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Effects of degree of deacetylation and cross-linking on physical characteristics, swelling and release behavior of chitosan microspheres

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Abstract

The effect of N-deacetylation in chitosan was studied by preparing chitosan samples with 48%, 62%, and 75% degree of deacetylation (DDA). The degree of deacetylation in prepared samples of chitosan was estimated by infrared and potentiometric methods of analysis. The degree of deacetylation was further verified by a method based on elemental analysis. The degree of deacetylation obtained by these methods was found to be almost the same, hence providing support that the methods used in determination of degree of deacetylation in chitosan samples are complementary. The degree of cross-linking, swelling and controlled release characteristics of microspheres showed dependence on degree of deacetylation and cross-linking in chitosan. The microspheres prepared with 62% degree of deacetylation and 6% (w/w) degree of cross-linking show an optimum degree of swelling, releasing 70% (w/w) of loaded centchroman in a controlled manner within a sustained period of 60 h. Varying the concentration of glutaraldehyde changed the degree of cross-linking in microspheres. The degree of deacetylation and cross-linking has controlled the loading and release profile of centchroman from prepared microspheres. The centchroman from microspheres was released in two stages. In the first stage, the centchroman was released in rapidly, in a burst, while in second stage the centchroman was released in a controlled manner. The centchroman release was diffusion controlled with zero order kinetics.
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Keywords: Chitosan; Degree of deacetylation; Glutaraldehyde; Centchroman; Cross-linked density

1. Introduction

Chitosan is a potentially useful polysaccharide, which is obtained by deacetylation of naturally occurring chitin derived from the wall of lower plants and skeletal of arthropods and mollusks (Jeuniaux, Voss-Foucart, Poulicek, & Bussers, 1989). Commercial grade chitin has been deacetylated in the presence of alkali (Chang, Tsai, Lee, & Fu, 1997), or enzyme (Arake & Ito, 1975) in a controlled manner, producing chitosan with different degree of deacetylation and physicochemical characterisitics (Errington, Harding, Varum, & Illum, 1993; Varum, Ottoy, & Smidsrod, 2001). Hence, efforts were made to evaluate various molecular parameters of chitosan before using it for specific applications (Mao, Troung-le, & Jans, 2001; Tarsi, Corbin, Pruzzo, & Muzzarelli, 1998). The non-toxic (Apsden, Mason, & Jones, 1997) and bio-adhesive nature of chitosan also supports its application as biomedical material (Mao et al., 2001) and carrier for drug delivery devices. But investigations have indicated that degree of deacetylation (Sang-Gyun et al., 2004) and molecular weight (Illum, Farraj, & Davis, 1994) of chitosan have significantly affected the role of chitosan in therapeutic and intelligent drug delivery systems (Thacharodi & Ruo, 1993). The degree of deacetylation also controls degree of crystallinity and hydrophobicity in

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chitosan due to variations in hydrophobic interactions. These hydrophobic interactions ultimately control the loading and release characteristics of chitosan matrices. The chitosan-based microspheres are able to encapsulate drugs in aqueous solution without using organic solvents (Vander Lubben et al., 2003). The degree of deacetylation of chitosan not only influences the properties of chitosan but also controls the degree of cross-linking in chitosan in the presence of any suitable cross-linker. The higher the degree of deacetylation in chitosan, the higher would be the degree of covalent cross-linking (Draget, 1996). The degree of deacetylation in chitosan has been determined by NMR (Hiral, Odani, & Nakajima, 1991), by the first derivative of UV-spectrophotometry (Tan, Khor, Tan, & Wong, 1998) and by X-ray diffraction (Zhang, Xue, Xue, Gao, & Zhang, 2005). But, infrared spectroscopy (Shigemasa, Matsuura, Sashiwa, & Saimoto, 1996), potentiometry (Broussignac, 1968) and elemental analysis (Kasaai, Arul, & Charlet, 2000) have been found to be easy and accurate in comparison to enzymatic (Nanjo, Katsumi, & Sakai, 1991), ninhydrin (Tien, Lacroix, Ispas-Szabo, & Mircea-Alexandru, 2003) and circular dichroism (Donald, 1987).

The preparation of microspheres involved cross-linking in chitosan with a molecule that has at least two reactive functional groups. The most common cross-linkers are glutaraldehyde (Aly, 1998) and glyoxal (Patel & Amiji, 1996). Although glutaraldehyde is known to be toxic, its fate in human body is not fully explained (Beauchamp, Stclair, Fenell, Clarke, & Morgan, 1992). The glutaraldehyde allows direct reaction in aqueous media under mild conditions without adding any reducer (Cheng et al., 1998). Naturally, occurring genipin (Mi, Tan, Liang, & Sung, 2003) and oxalic acid (Hirano, Yamaguchi, Fukui, & Iwata, 1990) have also been used as cross-linkers, but their biocompatibility in human systems has not yet been assessed completely.

The degree of cross-linking in microspheres was dependent on degree of deacetylation (Draget, 1996) but found to be independent of the molecular weight (Mi, Kuan, Shyu, Lee, & Chang, 2000) of chitosan. The concentration of glutaraldehyde (Donald, 1987; Mi et al., 2000; Arguelles-monal, Gaycoolea, Peniche, & Higuera-Ciapra, 1998) and reaction temperature (Mi et al., 2000) have also controlled the degree of cross-linking in chitosan microspheres. Thus, in the present investigation, an attempt was made to prepare glutaraldehyde cross-linked chitosan microspheres with different degree of deacetylation and cross-linked density. This was achieved by taking different concentrations of glutaraldehyde and using chitosan with different degree of deacetylation, thus controlling the cross-linked density in microspheres. The degree of deacetylation was estimated by different methods. Finally, prepared microspheres were characterized for controlled release parameters for centchroman.

2. Experimental

2.1. Chemicals used

The chitosan sample (α-chitosan, 48% DDA) was obtained from Sigma–Aldrich Chemical Company, USA and was purified by dissolving in 2% (w/w) acetic acid and passing through a filter under pressure to remove undissolved fraction of chitosan. The filtrate was subsequently precipitated in 1.0 M NaOH solution and dried at 30 °C under vacuum. The glutaraldehyde (25% w/w) solution, HCl, NaOH, KBr, acetic acid, and Rose Bengal were analytical grades chemicals (Loba Chemie, India) and used without further purification. The centchroman sample was received as gift sample from Torrin Pharmaceuticals Ltd., Ahmedabad, India and used after purification and crystallization.

