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Glutaraldehyde and glyoxal cross-linked chitosan microspheres for controlled delivery of centchroman

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Abstract—Glutaraldehyde and glyoxal cross-linked microspheres were prepared using chitosan with different molecular weights (MWs) and degrees of deacetylation (DDAs) for sustained release of centchroman under physiological conditions. The DDA in chitosan was determined by different methods, and the samples were categorized as chitosan with low (48%), medium (62%), and high (75%) DDA. The size and shape of the microspheres were determined by scanning electron microscopy (SEM), and hydrophobicity was determined by adsorption of Rose Bengal dye on microspheres cross-linked with glutaraldehyde or glyoxal. The effect of MW, DDA, and degree of cross-linking in microspheres was studied on the degree of swelling, as well as by the loading and release of centchroman. The glyoxal cross-linked microspheres were more compact and hydrophobic and showed better sustained release in companion to chitosan microspheres and glutaraldehyde cross-linked microspheres. The linear fractional release of centchroman with the square root of time indicated a Fickian behavior of centchroman, and the microspheres also showed zero-order release kinetics for centchroman.

Keywords: Chitosan; Glyoxal; Glutaraldehyde; Centchroman; Drug delivery; Cross-linking

1. Introduction

Chitosan is a naturally occurring biopolymer prepared by alkaline partial deacetylation of chitin obtained from the exoskeleton of shrimps,[1,2] fungi, insects, annelids, and mollusks.[1] Chitosan is also produced by the enzymatic[3] deacetylation of chitin. It is a biopolymer that is safe for industrial and biomedical applications.[4–7] The biodegradability[8] and bioadhesivity[9] of chitosan are useful properties in formulations of oral drug delivery devices, which need extended retention times in the mucosa of the gastrointestinal tract. The positive charge on chitosan generated under physiological conditions was found to be responsible for its enhanced bioadhesivity and site-specific applications[10–12] in controlled delivery systems. The main parameters that control the physicochemical properties[13–16] of chitosan, are molecular weight (MW)[17,18] and degree of deacetylation (DDA).[19–21] The DDA in chitosan also controls its biodegradability[22] and immunological activity.[23] The free amino groups in chitosan contribute toward its solubility in acidic media and reactivity with physical[24–26] and chemical cross-linkers.[26–28] Glutaraldehyde cross-linked chitosan microspheres have shown dynamic storage properties,[16] ion adsorption,[29,30] and immobilization of enzyme[30] and systems for controlled drug delivery.[31,32] Recently other cross-linkers such as genepin[33–35] and glyoxal[36–38] and other polymers[37] have been examined for their possible applications in the biomedical field, but few studies are reported in which glyoxal is used as the cross-linker in controlled delivery systems.[39] Therefore, in the present investigation, cross-linked chitosan microspheres were prepared at different concentrations of glyoxal and glutaraldehyde using chitosan with different DDAs. The physical properties of glyoxal and glutaraldehyde cross-linked microspheres were

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compared for loading and release kinetics of centchroman[†] from these microspheres. Centchroman is a non-steroidal contraceptive and exhibits antiestrogenic properties without affecting the hypothalamic–pituitary–ovarian axes. Centchroman has also shown anticancer activity; hence, investigations on a controlled delivery system for the drug[40] are considered worthwhile.

2. Experimental

2.1. Chemicals and reagents

Chitosan was purchased from Sigma–Aldrich Chemical Company (USA) and purified by dissolving in 2% (w/w) HOAc solution. The viscous solution was passed through a filter to remove an undissolved fraction of chitosan. The homogeneous filtrate was precipitated using 1.0 M NaOH and dried at 30 °C after washing with distilled water. Glutaraldehyde and glyoxal were obtained from Loba Chemie, India as 25% (w/w) and 4% (w/w) solutions. The glyoxal aggregates were removed from solution with an 8-μm mesh filter before using it as a cross-linker.

2.2. Determination of the molecular weight (MW) of chitosan

The MW of the procured chitosan sample was determined viscometrically using Eq. 1

$$[\eta]_{25\,^\circ\mathrm{C}} = 1.81 \times 10^{-3}\ \mathrm{cm^3\ g^{-1}}\, M_v^{0.93} \quad (\mathrm{HOAc}) \qquad (1)$$

2.3. Deacetylation in chitosan and its determination

To obtain chitosan with different DDAs, a 50 g chitosan sample with 48% (w/w) DDA was heated with 50 mL of a 40% (w/w) solution of sodium hydroxide at 80 °C for 4 h.[41] Similarly another sample of chitosan (48% w/w DDA) was refluxed for 8 h to obtain chitosan with higher DDA. The refluxed samples were separated, washed with hot and cold distilled water, and dried.

The DDAs in the original and alkali-treated chitosan samples were determined using potentiometric[42] and IR spectroscopic[43] methods of analysis and verified further with elemental analysis, which is based on structural units present in chitosan.[44]

For the potentiometric method of determination of DDA,[42] 0.025 g of chitosan was dissolved in 25 mL of HCl (1.75×10^{-3} M), and excess acid was back titrated with NaOH (1.80×10^{-2} M) using a pH meter (46 CL-Toshniwal, India). The differential volume (ΔV) between points of inflexions in the differential curve was used to calculate the DDA in chitosan using Eq. 2

$$\mathrm{DDA}\ (\%) = \left(\frac{203Q}{1+42Q}\right) \times 100 \quad \text{and} \quad Q = \frac{N\Delta V}{w} \qquad (2)$$

where w and N are the weight of chitosan and normality of alkali, respectively, used in titration.

In the IR method[43] for the determination of DDA, the ratio of absorbance of the amide I (1655 $\mathrm{cm^{-1}}$) to hydroxyl group (3450 $\mathrm{cm^{-1}}$) bands of chitosan was used to calculate the DDA using Eq. 3

$$\mathrm{DDA}\ (\%) = [1 - (A_{1655}/A_{3450})/1.33] \times 100 \qquad (3)$$

The DDAs determined by the potentiometric and IR methods were further verified by using the weight percent of carbon (W_C) and nitrogen (W_N) and fitting[45] the values in Eqs. 4 and 5

$$\mathrm{DDA}\ (\%) = \left(\frac{9600}{364 \times W_C + 2400}\right) \times 100 \qquad (4)$$

$$= \left(\frac{1400}{364 \times W_N}\right) \times 100 \qquad (5)$$

2.4. Preparation of glutaraldehyde and glyoxal cross-linked microspheres

The glutaraldehyde and glyoxal cross-linked chitosan microspheres were prepared dissolving a known amount of chitosan (2.0 g) in 200 mL of HOAc (2% w/w) under vigorous stirring for about 3 h at room temperature. To obtain microspheres, the solution was blown through a nozzle as fine droplets into a trough containing 250 mL (5% w/w) of a methanolic solution of NaOH (0.1 M). The chitosan microspheres that settled at the bottom of the trough were separated after 30 min and washed with distilled water. Since the reactivity of the cross-linker depends upon various factors, the cross-linking reaction was carried out at constant time (6 h) and temperature (25 °C) and varying concentration of cross-linkers from 2% to 12% (w/w) in a reaction vessel containing chitin microspheres. The cross-linking of microspheres with glutaraldehyde was carried out in neutral solution (pH 7) and under acidic conditions (pH 4) with glyoxal. Microspheres using chitosan with different DDAs and MWs were also prepared using glyoxal and glutaraldehyde as cross-linkers. To compare the effect of cross-linking, the non-cross-linked chitosan microspheres were also prepared. After 6 h, the microspheres were separated from the cross-linking vessel and dried at 30 °C after washing with distilled water.

