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Stabilization of Curcumin in Aqueous Chitosan-Tergitol-15-S-7 System

Palash Moni Saikia* and Pankaj Hazarika
Department of Chemistry, Darrang College, Tezpur-784001, Assam, India

ABSTRACT

The stability provided to curcumin by chitosan alone and in the presence of non ionic surfactant Tergitol-15-S-7 has been investigated by spectrophotometric analysis of the kinetics of degradation of curcumin. The interaction of curcumin with chitosan has been found to be exothermic and driven by hydrophobic interactions, hydrogen bond formation, and electrostatic interactions. The interaction of curcumin with chitosan, in presence of Tergitol-15-S-7 have been studied by monitoring the changes in the absorption and the fluorescence spectra at physiological *p*H (7.4). The apparent binding constants and the distribution of curcumin in the interior of chitosan have been evaluated by fluorescence quenching method. It has been observed from the fluorescence quenching techniques that curcumin is non-uniformly distributed in the colloidal chitosan solution. Curcumin is located mostly inside the hydrophobic interior of chitosan while a small fraction resides in the cationic centres of chitosan.

Key words: Curcumin; Stabilization; Chitosan; Tergotol-15-S-7; Surfactant; Binding constant

INTRODUCTION

Most natural dye molecules appears to defy implementation to model biological system due to poor aqueous solubility and fast metabolism. In order to improve the aqueous solubility, stability and bioavailability of the drug significant efforts have been put forward on impregnation of the water insoluble natural drug molecules onto biopolymeric pores.

Curcumin(1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, Scheme 1), the yellow pigment obtained from the Indian spice curry Turmeric (*Curcuma longa* Linn) is one of the most potent bioactive polyphenolic compound available in nature. Curcumin possesses remarkable therapeutic properties such as anticancer,antioxidant, anti-arthritic, antiamyloid, anti-ischaemic, and anti-inflammatory, and many other desirable clinical benefits. It was also established that curcumin has great potential to prevent protein aggregation in shattering diseases such as Alzheimer's and Parkinson's. Despite of its highly promising features as a health-promoting agent, poor aqueous solubility of curcumin(~11 ng/ml in plain aqueous buffer *p*H 5.0) in neutral aqueous medium remains a major trouble in its bioavailability, clinical efficiency and metabolism. However, curcumin is moderately soluble in aqueous solutions of high *p*H, both polar aprotic and polar protic solvents, and in micelles. But, curcumin decomposes in alkaline conditions. A number of attempts have been made to increase the aqueous solubility and stability and hence the bioavailability of curcumin through encapsulation of curcumin in phospholipids, cyclodextrine, hydrogel, liposomes, polymeric micelles, nanoparticles, etc.

*Corresponding author's Email: palashms@rediffmail.com

The curcumin solubility and stability can also be improved by chemical alteration, complexation or interaction with macromolecules. Curcumin binds to a variety of biopolymers and is known to retain its medicinal activity in the bound states.

Chitosan, [(1-4)-2-amino-2-deoxy-β-D-glucan], is a positively charged linear polysaccharide with unique physicochemical properties including its solid-state structure and the chain conformations in the dissolved state supporting numerous living organisms is also a food-grade polymer. The useful features of chitosan, e.g., its abundance, flexibility, nontoxicity, hydrophilicity, biocompatibility, biodegradability, antibacterial property, and high resistance to heatmakes chitosan suitable for biomedical application such as drug delivery, tissue engineering, wound dressing, etc. Because of the high content of amine functional groups, as well as the hydroxyl groups on the glucosamine unit, the protonated amine groups can attract metal anions, viz., molybdate, vanadate, palladate, chromate, cadmium, etc., and can absorb a number of dyes such as reactive, basic, acidic, disperse dyes in acidic (pH < 5) solutions. Chitosan also binds the herbal pigment curcumin with high affinity at considerably high *p*H (*p*H = 7.0-10.5) through its glucosamine unit. Moreover, chitosan effectively interacts with surfactants in aqueous solutions.

It is assumed that, the viscous transient network of chitosan entraps the curcumin moiety in the physiological *p*H of the medium. Incorporation of surfactants to the curcumin- chitosan system may alter the sensitivity of the chitosan and thus the nature of the interactions. However, there is no report showing the quantitative estimation of binding of curcumin with chitosan in presence of surfactants and its affect on the stability of curcumin in the system at physiological *p*H. UV-visible spectroscopy and fluorescence quenching are efficient tools applied to understand the interactions, distribution and localization of the dye, curcumin due to the molecular environment of chitosan polymer. Therefore, the present study aims to determine the extent of binding of aqueous curcumin with chitosan in absence and in the presence of Tergitol-15-S-7following the changes in the absorption and fluorescence of curcumin in different chitosan concentrations.