2.2. Molecular weight determination of chitosan

The molecular weight of chitosan samples was calculated with the following equation (Khan, Peh, & C'ing, 2000) using intrinsic viscosity (η) of chitosan and chitosan–acetic acid interaction parameters k (1.81×10^{-3} cm^3 g^{-1}) and α (0.93) at 25 °C.

$$[\eta]_{25\,^\circ\mathrm{C}} = kM_v^{\alpha} \text{ (acetic acid)}. \quad (1)$$

2.3. Deacetylation of chitosan

To obtain chitosan samples with different degree of deacetylation, 5.0 g chitosan sample with 48% degree of deacetylation was heated with 50 ml of 40% (w/w) solution of sodium hydroxide at 80 °C for 4 h under reflux (Takanori, Keisuke, & Yoshio, 1976). Similarly, in a second set of experiments, the sample was refluxed for 8 h to obtain chitosan with higher degree of deacetylation. Finally, the alkali treated chitosan was removed and washed with hot and cold water to remove impurities.

2.4. Determination of degree of deacetylation

The chitosan samples obtained after treatment with alkali (40% w/w) were characterized for degree of deacetylation by different methods. To determine the degree of deacetylation by potentiometric titration (Broussignac, 1968), 0.025 g chitosan sample was dissolved in 25 ml of 1.75×10^{-3} N HCl and excess HCl was back titrated with 1.80×10^{-2} N NaOH solution using pH meter (46 CL, Toshniwal, India). The differential and integral titration curves were drawn between solution pH and volume of alkali added, which produced an integral curve with two inflexions. The differential volume (ΔV) of alkali between first and second neutralization point corresponds to the acid consumed by amino groups present in the chitosan. The degree of deacetylation has been calculated using following equation

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

where, m is weight of chitosan sample and N is strength of NaOH used in titration.

The degree of deacetylation in alkali treated samples of chitosan was also determined by an IR method (Takanori et al., 1976). In this method, a thin film of chitosan was cast on glass plate from a solution obtained by dissolving 0.5 g chitosan in 20 ml of 2% (w/w) acetic acid. The film thus obtained was washed with water to remove acetic acid and used to record an infrared spectrum via a Perkin-Elmer 1600 FT-IR Spectrophotometer. The degree of deacetylation was evaluated by recording absorbance at 1655 cm^{-1} for amide-I and at 3450 cm^{-1} for OH group in chitosan. The absorbance of chitosan was used to calculate the degree of deacetylation (DDA) using following equation (Khan et al., 2000)

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

where factor 1.33 represents the ratio of A_{1655}/A_{3450} for fully N-acetylated chitosan.

The degree of deacetylation in prepared samples of chitosan was further verified by elemental analysis using a Heraeus Carlo Ebra 1108 Elemental Analyzer. Considering structural units of chitosan, the following derived relations (Gupta & Jabrail, 2006) between weight percent of elements (carbon and nitrogen) and degree of deacetylation (DDA) have been used

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

$$\mathrm{DDA} = \left(\frac{1400}{364 \times W_N}\right) \times 100\%, \tag{5}$$

where W_c and W_N are the weight percent of carbon and nitrogen in the samples.

The degree of deacetylation determined by these relationships was further verified using following relationship given by Kasaai et al. (2000).

$$\mathrm{DDA} = \left(1 - \frac{(C/N) - 5.145}{6.816 - 5.145}\right) \times 100\%, \tag{6}$$

where, C/N is the percent ratio of carbon and nitrogen in chitosan sample. The degree of deacetylation determined by these methods has been finally compared and used to verify the methods applied for determining the degree of deacetylation in chitosan.

3. Preparation of cross-linked chitosan microspheres

The glutaraldehyde cross-linked chitosan microspheres were prepared using chitosan of different degree of deacetylation and taking different concentrations of glutaraldehyde. To prepare microspheres, a calculated amount of chitosan (0.5 g) was dissolved in 200 ml of 2% (w/w) acetic acid under vigorous stirring for about 3 h at room temperature. The viscous solution of chitosan thus obtained was blown through a nozzle as fine droplets into a vessel containing 250 ml methanolic solution of NaOH (0.1 N) in which chitosan droplets were coacervated. After 30 min, the coacervated microspheres were separated by centrifuging the solution, and washing the settled microspheres with water removed the excess alkali from the microspheres. These microspheres were subsequently kept in 50 ml neutral solution of glutaraldehyde of known concentration (6% w/w). After 6 h, the microspheres were separated and vacuum dried at 30 °C after washing with hot and cold water. The microspheres using chitosan of different degree of deacetylation also prepared by similar method and microspheres with different degree of cross-linking were prepared taking different concentrations of glutaraldehyde in solution ranging from 2 to 12% (w/w).

3.1. Size and morphology of chitosan microspheres

To determine the effect of size and morphology of chitosan microspheres on loading and release characteristics of microspheres, the size of prepared microspheres was determined by Scanning Electron Microscope (SEM) after mounting microspheres on metal studs using double adhesive tapes and vacuum coating with gold. The size of microspheres varied because of varying the degree of cross-linking (Gupta & Jabrail, 2006), and degree of deacetylation; hence, SEM micrographs of microspheres with different degree of cross-linking and degree of deacetylation were obtained. To predict the surface characteristics of microspheres, the size parameters were used to calculate shape factor (S) using the following equation (Gonzalez-Rodriguez, Holgado, Sanchez-Lafuente, Rabasco, & Fini, 2002):

$$S = \frac{L^2}{4\pi A}, \tag{7}$$

where L is the perimeter and A the surface area of prepared microspheres. The higher the value of S above 0.80, the higher the surface roughness.

3.2. Degree of swelling (S_w) in cross-linked chitosan microspheres

The degree of swelling of prepared microspheres with different degree of deacetylation and cross-linking was determined by keeping 100 mg microspheres in 20 ml phosphate buffer solution (pH 7) and recording variations in their weight (W_t) in comparison to their initial weight (W_o). The percent degree of swelling is calculated using following equation as reported (Gupta & Ravikumar, 2000)

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

where W_t and W_o are the weights of microspheres at time t and at zero time of swelling in microspheres, respectively.

3.3. Surface hydrophobicity of the prepared microspheres

The glutaraldehyde cross-linking in chitosan microspheres produces surface hydrophobicity, which has been estimated by determining the amount of hydrophobic dye (Rose Bengal) adsorbed per unit area of the microspheres. To determine the hydrophobicity, a fixed amount of microspheres (0.1 g) of different size were kept separately in different vessels containing 10 ml Rose Bengal (0.1 M) for 2 h, and the amount of Rose Bengal adsorbed was determined by recording the absorbance ($\lambda_{max} = 549$ nm) of the solution after separating the microspheres. The partition quotient of the amount adsorbed to the initial amount of Rose Bengal taken was plotted against the size of the microspheres and the slope of resultant plot was used as a measure of degree of hydrophobicity of the microspheres (William, Barron, Maria, & Remunan-Lopez, 1998).

