pH on heparin release

pH responsive macromolecular devices have been most frequently used to design controlled release formulations for oral administration which remains the most clinically acceptable way of drug delivery. Oral administration of macromolecular drugs remains a significant challenge because they are susceptible to hydrolysis and digestion by the acid and enzymes in the gastrointestinal tract (GI). Also, the bioavailability of orally delivered drugs is very low due to poor membrane permeability [36]. Thus, to improve therapeutic efficiency and to reduce or eliminate side effects of oral controlled drugs, it is reasonable to deliver drugs to specific regions of the GI tract.

pH sensitive polymers are used as drug releasing carriers via loading in aqueous solutions. This system has an additional advantage that only physical interaction between the polymer and protein is used to entrap drug in the matrix. No chemical modification of drug occurs which otherwise would result in a decrease in the bioactivity of the heparin.

In the present investigation, the release dynamics of the heparin has been observed under varying pH conditions as found in GIT [e.g. stomach (gastric juice) 1.0, and small intestine 7.5–8.6]. The wide range of pH allows a specific drug to be delivered to a targeted site only. The results depicted in Fig. 9 indicate that in acidic and alkaline range the released amount of heparin decreases while it attains an optimum value at neutral pH 7.4. The observed results are consistent to the swelling results. In the acidic and alkaline pH, the microspheres did not swell sufficiently, and as a result loading decreased. Hence, under these conditions only the surface bound drug is mostly released. The results are quite obvious as the heparin polysaccharide chain gets degraded in the gastric juice. However, at physiological pH 7.4, the microspheres showed enhanced swelling and accordingly the drug release increases.

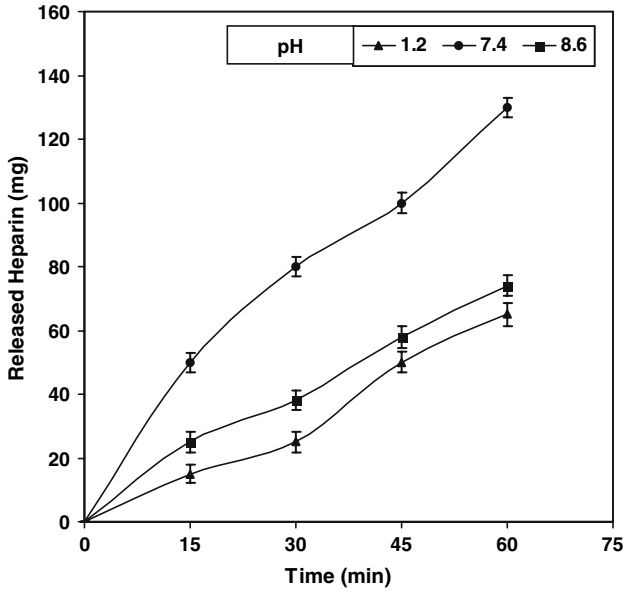

Fig. 9 Effect of pH of the release medium on the release profile of heparin from loaded microspheres of definite composition [starch] = 3 g, [epichlorohydrin] = 0.013 mM, % Loading = 60%, Temp. = 27 °C

Effect of physiological fluid on heparin release

The effect of nature of the medium on the release kinetics of heparin has been investigated by performing release experiments in various physiological fluids. The results are depicted in Fig. 10, which reveals that the release of heparin is significantly suppressed in physiological fluids in comparison to that in the neutral solution, PBS. The possible reason for the lower release of heparin in these fluids may be due to the presence of salt ions in the release medium which lowers the osmotic pressure in the system thus resulting in lower extent of swelling of loaded microspheres. Obviously the microspheres with suppressed swelling will result in less amount of released heparin.

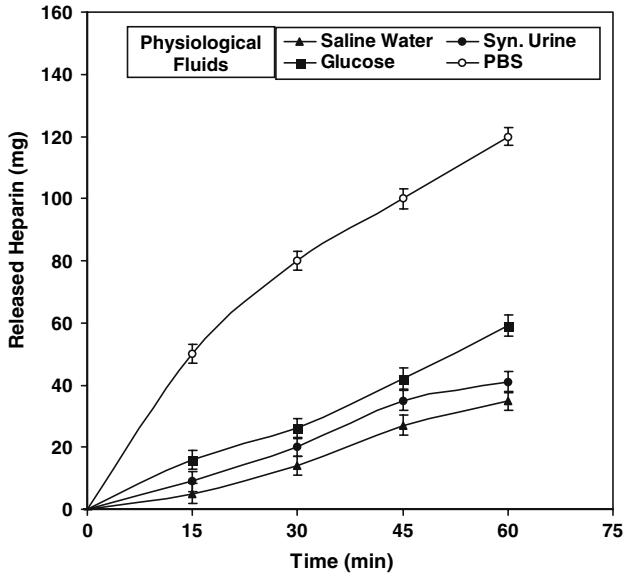

Fig. 10 Effect of simulated physiological fluids on the release profile of heparin from loaded microspheres of definite composition [starch] = 3 g, [epichlorohydrin] = 0013 mM, % Loading = 60%, pH = 7.4, Temp. = 27 °C