MATERIALS AND METHOD

Materials. Curcumin (purity ~ 95%) was obtained from Sigma-Aldrich and used as such. Chitosan with a degree of deacetylation of 80.5% (Molecular Weight 1,73000 gm/mol) was purchased from Sigma-Aldrich and used as such. Tergitol-15-S-7AR grade was also obtained Sigma-Aldrich and used as such. The *p*Hs of the systems was measured by using an Orion Multiparameter Kit after calibrating the instrument at *p*H 7.00 and 4.00. All other chemicals used were of analytical grade. Doubly distilled water has been used in preparation of all the solutions.

UV-Visible analysis. The absorbance readings of curcumin were taken from 200 to 600 nm using a Shimadzu UV-2550 UV-Vis double beam spectrophotometer. In the experiments for study of the kinetics of degradation of curcumin, the UV-Vis spectra were recorded over 60 min at 2 min intervals.

The binding process of curcumin with chitosan can be described by the following equilibrium:

$$curcumin + chitosan \leftrightharpoons [chitosan - curcumin] \quad (1)$$

The equilibrium constant K for the above equilibrium can be written as:

$$K_s \leftrightharpoons \frac{[chitosan-curcumin]}{[chitosan][curcumin]} \quad (2)$$

Assuming 1:1 complex formation between chitosan and curcumin, linear plot has been made by following the absorbance changes at a suitable wavelength, as a function of reciprocal concentration of chitosan or curcumin according to the equation, the modified Benesi- Hildebrand equation (3) given below

$$\frac{1}{\Delta A} = \frac{1}{K\Delta\varepsilon[\,curcumin]}\left(\frac{1}{[chitosan]}\right) + \frac{1}{\Delta\varepsilon[curcumin]} \qquad (3)$$

Here, ΔA and Δε correspond to the change in the absorbance and the molar extinction coefficient at the wavelength of the study (at 422 nm), respectively. [chitosan] and [curcumin] corresponds to the equlibrium concentrations of chitosan and curcumin, respectively.

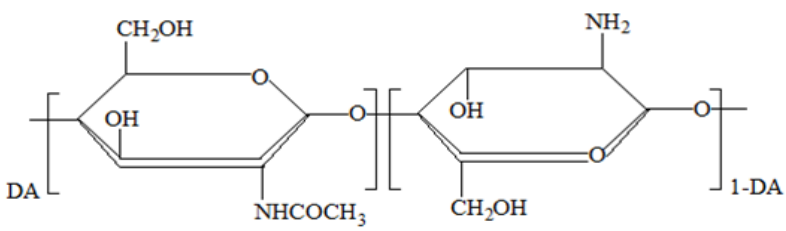
Fluorescence analysis. Fluorescence spectra were taken from 350 to 700 nm using a Hitachi F -2500 fluorescence spectrophotometer with the excitation and emission slit widths set at 5 nm. The excitation wavelength for each 0.025mM curcumin solution was 425 nm. The temperatures were maintained within ±1K. The binding constants can be estimated by following the fluorescence changes also. The fluorescence intensity of curcumin increases significantly in presence of chitosan. The changes in fluorescence intensity at 420nm due to curcumin were followed as a function of concentration of chitosan according to Eq. (4) to estimate the binding constant, K.

$$\frac{1}{F-F_o} = \frac{1}{F_{complex}-F_o} + \frac{1}{F_{complex}-F_o}\left(\frac{1}{K[citosan]}\right) \qquad (4)$$

Here, F_o and F are the respective fluorescence intensity from curcumin at a suitable wavelength in of 540-550nm in the absence and presence of chitosan.

RESULTS AND DISCUSSION

Binding of curcumin with chitosan. The aqueous solution of curcumin (2.5×10^{-5} mol dm^{-3}) containing 25% MeOH, shows the maximum absorption at 425 nm at *p*H 7.4 (phosphate buffer). Aqueous curcumin shows an absorption band at 425 nm and a shoulder at 365 nm. The absorption band at 425nm of curcumin is due to the enol form with conjugated π-bond system which is predominant in both solutions and in solid form of curcumin while the shoulder is due to the absorption by a symmetrical structure with the conjugation broken at the diketo groups as shown in Scheme 2. The absorption intensities of the 425 nm band of curcumin increase with increase in the chitosan concentration at the fixed *p*H of 7.4 (Figure 1).