3.4. Loading of centchroman on microspheres

The loading of centchroman on prepared microspheres was carried out by keeping 100 mg chitosan microspheres in 20 ml phosphate buffer solution (pH 5) containing a known amount of centchroman. The amount of centchroman in 20 ml loading solution was varied from 10 to 100 mg and loading time for centchroman was kept at 48 h. The amount of centchroman loaded on microspheres was determined by recording the absorbance of the loading solution at $\lambda_{max} = 275$ nm (after removing microspheres by filtration) using a Shimandzu UV-VIS-1601 PC spectrophotometer.

3.5. Release and diffusion coefficient of centchroman from chitosan microspheres

To investigate the drug release behavior of prepared microspheres at 25 °C, microspheres loaded with 100 mg centchroman were kept in 20 ml phosphate buffer solution (pH 7). The amount of centchroman released in solution at different time intervals was estimated recording absorbance at λ_{max}=275 nm of filtered release medium (1 ml) with replacement using Shimandzu UV-VIS-1601 PC spectrophotometer. The diffusion constant (D) of drug was determined from the initial slope of the curve drawn between M_t/M_α vs square root of release time (t), in which M_α and M_t was taken as the amount of drug released at infinite time and at time t from the microspheres.

4. Results and discussion

Although solubility of chitosan is influenced by its molecular weight, the extent of deacetylation in chitosan has also influenced its solubility and reactivity in much greater way, especially relative to pure chitin and cellulose. The degree of deacetylation increases the number of free amino groups, which modify hydrophobic interactions with water and helps in controlling the degree of cross-linking in the presence of any type of cross-linkers. The molecular weight of untreated and sodium hydroxide treated chitosan was determined viscometrically using Eq. (1), which was found to be 1134 kg mol^{-1}. Since the molecular weight of alkali treated chitosan was found to be same as that of untreated chitosan, hence giving an indication that no degradation of chitosan has taken place during deacetylation of original sample on treatment with alkali.

4.1. Degree of deacetylation

The original sample of chitosan ($\overline{M}_V = 1134$ kg mol^{-1}) with 48% (w/w) degree of deacetylation (L DDA) exhibited low solubility in acetic acid (2% w/w) and other mineral acids like hydrochloric acid (Hizaji & Amiji, 2003). However, alkali treated samples were slightly more soluble in acetic acid, which indicated that these samples possess more amino groups than untreated chitosan. The variation in degree of deacetylation in these samples has been determined by applying different methods of analysis as given below.

4.2. Potentiometric method

The degree of deacetylation in the original sample (L DDA) and samples treated with alkali for 4 h (M DDA) and 8 h (H DDA) was determined potentiometrically using Eq. (2). The differential volume (ΔV) between two neutralization points (Fig. 1) corresponds to the amount of amino groups present in the chitosan (Broussignac, 1968). The degree of deacetylation in the original sample was found

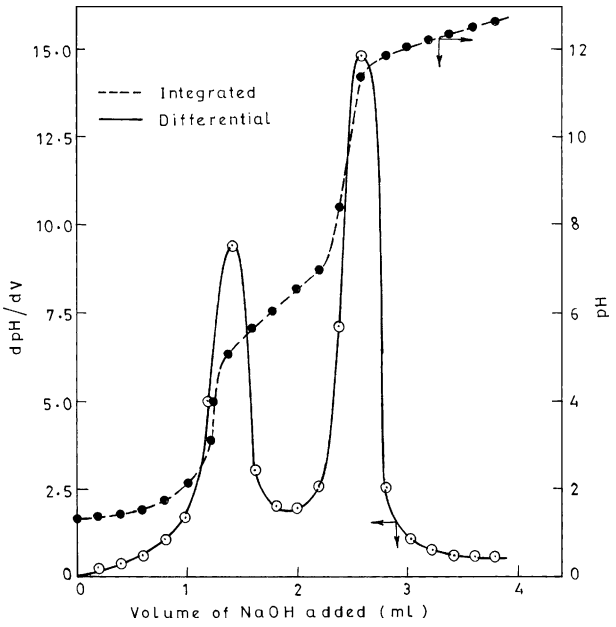

Fig. 1. Potentiometric determination of degree of deacetylation in chitosan. [Chitosan] = 0.025 g in 25 ml HCl of 1.75×10^{-3} N, [NaOH] = 1.75×10^{-2} N. $\overline{M}_V$ (Chitosan) = 1134 kg mol^{-1}.

to be 48% (w/w) and in samples treated for 4 and 8 h, the degree of deacetylation was 61.8% and 74.6%, respectively. Thus, on the bases of degree of deacetylation, these chitosan samples have been categorized as low (L DDA), medium (M DDA) and high degree of deacetylation (H DDA) as shown in Table 2. These results have indicated that treatment of chitosan with alkali has influenced the degree of deacetylation. Since reaction temperature (Mi et al., 2000) affects the rate of deacetylation, deacetylation in chitosan samples was carried out at fixed temperature (80 °C).

4.3. Infrared method

The degree of deacetylation in chitosan samples was also determined by an infrared technique using spectra recorded for all of the chitosan samples applied in the potentiometric titration method. The ratio of absorbance of amide-I at 1655 cm^{-1} to that of hydroxyl group at 3450 cm^{-1} in chitosan (Fig. 2) depends upon the degree of deacetylation in the chitosan (Oyrton, Monteiro, & Claudio, 1999) Eq. (3). The infrared spectra of all three samples of chitosan are recorded in transmittance mode. The transmittance for amide-I group ($\lambda = 1655$ cm^{-1}) in chitosan has shown an increasing trend from samples-1 to 3. The chitosan with low, medium and high degree of deacetylation has given percent transmittance (%T) as 31.1, 40.22, and 46.50, respectively, which on fitting in Eq. (3) has given degree of deacetylation as 48%, 62%, and 75% (w/w), respectively (Table 2). The degree of deacetylation calculated by infrared method was found to be very close to the values obtained by potentiometric titration method (Table 2).

4.4. Elemental analysis

The degree of deacetylation in chitosan samples has been determined further by elemental method Gupta and Jabrail, 2006 using Eqs. (4) and (5) (Table 2). The weight percent of carbon and nitrogen (Table 1) was used to calculate the degree of deacetylation using Eqs. (4) and (5), which was found to be in close agreement with the values obtained by the IR method (Table 2). The calculated degree of deacetylation was found to be very close to the values as obtained by potentiometric and infrared methods of analysis (Table 2). To verify the degree of deacetylation determined using Eqs. (4) and (5), the data were again fitted in Eq. (6) as suggested by Kasaai et al. (2000), which gives degree of deacetylation as 47.57%, 61.92%, and 74.73% (w/w). These values are similar to the values determined by Eqs. (4) and (5) as determined by potentiometric and infrared methods of analysis (Table 2). These investigations for determination of degree of deacetylation have clearly verified the methods used for determination of degree of deacetylation in chitosan samples. The degree of deacetylation in these chitosan samples was thus determined has been taken as 48%, 62%, and 75% (w/w) for further investigations, and categorized as L DDA (48% w/w), M DDA (62% w/w), and H DDA (75% w/w) chitosan (as shown in Table 2).