2.5. Determination of size and morphology of chitosan microspheres

The size and morphology of glutaraldehyde/glyoxal cross-linked chitosan microspheres (DDA 62% w/w,

[†] Centchroman [31477-60-8] is rel-1-[2-[4-[(3*R*,4*R*)-3,4-dihydro-7-methoxy-2,2-dimethyl-3-phenyl-2*H*-1-benzopyran-4-yl]phenoxy]ethyl]-pyrrolidine.

$\overline{M}_v$ 1134 kg mol^{-1}) was determined with a scanning electron microscope (SEM) after mounting microspheres on metal studs using double adhesive tape and vacuum coating with gold. The shape of the microspheres determined by SEM analysis was expressed in terms of shape factor (S) determined as ratio of the square of perimeter (L) and area (A) of a selected surface of about 20 microspheres[46] using Eq. 6

$$S = \frac{L^2}{4\pi A} \tag{6}$$

where L and A are the average perimeter and area of selected surface on microspheres, respectively.

2.6. Surface hydrophobicity of cross-linked microspheres

To determine the hydrophobic character of microspheres, the volume of Rose Bengal dye adsorbed per unit area of microspheres was determined by keeping 0.1 g chitosan microspheres of different sizes (specific surface area) separately in 10 mL of a 0.1 M solution of Rose Bengal dye for about 2 h to effect partitioning of dye at the interface. The volume of Rose Bengal dye adsorbed on microspheres of different specific surface area (A) was determined by recording the absorbance ($\lambda_{max} = 549$ nm) of the remaining solution after 2 h. The hydrophobicity of microspheres was calculated from the slope drawn between the partition quotient (Q) versus specific surface area (A) of microspheres[47] available for per milliliter adsorption of dye (μm^2/mL). The partition quotient (Q) is calculated as shown below

$$Q = \frac{\text{Volume of dye adsorbed on microspheres (mL)}}{\text{Volume of dye taken for adsorption (mL)}}$$

2.7. Mechanical properties and molecular weight of cross-linked microspheres

Texture analysis of microspheres was carried out by recording the compressive force necessary to maintain a constant penetration of a rigid sphere indenter at the surface of matrices. The indentate analysis was carried out using a texture $Taxt_2$ texture analyzer (Stable Micro System, Rheocomplan, France) with modified attachment to fit a 20-μm rigid sphere indenter. The modulus (E) was used to determine cross-linked MW (M_c) of the chitosan network[48] using Eq. 7

$$M_c = 3\frac{\rho RT}{E}(\phi_2)^{1/3} \tag{7}$$

where E = Young's modulus and ϕ_2 = the volume fraction of chitosan in the microspheres.

2.8. Degree of swelling in microspheres

The degree of swelling[36] in glutaraldehyde and glyoxal cross-linked chitosan microspheres was determined by keeping 100 mg of microspheres in 20 mL of buffered solution (pH 7) and using Eq. 8

$$S_w\ (\%) = \left(\frac{W_t - W_0}{W_0}\right) \times 100 \tag{8}$$

where W_t and W_0, respectively, are the weights of microspheres at different times of swelling and initial weights of the microspheres.

2.9. Centchroman loading onto microspheres

Loading of centchroman onto microspheres was carried out by keeping 100 mg of microspheres in 20 mL of phosphate-buffered solution containing a known amount of centchroman for 48 h. The centchroman loading in glutaraldehyde cross-linked microspheres was carried out at pH 5, whereas in glyoxal cross-linked microspheres, the loading was carried out at pH 4. The amount of centchroman loaded on micropheres was determined by recording the absorbance ($\lambda_{max} =$ 275 nm) of the remaining solution using a UV–vis (Shimadzu model 1601 PC) spectrophotometer.

2.10. Centchroman release from microspheres

The drug-release behavior of the microspheres was analyzed by estimating the amount of centchroman released at different intervals of time from 100 mg of microspheres in 20 mL of release media. The amount of drug released at each interval of 10 h was estimated by recording absorbance at $\lambda_{max} = 275$ nm with replacement using a UV–vis spectrophotometer and is presented as percent release from drug loaded on 100 mg of microspheres. The centchroman release trend from the microspheres was divided into burst and controlled steps of drug release. During the initial period of drug release, when the percent of drug release within a fixed interval of time was not constant and the microspheres were under the swelling process, the percent of drug release was termed 'burst release'. When the percent dug release within a fixed interval of time became constant, and microspheres attained equilibrium swelling, then the percent of drug release was termed 'controlled drug release'.

3. Results and discussion

3.1. General considerations

The physical and chemical properties of chitosan depend on its molecular weight (MW) and degree of deacetylation (DDA); hence, it is necessary to characterize chitosan for its MW and DDA before designing a controlled release system. The increase in DDA in chitosan increases its solubility in acid and also provides opportu-

nities for maximum cross-linking with cross-linking agents. The chitosan samples were characterized by their MWs, which were found to be 760, 1134, and 2227 kg mol^{-1}, respectively. These samples were categorized as low (LMW), medium (MMW), and high (HMW) chitosan according to their MWs.

3.2. Degree of deacetylation (DDA) in chitosan

The deacetylation in chitosan by alkali treatment was carried out at fixed temperature (80 °C) to avoid variation in the rate of deacetylation.[16] The alkali treatment did not show any variation in the MW of chitosan as verified by determining the MW of alkali-treated chitosan samples. The chemical properties of chitosan depend on DDA; hence, DDA in the original and alkali-treated chitosan samples was determined by methods reported in the literature. The DDA in samples determined by the potentiometric[42] method was found to be 48%, 62%, and 75% (w/w) (Table 1). The variation in DDA on treatment of chitosan with alkali was due to the conversion of the acetamido groups into free amino groups. The DDA in chitosan samples determined by the potentiometric method was further verified by recording their IR spectra (Fig. 1) of chitosan samples. Transmittances corresponding to the amide I ($\lambda = 1655\ \text{cm}^{-1}$) and hydroxyl group ($\lambda = 3450\ \text{cm}^{-1}$) bands of chitosan were used to determine the DDA, with the ratio of the absorbance of amide I ($\lambda = 1655\ \text{cm}^{-1}$) to that of hydroxyl group ($\lambda = 3450$) in Eq. 3. The transmittance of the amide I group was increased from 31.0% to 46.5% (Fig. 1) from the original sample of chitosan (Fig. 1A) to that of a chitosan sample treated for 8 h (Fig. 1C). The DDA in the chitosan samples determined by the IR method was found to be 48%, 62%, and 75% (w/w), respectively, as shown in Table 1. The DDAs determined by the IR method were found to be very close to those from the potentiometric method. To further verify the determinations, the DDAs were determined by elemental analysis[45] using Eqs. 4 and 5, from which the DDAs were found to be 48.0%, 61.98%, and 75.03% (w/w). Thus the DDAs determined by the elemental analysis method were found to be in agreement with values determined by the potentiometric and IR methods of analysis (Table 1). Depending upon these values, the samples were marked as chitosan with low (LDDA), medium (MDDA), and high DDA (HDDA) having DDAs as 48%, 62%, and 75% (w/w), respectively.