Scheme 1. Structure of chitosan

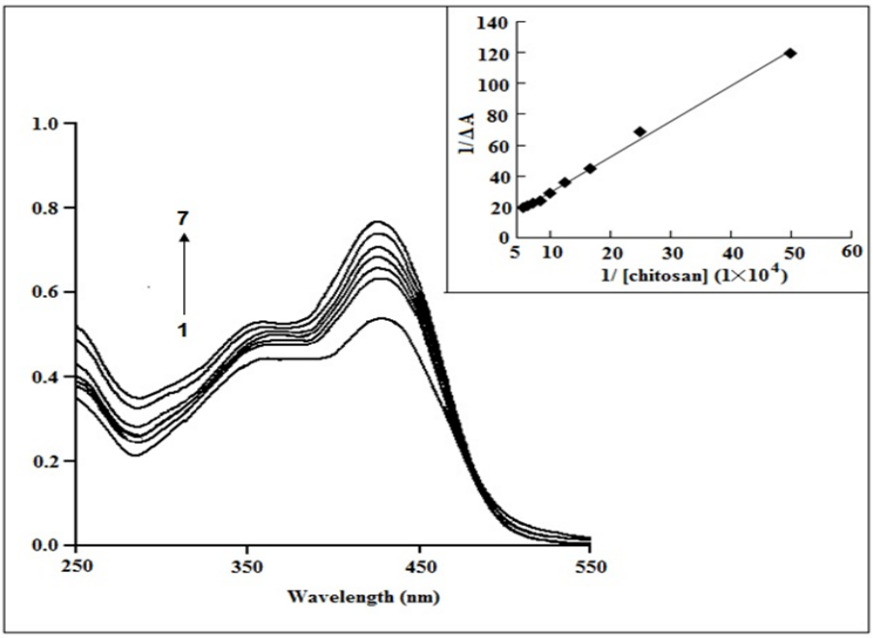
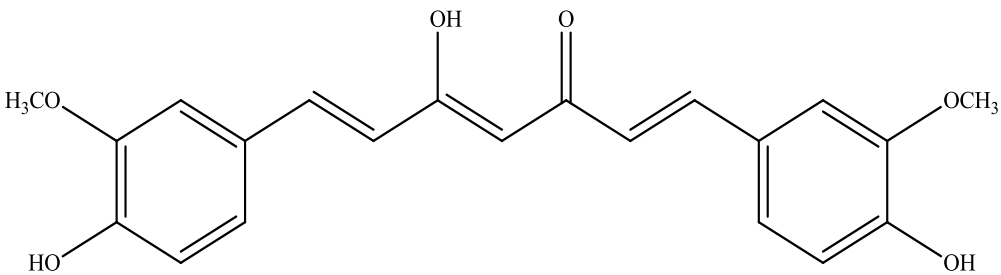


Figure 1. Absorption spectra of curcumin (2.5×10^{-5} mol dm^{-3})at *p*H 7.4 in presence of various concentrations of chitosan at 298(±0.1) K. [chitosan]: (1) 2μM, (2) 4μM, (3) 6 μM, (4) 8μM, (5) 10 μM, (6) 12 μM, (7) 14 μM. Inset: Plot of the determination of binding constant.

Although the spectra of curcumin bound to chitosan are similar to that in absence of chitosan, the intensities of the 425 nm band increases significantly on addition of chitosan which indicates that curcumin interacts with the polymer. Curcumin exists mainly as neutral form below *p*H 8.0.



Scheme 2. The enol form of curcumin

At the physiological *p*H range, the anionic fraction of curcumin is likely to interact with the cationic polymer. In solution of higher *p*H (pH > 6.5), the free amino groups of chitosan molecules become less protonated and the hydrophobic character along the chitosan chain becomes stronger. Therefore, the chitosan self-aggregates are formed in phosphate buffer solutions by intra and inter-molecular hydrophobic interactions.The agglomerates of chitosan that are formed may entrap the enol form of curcumin. Moreover, there may be intermolecular hydrogen bond formation between curcumin and the hydroxyl groups of the glucosamine unit of chitosan. The hydrogen bond interaction has also been pictured out betweencurcumin and phosphatidylcholine in addition to the hydrophobic interaction.The binding constant have been estimated following the changes in absorption intensity at 425 nm due to curcumin at various concentrations of chitosan, at *p*H 7.4 (phosphate buffer), varying the concentration of chitosan from 2μM to 16μM and keeping curcumin concentration at 25μM and fitting the data to the double reciprocal plot (Eq. (3)). Within this concentration range of chitosan the λ_{max} of curcumin slightly shifts from 425 nm to 421 nm. As shown in Figure 1 (Inset) fitting the data to equation (3) the linear plot with a squared correlation coefficient of 0.99 was obtained, from which the binding constant has been estimated as $2.01(\pm0.5)\times10^4\ M^{-1}$ at *p*H 7.4 and a temperature of 298K (±0.1) of the system.