4.5. Cross-linked chitosan microspheres and their physical characteristics

The data given in Table 3 clearly indicated that the size of microspheres was decreased from 110.0 to 27.28 μm on

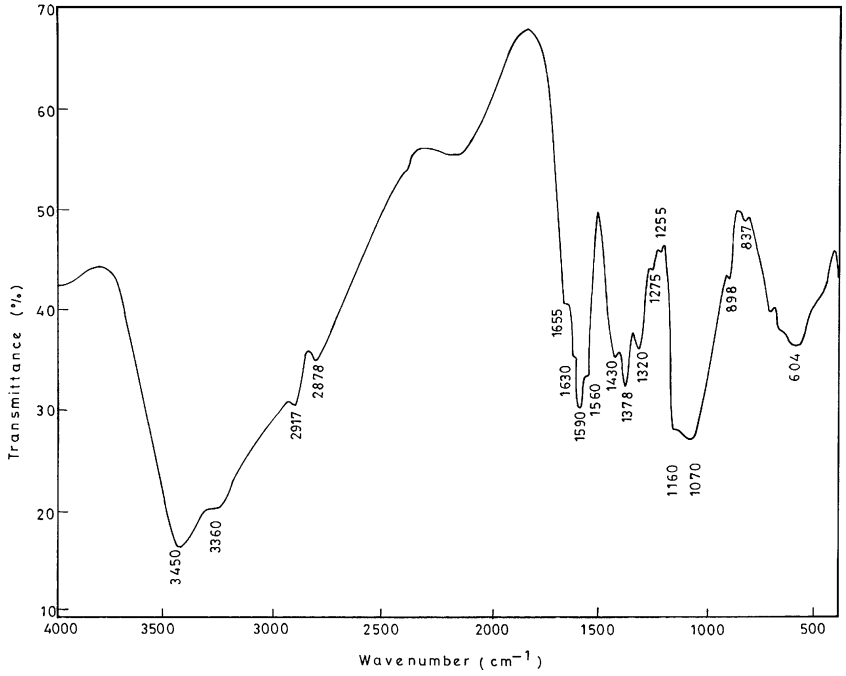

Fig. 2. FT-IR spectrum of chitosan for determining the degree of deacetylation using transmittance ratio of amide-I (1655 cm^{-1}) and hydroxyl group (3450 cm^{-1}).

Table 1
Elemental analysis of chitosan samples with different degree of deacetylation

Samples of chitosan	Weight percent of elements (Theoretical)			Weight percent of elements (Experimental)		
	N	C	H	N	C	H
Sample-1 (untreated)	8.01	48.35	6.89	8.03	48.36	6.90
Sample-2 (treated for 4 h)	6.20	35.94	5.21	6.22	35.96	5.22
Sample-3 (treated for 8 h)	5.12	28.57	4.21	5.13	28.56	4.22

Table 2
Degree of deacetylation in treated chitosan determined by different methods

Samples of chitosan	DDA potentiometrically Broussignac (1968) (%)	DDA IR method Oyrton et al. (1999) (%)	DDA elemental methods (%)	
			Mi et al. (2000)	Kasaai et al. (2000)
Sample-1 (L DDA) (untreated)	48.00	48.00	48.00 (47.90)[a]	47.57
Sample-2 (M DDA) (treated for 4 h)	61.80	62.00	61.98 (61.80)[a]	61.92
Sample-3 (HDDA) (treated for 8 h)	74.60	75.00	75.03 (74.97)[a]	74.73

[a] Values with Eq. (5).

Table 3
Physical characteristics of chitosan microspheres prepared with different degree of cross-linking and degree of deacetylation

Types of microspheres	DDA (%)	Cross- linking (%)	Size (ϕ) (μm)	Shape factor (S)	Hydrophobicity (dye adsorbed) (ml/μm^2)	S_w (%)	$D/10^{-11}$ cm^2 s^{-1}
1	48	0	112.0	0.915	0.037	295	1.490
2	62	0	119.0	0.922	0.029	300	1.579
3	75	0	132.0	0.926	0.026	322	1.620
3	62	2	110.0	0.918	0.031	290	0.808
4	62	4	76.74	0.872	0.038	270	0.104
5	62	6	39.78	0.832	0.166	250	0.024
6	62	12	27.28	0.714	0.220	150	0.007
7	48	6	76.14	0.869	0.080	282	0.334
8	75	6	18.75	0.687	0.195	213	0.003

increasing the degree of cross-linking from 2% to 12% (w/w). The non-cross-linked chitosan microspheres show a maximum size of 119.0 μm. The microspheres prepared 6% degree of cross-linking using chitosan with different degree of deacetylation also show variations in their size from 76.14 to 18.75 μm on varying the degree of deacetylation from 48% w/w (L DDA) to 75% w/w (H DDA) in the chitosan (Table 3).

The degree of cross-linking also has an effect on the morphology of the microspheres, as is clear from the SEM micrographs of microspheres prepared with pure chitosan (Fig. 3b) and cross-linked chitosan spheres (Fig. 3d). The microspheres prepared with pure chitosan were large in size (119 μm) with rough surface (Fig. 3a). With cross-linking, the size of microspheres became small (39.78 μm) and the surface became smooth (Fig. 3c). The degree of cross-linking and deacetylation has also shown an effect on surface roughness, as evidenced by a change in the shape factor (S) of the microspheres calculated using Eq. (6) (Table 3). The high value of surface factor (S), greater than 0.80, is an indication of surface roughness; whereas, S equal or lower than 0.80 is an indication of a smooth surface. The microspheres prepared with pure chitosan have shown a high value of shape factor (0.922) indicating a high degree of roughness (Fig. 3a). In comparison to microspheres prepared at 6% (w/w) degree of cross-linking result in a low value of shape factor (0.832), which is indicative of a smooth surface (Fig. 3c). The microspheres prepared with low degree of deacetylation (48% w/w DDA) have shown a rough surface ($S = 0.869$) in comparison to microspheres prepared with 75% DDA, which resulted in a low value of shape factor (0.687) at the same degree of cross-linking (6% w/w). The effect of the degree of deacetylation and cross-linking has also been observed on the hydrophobicity of the microspheres (William et al., 1998), as predicted on the basis of the value of the partition quotient (Q). Q is determined by the ratio of the volume of hydrophobic dye (Rose Bengal) adsorbed per unit area of the microspheres to the initial volume of Rose Bengal taken in the vessel. The volume of Rose Bengal adsorbed per unit area of the microspheres prepared with pure chitosan has been found to be low (0.029 ml μm^{-2}) in comparison to cross-linked microspheres (0.166 ml μm^{-2}) prepared at 6% (w/w) degree of cross-linking (Table 3 and Fig. 4). The hydrophobicity in microspheres has shown an increasing trend on increasing the degree of cross-linking. Although chitosan with high degree of deacetylation (75% w/w H DDA) is hydrophilic