3.3. Cross-linked microspheres and their physical characteristics

In addition to DDA in chitosan, the degree of cross-linking also controls the properties of chitosan. Glutaraldehyde has frequently been used as a cross-linker, but its reactivity in comparison with glyoxal in cross-linking chitosan microspheres is interesting and useful. The chitosan microspheres cross-linked with glutaraldehyde or glyoxal were characterized for their size by scanning electron microscopy (SEM), which shows a decreasing trend in the size of microspheres from 119 to 31.60 μm for non-cross-linked and glyoxal cross-linked chitosan microspheres. The microspheres prepared in the presence of glutaraldehyde were found to be larger in size (39.78 μm), which clearly indicates that glyoxal cross-linked microspheres are more compact due to the high degree of cross-linking in comparison with glutaraldehyde cross-linked microspheres. This was further verified with SEM micrographs (Fig. 2a, c, and e). The microspheres prepared with glyoxal were smooth in morphology (Fig. 2f) in comparison with those of pure chitosan (Fig. 2b) and those cross-linked with glutaraldehyde (Fig. 2d). The cross-linking also shows an effect on the shape of microspheres as inferred from the shape factor.[48] The shape factor (S) was decreased from 0.922 to 0.795 for pure chitosan and glyoxal cross-linked chitosan microspheres, which indicates that microspheres prepared with pure chitosan were more spherical than glyoxal cross-linked microspheres. The shape factor for glutaraldehyde cross-linked microspheres was found to be 0.832, which clearly indicates that these are more spherical than glyoxal cross-linked microspheres. Since the degree of cross-linking in glyoxal cross-linked microspheres was larger, they could not become spherical in comparison with pure chitosan and glutaraldehyde cross-linked chitosan microspheres. The shape factor (S) has also shown variation with MW of chitosan. The microspheres with LMW chitosan are more spherical than those of HMW chitosan. The microspheres with HDDA (75% w/w) are less spherical than those with LDDA (48% w/w). The chitosan is hydrophilic in nature in acidic media due to the presence of the amino groups, but hydrophilicity and solubility of

Table 1. Determination of the degree of deacetylation (DDA) in chitosan samples[a]

Chitosan samples	Potentiometric method (%)	IR method (%)	Elemental analysis method		
			C (%)	N (%)	DDA (%)
Sample 1	48.0	48.0	48.36	8.03	48.80
Sample 2	61.8	62.0	35.36	6.22	61.98
Sample 3	74.6	75.0	28.56	5.13	75.03

[a] $\overline{M}_v$ of chitosan = 1134 kg mol^{-1}, original chitosan (Sample 1), 4 h (Sample 2), and 8 h (Sample 3) alkali (40% w/w) treated chitosan, degree of deacetylation (DDA).

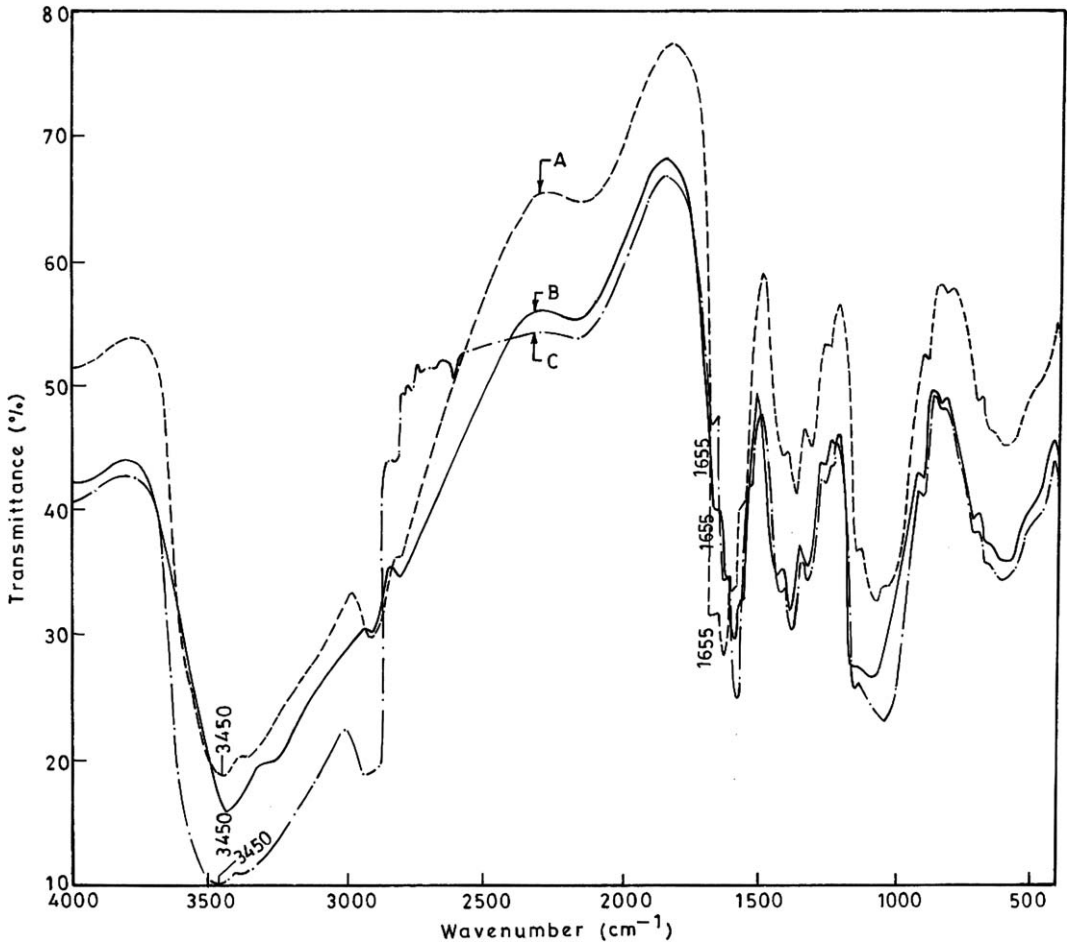

Figure 1. FTIR spectra of chitosan samples ($\overline{M}_v$ 1134 kg mol^{-1}) for determining DDA.

chitosan is changed on covalent cross-linking with dialdehydes. However, the variations in hydrophilicity depend on the type of cross-linker and chitosan used for cross-linking. Cross-linking with glutaraldehyde/glyoxal increases the hydrophobic character in chitosan. The degree of hydrophobicity in microspheres was estimated with the volume of hydrophobic dye adsorbed per unit area of microspheres, which was obtained from the slope of a plot drawn between the partition quotient (Q) of the dye and the specific surface area of the microspheres (A) as shown in Figures 3 and 4. The volume of Rose Bengal dye adsorbed per unit area of pure chitosan microspheres was found to be lowest (0.029 mL μm^{-2}) in comparison with glutaraldehyde (0.166 mL μm^{-2}) and glyoxal (0.186 mL μm^{-2}) cross-linked microspheres (Fig. 3), which clearly indicates that microspheres prepared with pure chitosan are more hydrophilic than microspheres prepared with glyoxal as cross-linker. This gives an indication that the degree of cross-linking in chitosan microspheres has increased the hydrophobic character. The hydrophobic character in the microspheres also varies with DDA as clear from the trends shown by microspheres prepared with different DDAs (Fig. 4). The non-cross-linked microspheres prepared with HDDA (75% w/w) microspheres were less hydrophobic than those prepared from LDDA (48% w/w) material. The microspheres prepared using chitosan with different MWs have shown similar hydrophobicity, which indicates that MW has no significant effect on hydrophobic interactions that arise from similar chemical composition. The cross-linking in microspheres was carried out under controlled pH for cross-linking through a Schiff base reaction[38,49] rather than acetalization.[50] The IR spectra have shown a strong absorption band at 1660 cm^{-1} (—C=N—) in both the glutaraldehyde and glyoxal cross-linked microspheres, but a band corresponding to R–O–R stretching (1910 cm^{-1}) was missing, which provides proof that cross-linking in chitosan had taken place through a Schiff base reaction and not via acetalization.