Chemical stability of drug

In order to ascertain the chemical stability of heparin, the UV spectral study of pure heparin in solution and released heparin in released medium were scanned in the range 220–330 nm as shown in Fig. 11. The two spectra clearly show that there is almost no change in the absorption pattern of the spectra. This clearly suggests that the chemical activity of the drug is retained during the loading and drying process of the microspheres.

Effect of α-amylase on the degradation of microspheres

The influence of α-amylase concentration on the degradation profile of crosslinked starch microspheres has been investigated by using various concentrations of enzyme solution in the concentration range 6.5–19.5 IU/mL. The results are presented in Fig. 12, which clearly shows that percent degradation of crosslinked microspheres is greater when higher α-amylase concentration is used. The observed results may be explained by the fact that in an enzyme solution of higher α-amylase activity, greater number of enzyme molecules invade the microsphere surface and as a consequence degradation will also be faster.

Conclusions

Crosslinked starch microspheres form a swelling-controlled release system, which effectively delivers heparin into the release medium. It is found that release profiles of heparin are greatly influenced by percent loading of heparin and on varying the concentration of starch and crosslinker.

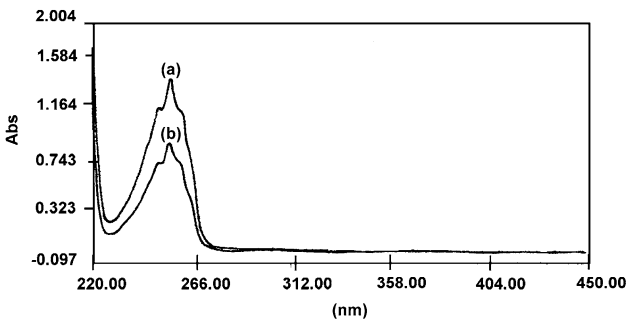

Fig. 11 UV spectra of (**a**) pure drug and, (**b**) released drug in PBS

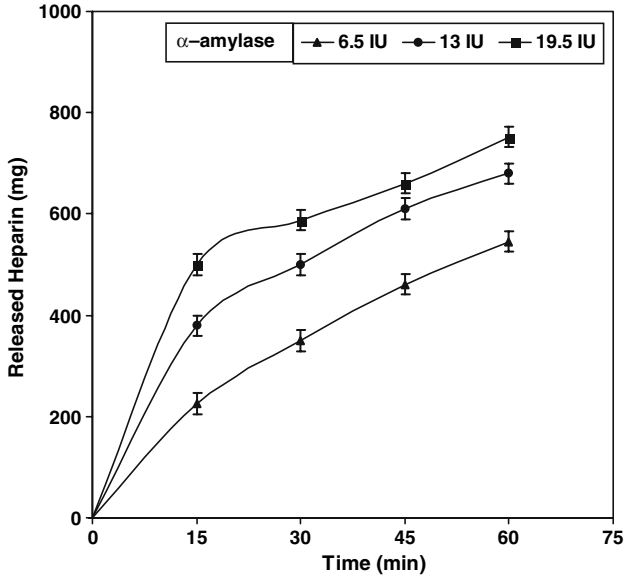

Fig. 12 Effect of α-amylase concentration of the release medium on the release profile of heparin from loaded microspheres of definite composition [starch] = 3 g, [epichlorohydrin] = 0.013 mM, % Loading = 60%, pH = 7.4, Temp. = 27 °C

With increase in percent loading of drug onto the microspheres, the release of heparin increases up to 60% but beyond this a decrease is observed. When the concentration of starch increases in the range 1–3 g, the released amount of heparin also increases. However, a decrease is noticed beyond 3 g of starch content in the microspheres.

The released amount of heparin is also affected on varying the concentration of crosslinked (epichlorohydrin) in the range 0.006–0.038 mM. An initial increase is observed up to 0.013 mM while beyond that a decrease is noticed. An optimum drug is released near physiological pH (7.4) while it decreases in both the acidic and alkaline ranges.

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