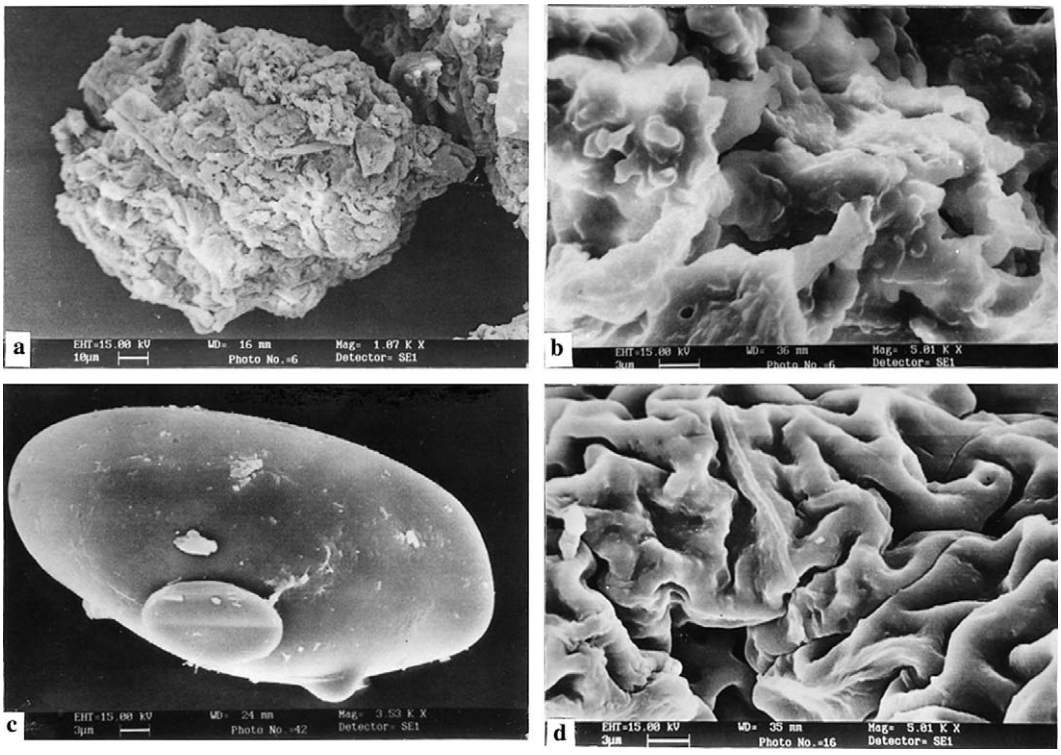

Fig. 3. Scanning electrons micrographs of pure (a and b) and cross-linked chitosan microspheres (c and d) for their size and morphology.

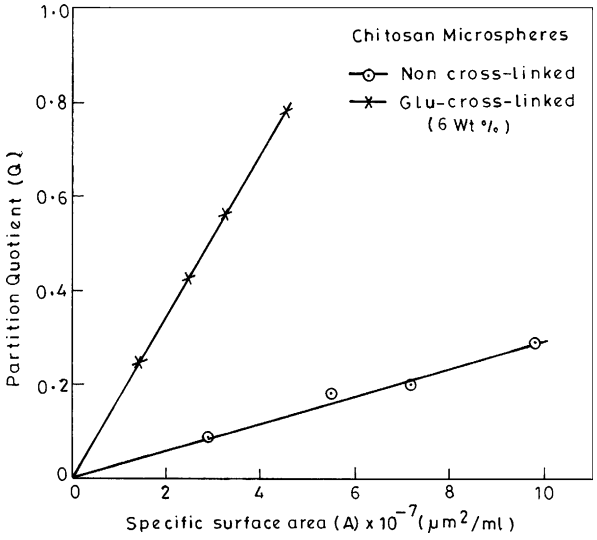

Fig. 4. Determination of hydrophobicity of pure chitosan and cross-linked chitosan microspheres by hydrophobic dye adsorption method.

(0.026 ml μm^{-2}) in comparison to chitosan with low degree (48% w/w) deacetylation (0.037 ml μm^{-2}) but order of hydrophobicity has reversed on cross-linking the chitosan with glutaraldehyde. The microspheres prepared with a high degree of deacetylation (75% w/w DDA) were more hydrophobic (0.195 ml μm^{-2}) than with a low degree of deacetylation (48% w/w DDA), which adsorbed a low volume of dye on microspheres (0.08 ml μm^{-2}); hence, these microspheres were more hydrophilic (Table 3). Thus, degree of cross-linking and deacetylation has shown significant effect on size, shape, morphology, and hydrophobicity of the microspheres. These characteristics of microspheres would influence, ultimately, the parameters that are important for loading and release of drug from the microspheres.

4.6. Degree of swelling (S_w) and diffusion coefficient (D)

The degree of swelling (S_w) in chitosan microspheres and diffusion coefficient (D) of drug in microspheres controlled the loading and release characteristics of prepared microspheres, hence these parameters were evaluated as given in Table 3. The microspheres prepared with pure chitosan reveal a maximum degree of swelling of 300% (Table 3) in comparison to microspheres prepared with different concentrations of glutaraldehyde, or with those from chitosan with different degrees of deacetylation. The maximum degree of swelling decreased to 150% on increasing the concentration of glutaraldehyde from 2 to 12% (w/w) in microspheres (Table 3). The optimized microspheres were prepared with a degree of cross-linking (6% w/w) and showed a maximum degree of swelling of 250% (w/w) using chitosan with 62% (w/w) degree of deacetylation (Table 3). However, microspheres from chitosan with 75% (w/w) of DDA resulted in the lowest degree of swelling (213% w/w) at the same degree of cross-linking (6% w/w), which was due to a high degree of cross-linking with available amino groups (Draget, 1996) in chitosan with 75%w/w DDA. The variation in degree of cross-linking with concentration of glutaraldehyde and degree of

deacetylation in chitosan has ultimately influenced the compactness of matrices and its hydrophobicity. This likely controlled the degree of swelling (S_w) and diffusivity of centchroman in these microspheres, as evidenced by the observed variations in the value of diffusion coefficient (D) determined as a function of degree of cross-linking and degree of deacetylation in chitosan microspheres (Table 3).

4.7. Loading of centchroman on chitosan microspheres

The experimental data on physical characteristics of cross-linked chitosan microspheres have clearly indicated that the degree of cross-linking and deacetylation have significantly influenced the physico-chemical properties of the microspheres, hence the loading of centchroman in the microspheres has been influenced significantly.