3.4. Degree of swelling in cross-linked microspheres

The degree of swelling is a significant characteristic of cross-linked microspheres that controls the loading and release profile of the drug. The hydrophobicity in

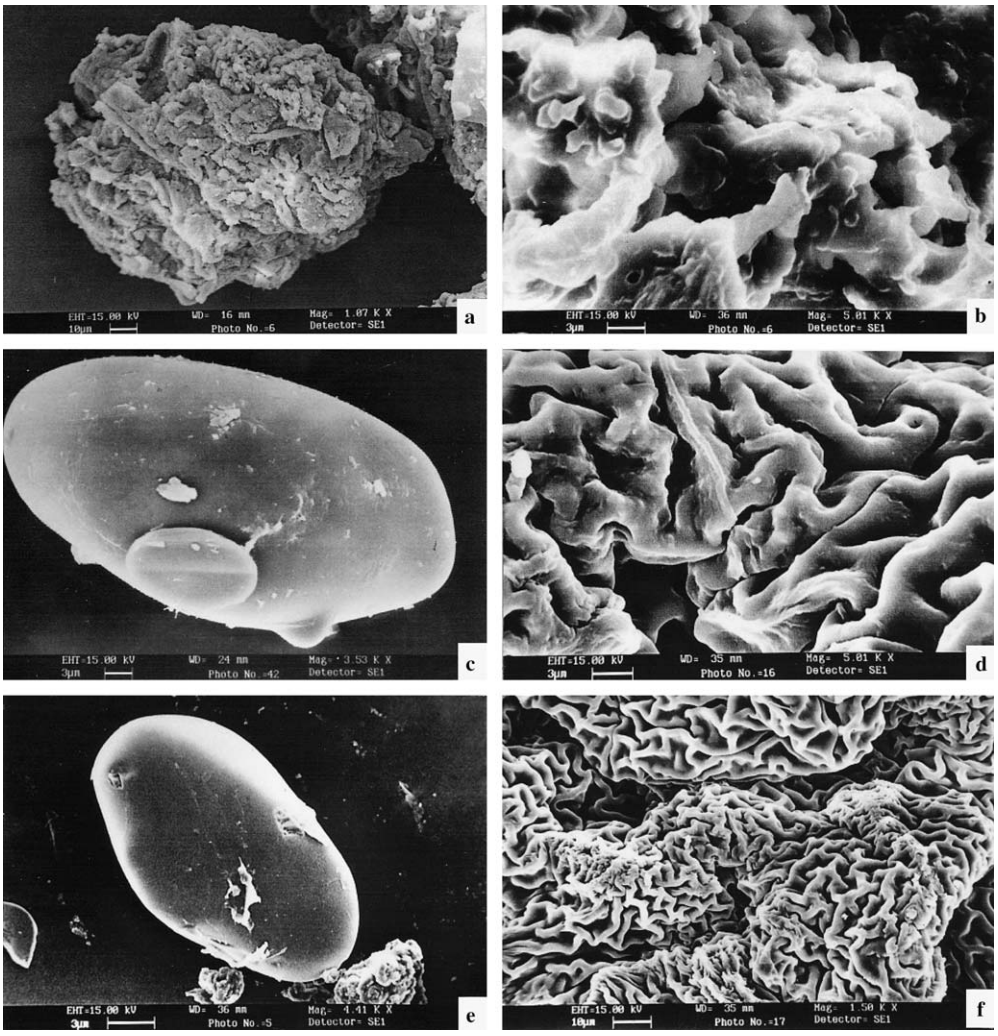

Figure 2. Scanning electrons micrographs of pure (a, b), glutaraldehyde (c, d), and glyoxal (e, f) cross-linked chitosan microspheres. $\overline{M}_v$ (1134 kg mol^{-1}), DDA (62% w/w).

microspheres and size of cross-link MW (M_c) control the degree of swelling. The chitosan microspheres (62% w/w DDA) without any cross-linker have shown a maximum degree of swelling of 300% within a period of 20 h (Fig. 5), and then afterward, the degree of swelling decreased due to the dissolution of the chitosan. The degree of swelling in the microspheres changed significantly on cross-linking with glutaraldehyde or glyoxal. To compare the effect of cross-linking and type of cross-linkers on the degree of swelling, the microspheres were prepared using chitosan with constant DDA (62% w/w DDA) and MW (1134 kg mol^{-1}). The degree of swelling in the microspheres has shown a decreasing trend on cross-linking with glutaraldehyde or glyoxal (Table 2). The microspheres prepared with glyoxal have shown an overall low degree of swelling in comparison with microspheres prepared with glutaraldehyde as the cross-linker. The glyoxal cross-linked microspheres have shown a maximum degree of swelling of 200% within a period of 30 h, whereas glutaraldehyde cross-linked microspheres have shown a maximum swelling of 250% within a period of 40 h (Table 2).

The variation in degree of swelling (% S_w) on varying the degree of cross-linking was due to the increased hydrophobicity in the microspheres as clear from the volume of hydrophobic dye adsorbed per unit area of microspheres, which was found to be highest (0.186 mL μm^{-2}) with glyoxal cross-linked microspheres in comparison with chitosan (0.029 mL μm^{-2}) and glutaraldehyde cross-linked microspheres (0.166 mL μm^{-2}) (Table 2 and Fig. 3). The cross-linked molecular weight (M_c) in glyoxal cross-linked microspheres was found to be 276.3 kg mol^{-1} in comparison with chitosan (1006.8 kg mol^{-1}) and glutaraldehyde cross-linked microspheres (184.2 kg mol^{-1}), which clearly indicates that hydrophobic interactions are stronger in glyoxal cross-linked microspheres (Table 2) in comparison with glutaraldehyde cross-linked microspheres. Otherwise, the degree of swelling would have been higher with glyoxal cross-linked microspheres (Table 2 and Fig. 3).