The binding between curcumin and chitosan has also been studied by fluorescence measurements. The fluorescence of curcumin is mainly dependent on the medium. Curcumin exhibits a very weak fluorescence band at ~ 550 nm in aqueous buffer solutions containing 25% MeOH after excitation at 425 nm. It has been reported that in hydrophobic macromolecular environment, the fluorescence intensity significantly increases with a Stokes shift of about ~ 80 nm.However, on the addition of increasing amount of chitosan at a fixed concentration of curcumin ($2.5\times10^{-5}\ M^{-1}$), the fluorescence spectrum becomes sharp and fluorescence intensity considerably increases with a slight hypsochromic shift from 550 nm to 539 nm due to binding of curcumin with chitosan (Figure 2). It has been well documented that in aqueous buffer medium ($2.5\times10^{-5}\ M^{-1}$) curcumin containing 25% MeOH alone cannot fluoresce significantly.The hydrophobic regions are available within chitosan molecule in aqueous solution into which a fraction of curcumin partitions and fluoresce considerably. The number of hydrophobic regions per unit volume increases as more chitosan is added to the solutions and protects curcumin from the fluorescence quenching in aqueous surroundings. As a consequence, when the concentration of chitosan increases in the bulk solution the fluorescence intensity of chitosan bound curcumin increases spectacularly. The slight spectral shift in the λ_{em} has been observed upon the complexation of curcumin with chitosan as the microenvironment of curcumin was changed. The dye is supposes to reside in the slightly nonpolar region of the polymer where the polarity and so the dielectric constant of the microenvironment are much lower than that in bulk water.The binding constant for the binding of curcumin with chitosan was estimated by following fluorescence intensity changes at 540 nm, after excitation at 425 nm for solutions containing curcumin with varying chitosan concentrations from 0.02mM to 0.2mM at *p*H 7.4 (phosphate buffer) at 298 (±0.1) K. For the above mentioned equilibrium (4), the binding constant K has been estimated as $2.25(\pm0.5)\times10^4\ M^{-1}$.

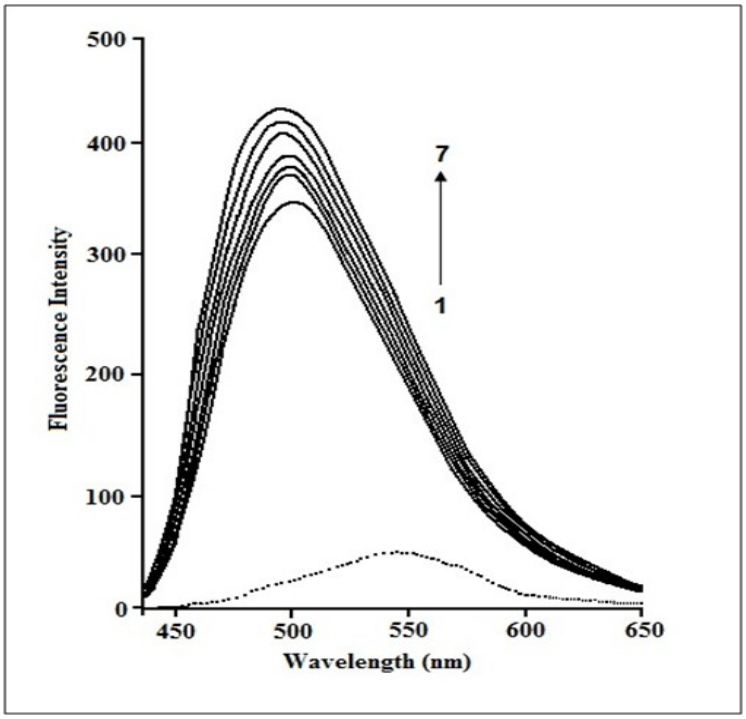


Figure 2. Fluorescence spectra of curcumin (2.5×10^{-5} mol dm^{-3}) at *p*H 7.4 in presence of various concentrations of chitosan at 298(±0.1) K. [chitosan]: (1) 2μM, (2) 4μM, (3) 6 μM, (4) 8μM, (5) 10 μM, (6) 12 μM, (7) 14 μM.k