The loading of centchroman in microspheres was carried out keeping 100 mg microspheres in 20 ml phosphate buffered solution (pH 5) of centchroman for 48 h. To verify the effect of initial concentration of centchroman, the loading was carried by varying the concentration of centchroman from 10 mg to 100 mg in 20 ml loading solution. The microspheres prepared with pure chitosan (Table 4 and Fig. 5) resulted in a maximum loading (L_{max}) of 15 mg per 100 mg microspheres. At a 6% w/w degree of cross-linking, loading has increased to 37.5 mg per 100 mg microspheres. However, with a further increase in degree of cross-linking beyond 6% w/w, the loading of centchroman per 100 mg microspheres showed a decreasing trend (Table 4 and Fig. 5). At high degree of cross-linking (12% w/w), the extent of maximum loading of centchroman has decreased to 30 mg per 100 mg microspheres (Table 3, Fig. 5). This decreasing trend in maximum loading (L_{max}) for centchroman at high degree of cross-linking (12% w/w) was due to the decrease in pore size and the increase in hydrophobic character of the microspheres. This ultimately decreased the diffusivity of centchroman, as indicated from the diffusion constant (D) determined at high degree of cross-linking (12% w/w) in chitosan microspheres. The microspheres prepared at 12% (w/w) cross-linking have shown low degree of swelling (150% w/w), which is responsible for the decrease in loading capacity of microspheres. The microspheres prepared at 6% (w/w) degree of cross-linking have shown high loading capacity for centchroman (37.5 mg per 100 mg microsphere). The loading of centchroman on microspheres prepared with chitosan of different degree of deacetylation was also evaluated (Table 4 and Fig. 6). These results have clearly indicated that loading capacity of microspheres has shown a dependence on degree of deacetylation in chitosan. The maximum loading capacity for centchroman was increased on increasing the degree of deacetylation from 48% to 62% (w/w) (Table 4 and Fig. 6) but shown a decreasing trend on further increasing the degree of deacetylation beyond 62% (w/w). The initial increase in maximum loading capacity (L_{max}) for centchroman was due to the formation of optimum size cross-links in chitosan, which facilitated the penetration and retention of drug in chitosan microspheres but on further increasing the degree of deacetylation beyond 62% w/w, the size of cross-links in microspheres was decreased to a minimum, which ultimately has decreased pore size and hydrophilic character of microspheres. The decrease in pore size has decreased the loading capacity for centchroman (32.0 mg per 100 mg microspheres) due to the decrease in diffusivity

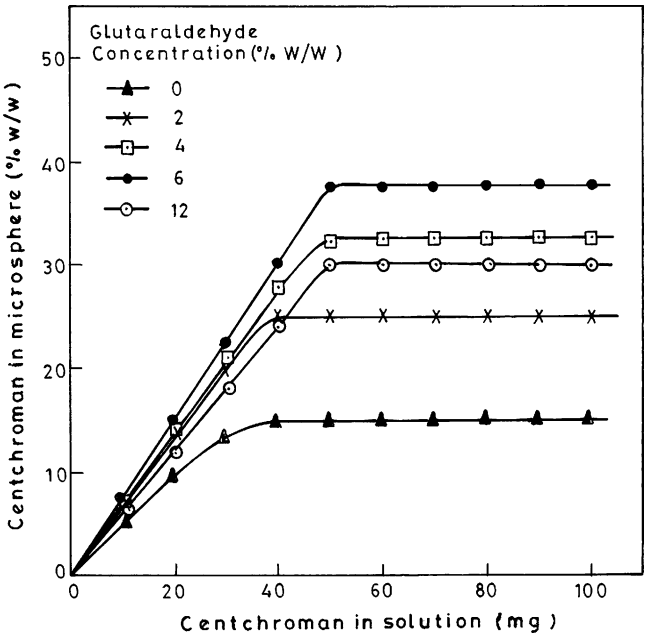

Fig. 5. Loading of centchroman on cross-linked microspheres prepared at different concentrations of glutaraldehyde. Degree of deacetylation = 62% w/w. $\overline{M}_V$ (Chitosan) = 1134 kg mol^{-1}. Loading time = 48 h, Loading media = 100 mg microspheres in 20 ml phosphate buffered solution of centchroman (pH 5), T = 25 °C.

Table 4
Loading and release characteristics of chitosan microspheres prepared with different degree of cross-linking and degree of deacetylation

Types of microspheres	DDA (%)	Cross- linking (%)	Loading L_{max} (mg)	Burst release (mg)	Controlled release (mg)	Controlled release time (h)
1	62	0	15.0	10.10	4.90	10
2	62	2	25.0	12.40	12.60	20
3	62	4	32.5	10.75	21.25	50
4	62	6	37.5	11.10	26.40	60
5	62	12	30.0	19.58	9.72	20
6	48	6	27.0	19.25	7.79	10
7	75	6	32.0	14.84	16.76	40

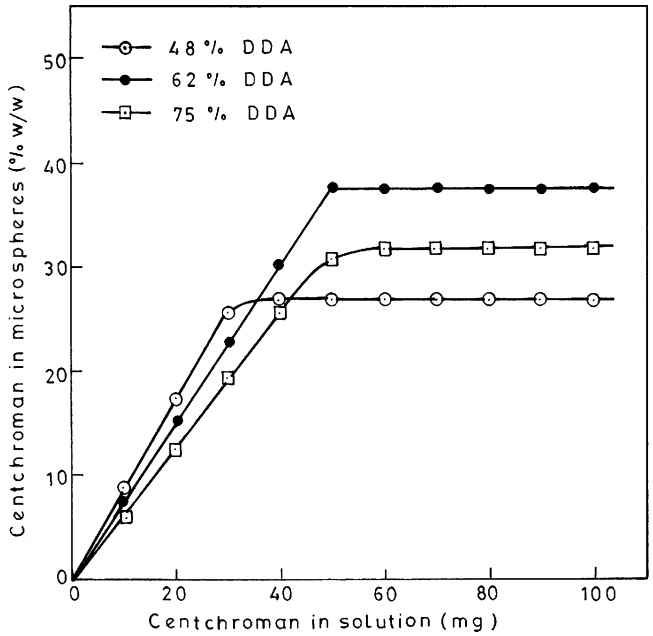

Fig. 6. Loading of centchroman on cross-linked microspheres prepared with chitosan of different degree of deacetylation. Degree of cross-linking = 6% w/w, Loading time = 48 h. Loading media = 100 mg microspheres in 20 ml phosphate buffered solution of centchroman, $\overline{M}_V$ (chitosan) = 1134 kg mol^{-1}, (pH 5), $T = 25$ °C.