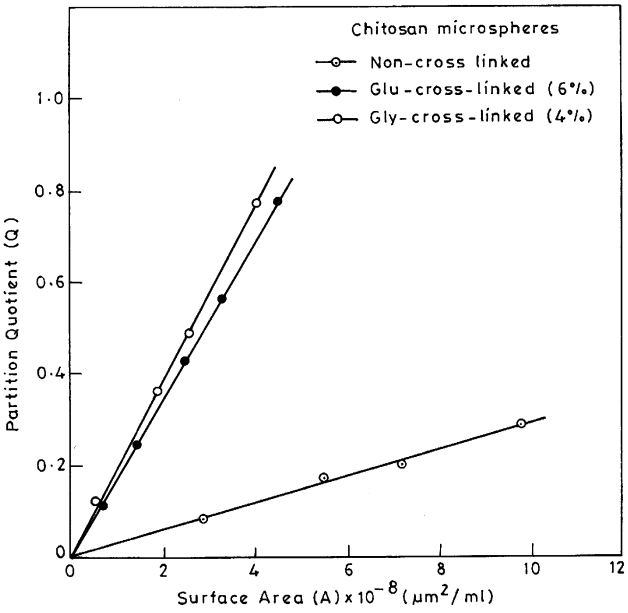

Figure 3. Determination of the degree of hydrophobicity by dye adsorption on pure chitosan, glutaraldehyde (6% w/w), and glyoxal (4% w/w) cross-linked chitosan microspheres. $\overline{M}_v$ (1134 kg mol^{-1}), DDA (62% w/w).

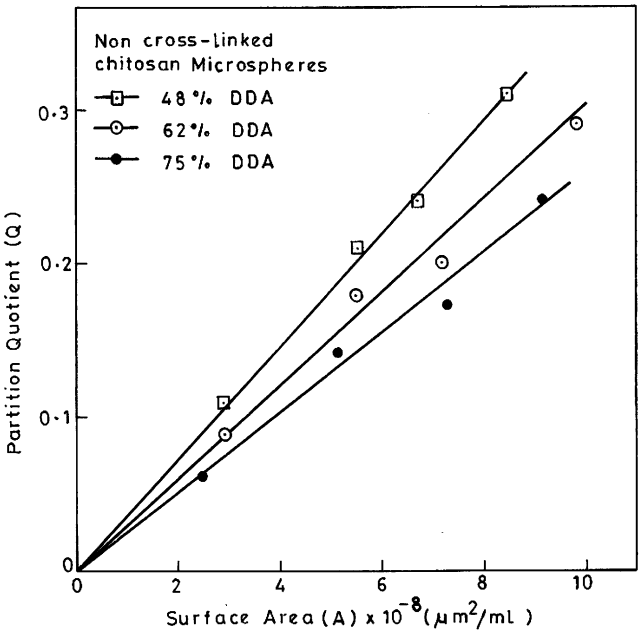

Figure 4. Determination of the degree of hydrophobicity by dye adsorption method in microspheres with different DDAs. $\overline{M}_v$ (1134 kg mol^{-1}).

3.5. Effect of molecular weight (MW) and degree of deacetylation (DDA) on the degree of swelling

The effect of chitosan molecular weight on the degree of swelling in glutaraldehyde/glyoxal cross-linked microspheres was studied using chitosan microspheres prepared with different MWs at constant concentration

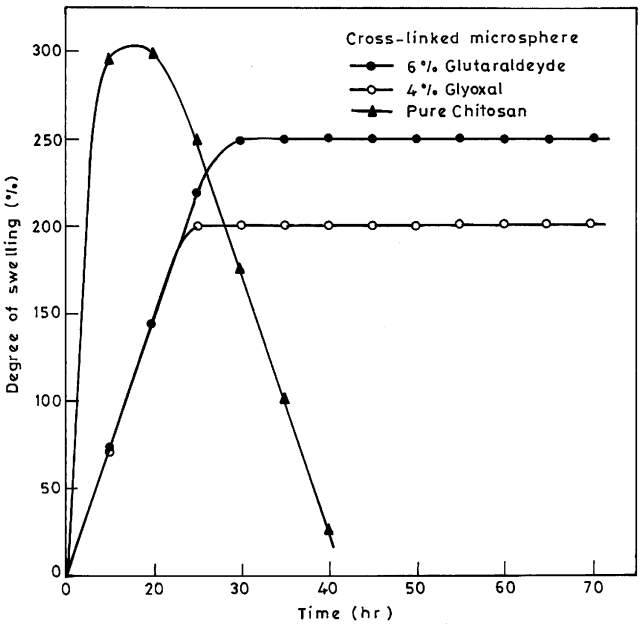

Figure 5. Degree of swelling in pure chitosan, glutaraldehyde (6% w/w), and glyoxal (4% w/w) cross-linked chitosan microspheres. $\overline{M}_v$ (1134 kg mol^{-1}), DDA (62% w/w).

of glutaraldehyde (6% w/w) and glyoxal (4% w/w). The microspheres prepared with LMW chitosan (760 kg mol^{-1}) have shown a high degree of swelling in comparison with microspheres prepared with MMW chitosan (1134 kg mol^{-1}) and HMW chitosan (2224 kg mol^{-1}), but the overall degree of swelling in glyoxal cross-linked microspheres remained low in comparison with glutaraldehyde cross-linked microspheres (Table 3). The high degree of swelling in glutaraldehyde cross-linked microspheres was due to low hydrophobicity (0.164 mL μm^{-2}) in comparison with glyoxal cross-linked microspheres (0.186 mL μm^{-2}). The MW of cross-links (M_c) in glyoxal cross-linked microspheres has been found to be high (253.6 kg mol^{-1}) in comparison with glutaraldehyde (216.32 kg mol^{-1}) cross-linked microspheres, which clearly indicates that hydrophobic character is more predominant in glyoxal cross-linked material (Table 3). The microspheres prepared with HMW chitosan show a low degree of swelling both with glutaraldehyde (150% w/w) and glyoxal (122% w/w) cross linkers (Table 3). The MWs of cross-links (M_c) were found low in comparison with LMW and MMW chitosan microspheres (Table 3). The low degree of swelling and low MW of cross-links (M_c) in microspheres prepared with HMW chitosan have been attributed to the high degree of cross-linking in these microspheres. The degree of hydrophobicity in microspheres prepared with different MWs was found to be constant (Table 3).

The swelling behavior of chitosan microspheres with different DDAs was also studied. The chitosan microspheres with low DDA (48% w/w) have shown a high

Table 2. Effect of the degree of cross-linking on the physical characteristics of chitosan microspheres[a]

Types of chitosan microspheres	Degree of swelling (S_w) (%)		Adsorption[b] of dye (mL μm^{-2})		MW of cross-links (M_c) (kg mol^{-1})	
Cross-linker (% w/w)	Glu	Gly	Glu	Gly	Glu	Gly
0	300	300.0	0.029	0.029	1006.8	1006.8
2	290	218.0	0.031	0.042	935.8	685.5
4	270	200.0	0.038	0.186	303.2	276.3
6	250	192.5	0.166	0.298	184.7	156.8
12	150	145.0	0.220	0.360	166.4	144.1

[a] Chitosan $\overline{M}_v$ 1134 kg mol^{-1}, DDA 62% w/w.
[b] Rose Bengal dye (hydrophobicity).