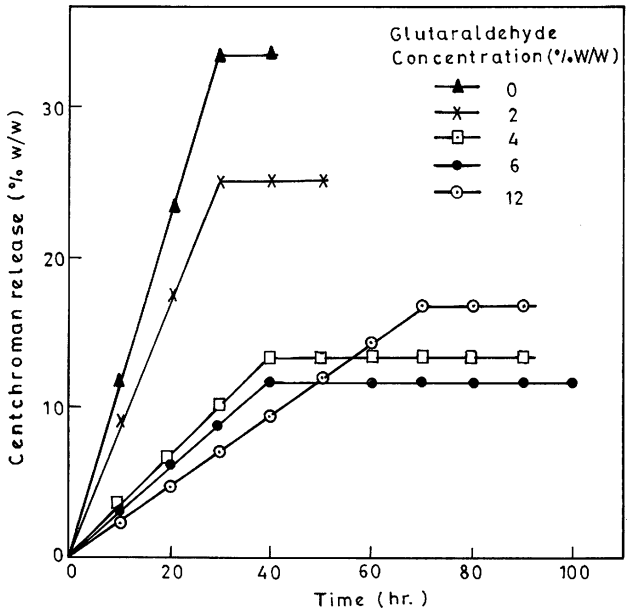

Fig. 7. Release of centchroman from cross-linked microspheres prepared at different concentrations of glutaraldehyde. Degree of deacetylation = 62% w/w $\overline{M}_V$ (Chitosan)=1134 kg mol^{-1}, release media = 100 mg drug loaded microspheres in 20 ml phosphate buffered solution (pH 7). $T = 25$ °C.

of the drugs in microspheres. This data is shown in Table 4 and Fig. 6 for the microspheres prepared with low degree of deacetylation (48% w/w DDA) have also shown low capacity for loading (27 mg per 10 mg microspheres). This trend was due to the low degree of cross-linking and formation of large size pores in the microspheres, which significantly decreased the retention capacity of microspheres (Table 4 and Fig. 6).

4.8. Release of centchroman from chitosan microspheres

The release profile of centchroman has indicated that the drug release was dependent on the degree of cross-linking in chitosan microspheres (Table 4 and Fig. 7). The total drug release from microspheres occurred in two steps. In the first step, the amount of centchroman released (during the first fixed time interval of 10 h) was variable, but in the second step of drug release, the amount of drug released within fixed time interval (10 h) was almost constant, as is clear from the trends shown in Table 4 and Fig. 7. The microspheres prepared with other variations have shown similar trends but variation in controlled and burst release time and amount of drug released in these steps was noticed. The microspheres prepared with pure chitosan have shown a burst-release of 67.36% (w/w) within first step of 30 h and 32% w/w of centchroman was released in controlled manner in second step of 10 h (Table 4 and Fig. 7) but on increasing the degree of cross-linking, the amount and time of drug release in these steps was changed significantly (Table 4 and Fig. 7). The microspheres prepared at 6% (w/w) degree of cross-linking have shown a burst-release of 29.6% (w/w) within a period 40 h and 70% of centchroman was released in controlled manner in second step of 60 h. But, on increasing the degree of cross-linking beyond 6% (w/w), the amount of drug released in burst manner was 65% (w/w) within a prolonged period of 70 h and small amount of centchroman (32.4% w/w) was released in controlled step of 20 h (Table 4 and Fig. 7). The data shown in Table 4 and Fig. 7 have given a clear indication that microspheres with 6% (w/w) degree of cross-linking were capable of releasing a substantial amount of loaded centchroman (70% w/w) in a controlled manner within a sustained period of 60 h. This is in clear contrast to microspheres prepared with pure chitosan or with low and high degree of cross-linking with glutaraldehyde. The analysis of drug release has indicated that, initially, the drug release has followed first order kinetics and followed by zero order kinetics (Bezemer et al., 2000) in a controlled drug release step.

The release characteristics of chitosan microspheres with different degree of deacetylation were evaluated by analyzing the release patterns of centchroman from these microspheres. The microspheres prepared with low degree of deacetylation (48% w/w DDA) have shown a burst release of 71% (w/w) within first release step of 40 h, with about 28.8% (w/w) centchroman released in a controlled manner within a period of 10 h (Table 4 and Fig. 8). The high burst-release (71% w/w) in these microspheres was due to a low degree of cross-linking in chitosan with 48% w/w DDA. On increasing the degree of deacetylation to 62% (w/w) (M DDA), the release pattern of centchroman was improved significantly. In these microspheres, 70% (w/w)

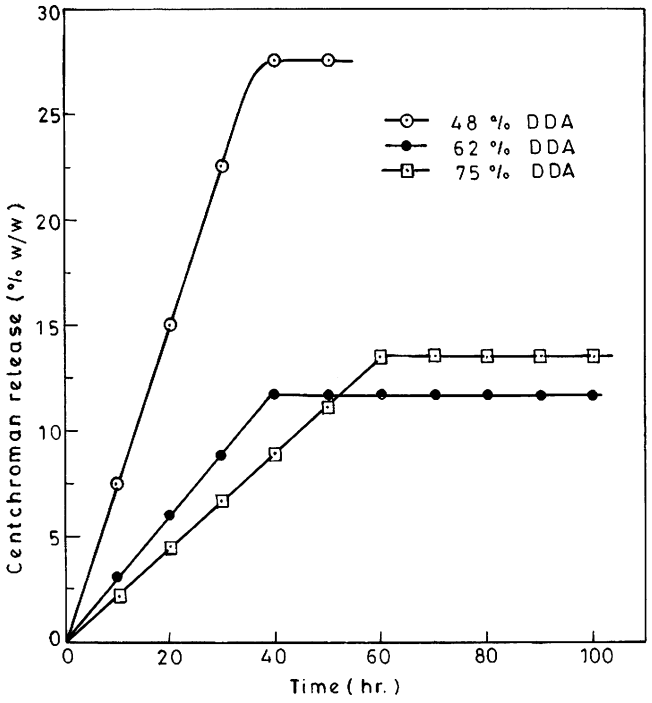

Fig. 8. Release of centchroman from cross-linked microspheres prepared with chitosan of different degree of deacetylation. (L DDA = 48% w/w, M DDA = 62% w/w, and H DDA 75% w/w), Degree of cross-linking = 6% w/w. Loading time = 48 h, Centchroman loaded per 100 mg microspheres = 27 mg (L DDA), 37.5 mg (M DDA), 32 mg (H DDA), $\overline{M}_V$ (chitosan) = 1134 kg mol^{-1}, Release media = 100 mg drug loaded microspheres in 20 ml phosphate buffered solution (pH 7), $T = 25$ °C.