Table 3. Effect of $\overline{M}_v$ and DDA on the physical characteristics of chitosan microspheres[a]

Types of chitosan microspheres		Degree of swelling (S_w) (%)		Adsorption[b] of dye (mL μm^{-2})		MW of cross-links (M_c) (kg mol^{-1})	
$\overline{M}_v$ (kg mol^{-1})	DDA (% w/w)	Glu	Gly	Glu	Gly	Glu	Gly
760	62.0	287.5	237.5	0.164	0.186	216.30	253.80
1134	62.0	250.0	200.0	0.166	0.187	184.70	244.00
2224	62.0	150.0	122.5	0.164	0.186	179.00	189.20
1134	48.0	282.0	212.0	0.080	0.186	332.97	345.72
1134	75.0	213.0	212.0	0.195	2.100	172.59	270.84

[a] Degree of cross-linking in microspheres with 6% w/w glutaraldehyde (Glu) and 4% w/w glyoxal (Gly).
[b] Rose Bengal dye (hydrophobicity).

degree of swelling in comparison with microspheres prepared with a high degree (75% w/w) of deacetylation (Table 3), which was due to the variations in the degree of cross-linking. The microspheres with low DDA were having a high MW of cross-links (M_c), but their hydrophobicity was low (0.08 mL μm^{-2}) due to poor cross-linking in these microspheres (Table 3). The amino groups in chitosan microspheres with low DDAs (48% w/w) were low, which reduced the degree of cross-linking with glutaraldehyde and glyoxal; hence, chitosan microspheres prepared with low DDAs have shown a high degree of swelling. In these microspheres, the hydrophobic character of the cross-linker could not dominate over high MW of cross-links (M_c); hence, the degree of swelling was found to be high. That the hydrophobic character of chitosan microspheres was changed on cross-linking with glutaraldehyde or glyoxal is clear from the degree of hydrophobicity shown by pure chitosan microspheres with different DDAs (Fig. 4). The non-cross-linked chitosan microspheres with low DDAs (48% DDA) have shown a high degree of hydrophobicity in comparison with microspheres with a high degree (75% w/w) of deacetylation. The opposite trend of pure chitosan microspheres (Fig. 4) with cross-linked microspheres was due to the presence of sufficient free hydrophilic amino groups in the chitosan (Fig. 4), which were otherwise consumed on cross-linking with glutaraldehyde or glyoxal (Table 3).

The microspheres prepared using chitosan with a MW of 1134 kg mol^{-1} and a DDA of 62% (w/w) and cross-linking with glyoxal (4% w/w) and glutaraldehyde (6% w/w) have shown maximum equilibrium swelling of 200% and 250% without any degradation and dissolution for times up to 70 h (Fig. 5). Hence these microspheres are considered suitable for prolonged delivery of drugs.

3.6. Effect of molecular weight (MW), degree of deacetylation (DDA), and cross-linking on the loading of centchroman in microspheres

The effect of chitosan MW, DDA, and cross-linking on centchroman loading capacity of microspheres was studied (Tables 4 and 5 and Fig. 6) at pH 4 (glyoxal) and pH 5 (glutaraldehyde). The variation in loading pH was made to obtain maximum loading in cross-linked microspheres. MMW chitosan microspheres cross-linked with glyoxal (4% w/w) have shown maximum loading (40.5% w/w) in comparison with pure chitosan (15% w/w) and microspheres cross-linked with glutaraldehyde (37.5% w/w). The microspheres prepared with LMW and HMW chitosan have shown lower loading of centchroman (Table 4). The microspheres prepared with LMW chitosan have shown a high degree of swelling due to the high MW (M_c) of cross-links (Table 3), which decreased drug retention capacity. Hence the loading of centchroman in these microspheres was found to be low. The glyoxal cross-linked microspheres with LMW chitosan (Table 4) have shown low capacity for centchroman loading (28% w/w) in comparison with glutaraldehyde cross-linked microspheres (33.5% w/w), which was due to the large size of the cross-links (M_c)

Table 4. Effect of MW and DDA of chitosan microspheres on loading, release, and diffusion coefficient (D) of centchroman[a]

Types of chitosan microspheres		Loading of centchroman (%)		Controlled release of centchroman (%)		$D_{\text{diffusion constant}}$ (10^{-12} cm^2 s^{-1})	
$\overline{M}_v$ (kg mol^{-1})	DDA (% w/w)	Glu	Gly	Glu	Gly	Glu	Gly
760	62.0	33.5	28.0	50.10	60.00	2.889	1.190
1134	62.0	37.5	40.5	70.40	77.78	0.240	0.150
2224	62.0	30.0	25.0	32.20	33.20	0.047	0.028
1134	48.0	27.0	29.0	27.48	60.00	3.340	1.420
1134	75.0	32.0	35.0	52.38	45.86	0.021	0.021

[a] Glu = glutaraldehyde; Gly = glyoxal.

Table 5. Effect of the degree of cross-linking in chitosan microspheres on loading, release, and diffusion coefficient (D) of centchroman[a]

Types of chitosan microspheres	Loading of centchroman (%)		Controlled release of centchroman (%)		$D_{\text{diffusion constant}}$ (10^{-12} cm^2 s^{-1})	
Degree of cross linking (% w/w)	Glu	Gly	Glu	Gly	Glu	Gly
0	15.0	15.0	32.67	32.67	15.79	15.790
2	25.0	30.0	48.00	51.67	8.08	5.380
4	32.5	40.5	65.38	77.78	1.08	0.150
6	37.5	35.0	70.40	57.14	0.24	0.096
12	30.0	28.0	32.40	30.71	0.07	0.022

[a] Glu = glutaraldehyde; Gly = glyoxal.

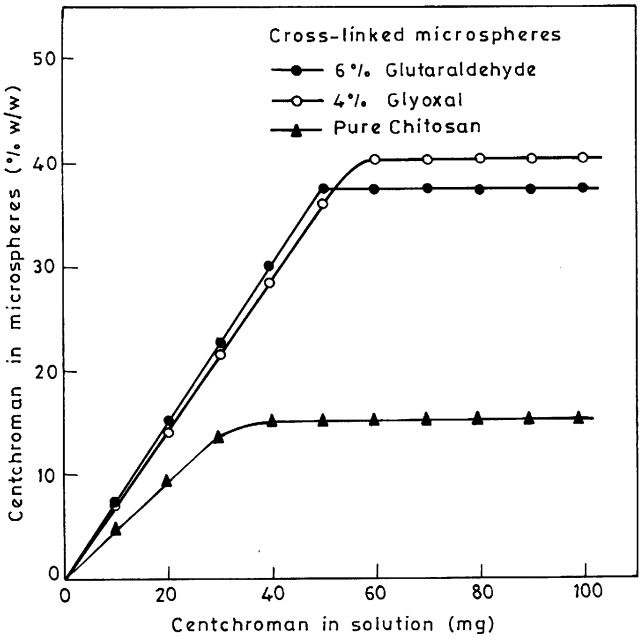

Figure 6. Loading of centchroman on pure chitosan, glutaraldehyde (6% w/w), and glyoxal (4% w/w) cross-linked chitosan microspheres, $\overline{M}_v$ (1134 kg mol^{-1}), DDA (62% w/w), loading time 48 h, load media: 100 mg of microspheres in 20 mL of a buffered solution of centchroman, pH 5 (glutaraldehyde) and pH 4 (glyoxal), at 25 °C.

in glyoxal cross-linked microspheres (253.6 kg mol^{-1}) in comparison with glutaraldehyde cross-linked microspheres (216.32 kg mol^{-1}). Hence the overall loading with glutaraldehyde cross-linked microspheres was more (37.5% w/w) than that observed with glyoxal cross-linked microspheres (28.0% w/w). A similar trend was shown by microspheres prepared with HMW chitosan (Table 4); hence, variation in loading capacity of microspheres for centchroman could be explained on similar criteria. But the trend of centchroman loading in microspheres prepared with MMW chitosan (1134 kg mol^{-1}) was found to be different from that with LMW and HMW chitosan microspheres cross-linked with glutaraldehyde and glyoxal (Table 4 and Fig. 6). The high drug loading capacity of glyoxal cross-linked microspheres was attributed to the optimum size of the cross-links (244 kg mol^{-1}) and the degree of swelling (200%) in comparison with glutaraldehyde cross-linked microspheres (Tables 3 and 5). The DDA in chitosan microspheres has also shown variations on loading capacity for centchroman (Table 4). The glyoxal cross-linked chitosan microspheres (DDA, 48% and 75% w/w) have shown high centchroman loading capacity in comparison with glutaraldehyde cross-linked chitosan microspheres with the same DDA (Table 4). The variation in loading trend for centchroman in chitosan microspheres versus DDA and MWs (Table 4) could be explained due to substantial variations in the chemical properties of the prepared microspheres. Microspheres prepared with MDDA chitosan (DDA, 62% w/w) have shown maximum loading for centchroman in the presence of both glyoxal and glutaraldehyde cross-linkers (Table 4).

The effect of the degree of cross-linking on centchroman loading capacity of microspheres was also studied by varying the concentration of glyoxal and glutaraldehyde in the microspheres (Table 5). The loading of centchroman was increased up to a cross-linking of 6%

(w/w) with glutaraldehyde and up to 4% (w/w) cross-linking with glyoxal (Table 5), and then a decreasing trend was found in both cross-linkers. The initial increasing trend in the centchroman loading capacity of microspheres with the increase in concentration of cross-linkers was attributed to the increase in MW of cross-links (M_c) and degree of hydrophobicity, which substantially increased drug retention capacity in both types of microspheres (Tables 2 and 5). The microspheres with 4% (w/w) glyoxal have shown optimum loading of 40.5% (w/w), whereas the glutaraldehyde cross-linked microspheres have shown a maximum loading of 37.5% (w/w) at 6% (w/w) degree of cross-linking (Table 5).

3.7. Effect of molecular weight (MW), degree of deacetylation (DDA), and cross-linking on the release of centchroman

The release characteristic of microspheres was studied using 100 mg of centchroman-loaded microspheres in 20 mL of phosphate-buffered solution (pH 7). The non-cross-linked chitosan microspheres showed a burst release of 67.3% w/w of loaded centchroman within a period of 30 h (Fig. 7), and the remaining drug (32.6% w/w) was released in controlled manner within a period of 10 h. The release behavior of glutaraldehyde and glyoxal cross-linked microspheres was compared using glutaraldehyde (6% w/w) and glyoxal (4% w/w) cross-linked microspheres obtained from chitosan with constant MW (1334 kg mol^{-1}) and DDA (62% w/w). The glutaraldehyde cross-linked microspheres released 29.60% (w/w) of loaded centchroman in a burst release manner within an initial period of 40 h (Fig. 7), and the remaining 70.40% (w/w) centchroman was released in a controlled manner in the next 60 h of drug release (Table 5 and Fig. 7), which clearly indicates that cross-linking in chitosan microspheres has modified the drug-release time, and a substantial amount of loaded centchroman (70.40% w/w) was released in a controlled manner (Table 5 and Fig. 7). To compare the release pattern of glutaraldehyde with glyoxal, the release pattern of glyoxal cross-linked (4% w/w) microspheres has been analyzed. The glyoxal cross-linked microspheres released a substantial amount of drug (77.78% w/w) in a controlled manner within a period of 70 h, and the remaining 22.22% of loaded drug was burst released within a period of 30 h (Fig. 7). The comparison of the release pattern of pure chitosan with that of cross-linked chitosan microspheres has made it clear that loaded drug from microspheres is released in two steps, and the amount of drug released in these steps shows a variation on varying the degree of cross-linking and the type of cross-linkers. The glyoxal cross-linked microspheres showed significant improvement on the controlled step of drug release (Table 5 and Fig. 7). The effect of the MW of chitosan on drug-release behavior was studied using glutaraldehyde (6% w/w) and glyoxal (4% w/w) cross-linked microspheres (Table 4). The glutaraldehyde cross-linked microspheres with LMW chitosan (760 kg mol^{-1}) have shown a burst release of 49.90% of loaded centchroman within a period of 30 h, and 32% (w/w) was released in controlled manner. On the other hand, glyoxal cross-linked microspheres with LMW chitosan released 40.0% (w/w) loaded centchroman in a burst release manner within a initial period of 30 h, and the remaining 60% w/w was released in controlled manner (Table 4). But on increasing the MW of chitosan (MMW, 1134 kg mol^{-1}), the burst release of centchroman became 29.60% (w/w) and 22.22% (w/w) in glutaraldehyde and glyoxal cross-linked microspheres, respectively (Table 5), and controlled release of centchroman was increased to 70.40% (w/w) and 77.78% (w/w) from these microspheres (Table 4). On further increasing the MW of chitosan (HMW, 2224 kg mol^{-1}), the amount of centchroman released in a controlled manner was decreased. The HMW chitosan microspheres cross-linked with glutaraldehyde showed a 32% (w/w) drug release in a controlled manner, whereas glyoxal cross-linked microspheres showed a 33.20% (w/w) drug release in a controlled manner (Table 4). These observations clearly indicate that the variation in the

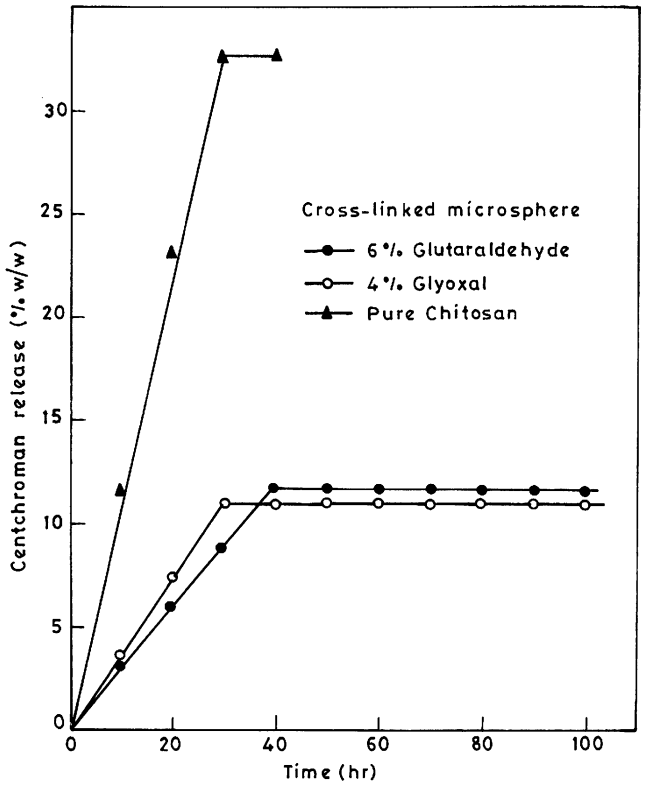

Figure 7. Centchroman release from pure chitosan and cross-linked chitosan microspheres. $\overline{M}_v$ (1134 kg mol^{-1}), DDA (62% w/w), release media: 100 mg drug-loaded microspheres in 20 mg of a buffered solution, pH 7, at 25 °C.