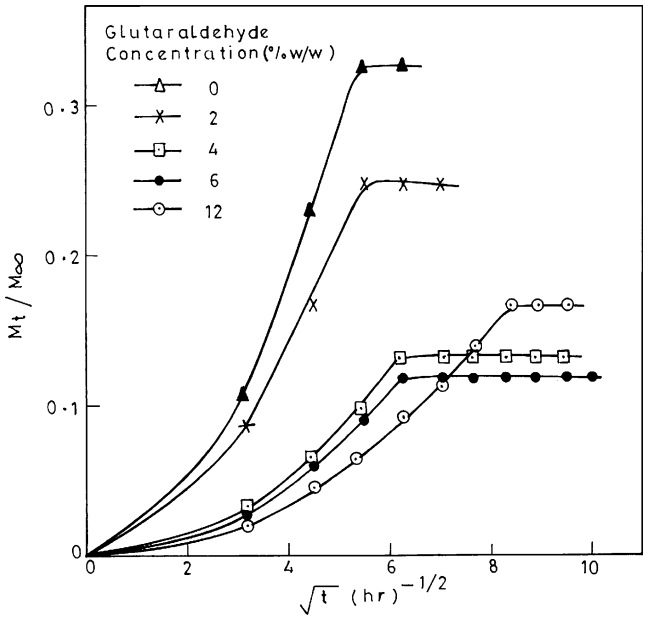

Fig. 9. Fractional release of centchroman from cross-linked microspheres prepared at different concentrations of glutaraldehyde. Degree of deacetylation = 62% w/w. $\overline{M}_V$ (Chitosan) = 1134 kg mol^{-1}. Centchroman loaded per 100 mg microspheres = 15 mg (0% Glu), 25 mg (2% Glu), 37.5 mg (6% Glu), and 30 mg (12% Glu). Release media = 100 mg drug loaded microspheres in 20 ml phosphate buffered solution (pH 7), $T = 25$ °C.

of drug was released in a controlled manner within a period of 60 h (Table 4 and Fig. 8). On increasing the degree of deacetylation beyond 62% (w/w), the release pattern of centchroman was changed significantly. In microspheres with 75% (w/w) of degree of deacetylation, 46% (w/w) of centchroman was burst released in first step of 60 h and 52.4% (w/w) of centchroman was released in controlled manner in second step of 40 h. These observations have clearly indicated that microspheres prepared using chitosan with 62%w/w of degree of deacetylation were suitable for sustained release of centchroman in comparison to microspheres prepared using chitosan with low (48% w/w DDA) and high degree of deacetylation (75% w/w DDA). To determine the mechanism of drug release from the microspheres prepared at different degree of cross-linking, plot was drawn (Fig. 9) between fractional releases of centchroman (M_t/M_α) vs under root of release time (t) using following power law equation (Kim, Bae, & Okano, 1992):

$$\frac{M_t}{M_\infty} = \left(\frac{16Dt}{\Pi r^2}\right)^n, \tag{9}$$

where D is diffusion constant, r is radius of chitosan microspheres and constant n is drug release mechanism dependant constant.

The data have shown a linear variation between M_t/M_α vs $\sqrt{t}$, which clearly indicated that drug released from these microspheres was Fickian-controlled. The value of constant (n) was 0.5. Li and Xu (Li & Xu, 2002) reported a similar mechanism for the release of drug from chitosan

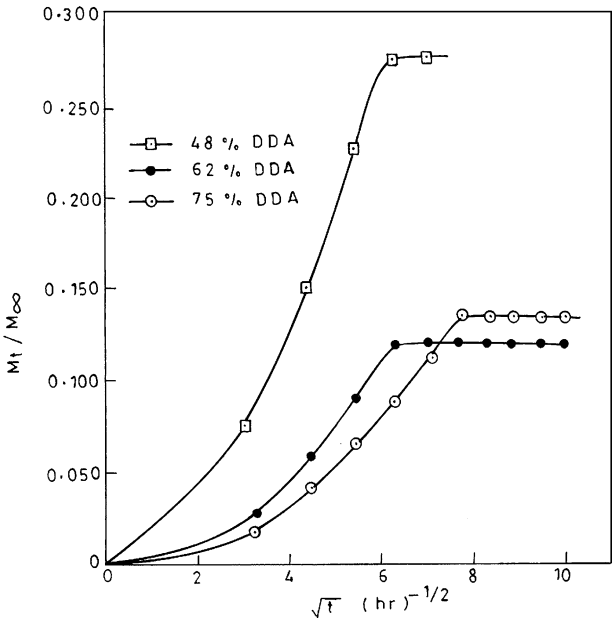

Fig. 10. Fractional Release of centchroman from cross-linked microspheres prepared with chitosan of different degree of deacetylation (L DDA = 48% w/w, MDDA = 62% w/w, and H DDA = 75% w/w), Degree of cross-linking = 6% w/w, Centchroman loaded per 100 mg microspheres = 27 mg (L DDA), 37.5 mg (M DDA), 32 mg (H DDA), $\overline{M}_V$ (chitosan) = 1134 kg mol^{-1}, Release media=10 0mg drug loaded microspheres in 20 ml phosphate buffered solution (pH 7), $T = 25$ °C.

gel. However, at later periods, the release mechanism of centchroman from microspheres became anomalous (Siepmann & Peppas, 2004) due to structural variations in microspheres on swelling and degradation. The mechanism of drug release from microspheres with different degree of deacetylation was also evaluated on the basis of trends shown by fractional releases of centchroman in the plot drawn (Fig. 10) between M_t/M_α vs $\sqrt{t}$, which supported a Fickian behavior. The trend of centchroman release from microspheres was linear in the beginning indicating a diffusion controlled mechanism of drug release but after structural variations in the microspheres, the release mechanism of centchroman became anomalous (Siepmann & Peppas, 2004) as found with microspheres obtained at different degree of cross-linking (Fig. 9). From the observed patterns of drug release from microspheres with different degree of deacetylation (Fig. 10, Table 4), it became clear that the release of centchroman has followed a first order kinetics in the beginning but became zero order after equilibrium degree of swelling and burst-release of centcroman from these microspheres.

5. Conclusion

Microspheres for controlled delivery of centchroman were prepared at different degree of cross-linking, using chitosan with different degree of deacetylation. The prepared microspheres were characterized for degree of swelling and surface morphology. The loading and release characteristics of microspheres were evaluated as a function of degree of cross-linking and deacetylation. The microspheres prepared with low degree of deacetylation (48% w/w DDA) showed high burst-release of centchroman (71% w/w) within a first step of drug release. About 28.8%w/w of centchroman was released in a controlled manner in second step of drug release of 10 h. However, microspheres prepared using chitosan with 62% (w/w) degree of deacetylation (M DDA) have shown significant improvements in releasing centchroman in controlled manner within a period of 60 h. In contrast, microspheres prepared using chitosan with 75% (w/w) DDA showed poor control characteristics. The effect of degree of cross-linking on controlled characteristics of prepared microspheres was studied successfully. The microspheres prepared at 6% (w/w) degree of cross-linking have shown better characteristics in comparison to those prepared with low and high degree of cross-linking. These investigations have clearly suggested that loading and release characteristics of chitosan microspheres were dependent on pore size and degree of hydrophobicity produced in microspheres by cross-linking and degree of acetylation.

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