drug-release pattern in microspheres prepared with different MWs of chitosan is due to the variation in the MW of cross-links (M_c) that controlled the diffusion constant (D) of centchroman from the microspheres (Table 4). On varying the MW of chitosan, the hydrophobicity of the microspheres was constant (Table 3), but the DDA and cross-linking changed the degree of hydrophobicity significantly (Tables 2 and 3), both of which controlled the release pattern of centchroman from these microspheres (Tables 4 and 5). The release behavior of chitosan microspheres with constant MW (1134 kg mol^{-1}) and different DDAs was also studied (Table 4). On increasing the DDA from 48% to 62% (w/w), the burst release of centchroman in glutaraldehyde and glyoxal cross-linked microspheres decreased, and the controlled release of centchroman was increased (Table 4). However, on increasing the DDA from 62% to 75% (w/w), the amount of centchroman released in a controlled manner decreased with both cross-linkers, but glutaraldehyde cross-linked microspheres have shown better controlled release (52.38% w/w) than glyoxal cross-linked microspheres (45.86% w/w) with the same DDA (Table 4). The variation in DDA has not only affected the cross-linked MW (M_c), but the degree of hydrophobicity that controls the drug-release behavior of the microspheres was also influenced significantly (Table 3). The release characteristic of chitosan microspheres with constant MW (1134 kg mol^{-1}) and DDA (62% w/w) and different amounts of glutaraldehyde and glyoxal was evaluated (Table 5). On increasing the degree of cross-linking in the microspheres, the controlled release of centchroman was improved up to 6% (w/w) of glutaraldehyde and 4% (w/w) of glyoxal, but on further increasing the concentration of these cross-linkers, the centchroman released in a burst step was increased (Table 5). The variation in degree of cross-linking has varied the MW of cross-links (M_c) and degree of hydrophobicity in the microspheres (Table 2). From these experimental variations, the characteristics of optimized microspheres are shown in Table 6, which clearly indicated that glyoxal cross-linked microspheres have shown better controlled release of centchroman than the glutaraldehyde cross-linked microspheres.

To ascertain the release kinetics and mechanism of centchroman from cross-linked microspheres, the pattern of fractional release of centchroman (M_t/M_∞) was analyzed by plotting a graph between fractional releases of centchroman (M_t/M_∞) versus the square root of release time ($\sqrt{t}$) for microspheres prepared under different experimental conditions.[51] The fractional release of centchroman (M_t/M_∞) initially increased rapidly with chitosan microspheres, but glyoxal and glutaraldehyde cross-linked microspheres initially showed a slow fractional release of centchroman (Fig. 8). The fractional release of centchroman was constant after 30 h from chitosan and glyoxal cross-linked microspheres, and after 40 h with glutaraldehyde cross-linked microspheres. The time of controlled release of centchroman was maximum with glyoxal cross-linked microspheres (70 h) and minimum (10 h) with pure chitosan microspheres (Fig. 8). The initial release of centchroman from these microspheres was Fickian with regard to dif-

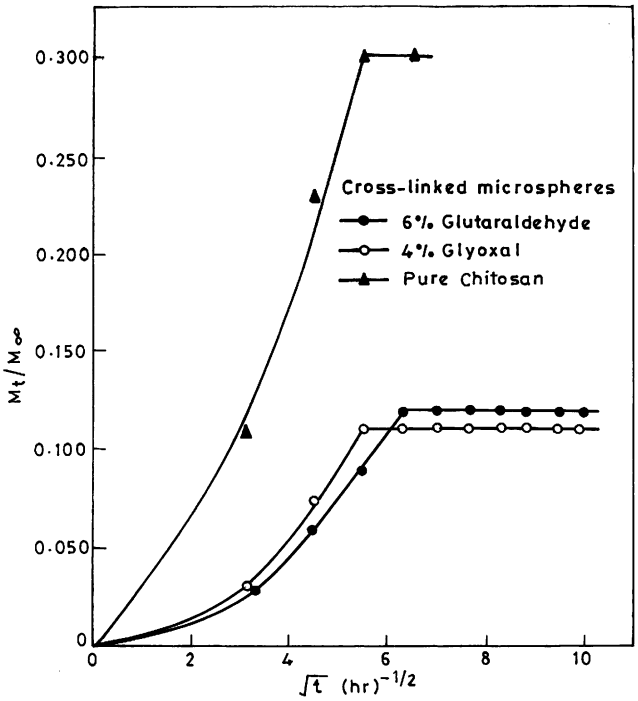

Figure 8. Fractional release of centchroman from pure chitosan, glutaraldehyde (6% w/w), and glyoxal (4% w/w) cross-linked chitosan microspheres. $\overline{M}_v$ (1134 kg mol^{-1}), DDA (62% w/w), release media: 100 mg centchroman-loaded microspheres in 20 mL of a buffered solution, pH 7, at 25 °C.

Table 6. Physical characteristics of optimized chitosan cross-linked microspheres[a]

Types of microspheres[b]	Size (μm)	Shape factor (S)	Specific surface area (μm^2/g) × 10^{-8}	No. of microspheres (per g)	M_c (kg mol^{-1})	Dye[c] adsorbed (mL μm^{-2})	Swelling (% S_w)
CH	119	0.922	5.50	14590	1006.0	0.029	300
Glu-CH	39.8	0.832	4.60	92000	184.6	0.166	250
Gly-CH	31.6	0.795	4.10	136670	276.3	0.186	200

[a] $\overline{M}_v$ of chitosan (CH) 1134 kg mol^{-1}; DDA (CH) 62% w/w, Glu-CH (6% glutaraldehyde), Gly-CH (4% w/w glyoxal).
[b] CH = chitosan; Glu-CH = glutaraldehyde cross-linked chitosan; Gly-CH = glyoxal cross-linked chitosan.
[c] Rose Bengal (hydrophobicity).

fusion constant (n) of 0.5 and became non-Fickian in the later stage of drug release, which was attributed to structural variation in microspheres on swelling and dissolution of chitosan. The fractional release of centchroman (M_t/M_∞) from chitosan microspheres with different MWs, DDA, and cross-linking was also evaluated using drug-release data and analyzed for release pattern (Fig. 8) and diffusion constants (Tables 4 and 5). The centchroman release from these microspheres initially followed first-order kinetics, but after equilibrium swelling, the drug release showed zero-order kinetics.[52] The initial slope of the fractional plot (Fig. 8) was used to calculate the diffusion constant (D) of centchroman from microspheres prepared using chitosan with different degrees of MW and DDA and also with different concentrations of cross-linkers (Tables 4 and 5). The diffusion constant (D) for centchroman in glyoxal cross-linked microspheres was found to be low in comparison with pure chitosan and glutaraldehyde cross-linked microspheres.

4. Conclusions

The effects of chitosan MW, DDA, and cross-linking in microspheres were studied, which indicate that chitosan with medium MW (1134 kg mol^{-1}) and 62% (w/w) DDA is useful to produce microspheres with improved controlled release characteristics for centchroman at 6% (w/w) degree of cross-linking with glutaraldehyde and 4% (w/w) degree of cross-linking with glyoxal. The microspheres prepared with glyoxal were more hydrophobic, non-spherical, and smaller in size due to the high degree of cross-linking. These microspheres increased the controlled release period for centchroman in comparison with chitosan and glutaraldehyde cross-linked chitosan microspheres.

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