Curcumin in chitosan-Tergitol-15-S-7 surfactant system. The spectra of 2.5μM aqueous curcumin induced by chitosan in presence of 1.0 mM Tergitol-15-S-7 at *p*H 7.4 and 298 (± 0.1) K have been shown in the Figure 3. The intensity of 420 nm band increases significantly with corresponding increase in the concentration of chitosan in presence of Tergitol-15-S-7. The binding constant of curcumin-nonionic surfactant-polymer system has been determined by monitoring the change in absorbance values of the aqueous curcumin at progressively increasing concentration of chitosan, in fixed concentration of Tergitol-15-S-7. The binding constant has been found to be 2.19(±0.5) $\times10^5$ M^{-1} which indicates that the curcumin-chitosan binding in the presence of Tergitol-15-S-7 is about ten times stronger than that in absence of the surfactant. This implies that there is greater hydrophobic interaction between the dye and the chitosan-Tergitol-15-S-7. In chitosan- Tergitol-15-S-7 system the positively charged polymer interacts with Tergitol-15-S-7 by electrostatic means. The electronegative oxygen atoms of the PEO chains of Tergitol-15-S-7 might associates with the electropositive chitosan chains. The excess polymer chains left after association with Tergitol-15-S-7 may form a transient network which is also available to interact with curcumin. As a result, the hydrophobic interaction of curcumin with chitosan in the presence of Tergitol-15-S-7 is more pronounced.

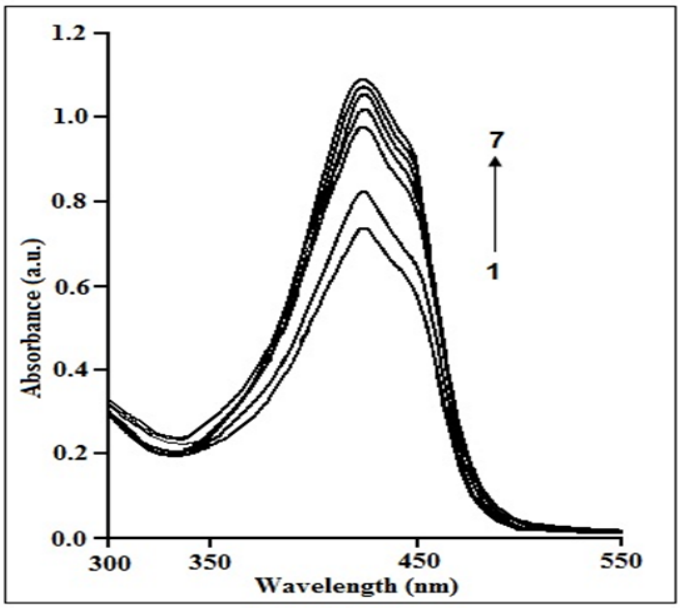


Figure 5. Absorption spectra of curcumin (2.5×10^{-5} mol dm^{-3})at *p*H 7.4 in varying concentrations of chitosan in presence of 1×10^{-3} mol dm^{-3} TW80 at 298(±0.1) K. [chitosan]: (1) 2μM, (2) 4μM, (3) 6 μM, (4) 8μM, (5) 10 μM, (6) 12 μM, (7) 14 μM.

The fluorescence studies have also been carried out for the aqueous curcumin in non-ionic surfactant-chitosan system and from the results obtained the binding constant have been determined. The fluorescence intensity of the aqueous curcumin containing 25% MeOH increases effectively with increasing concentration of chitosan, with a blue shift from 550 nm to 490 nm, in the presence of Tergitol-15-S-7. This large blue shift is due to change in micro-environment from a polar field to a less polar field. Thus, chitosan- Tergitol-15-S-7 system is more effective in binding curcumin molecule the surfactant free chitosan medium under physiological *p*H.

Nature of binding sites in chitosan. To understand the nature of binding sites of the curcumin -chitosan binding, experiments were carried out by following quenching studies using two quenchers, viz., acrylamide, a hydrophobic quencher and potassium iodide, a hydrophilic quencher. The concentrations of the quenchers were varied from 0 to 0.15 M, keeping the ionic strength constant. Small aliquots of quencher stocks have been added to curcumin samples and fluorescence spectra were recorded after each addition. The fluorescence intensity of chitosan bound curcumin decreased regularly with the increase in the concentration of the quenchers with no shift in λ_{em}.

The steady-state fluorescence quenching data obtained with the two different quenchers have been analysed according to the well known Stern-Volmer equation in order to obtain quantitative quenching parameters

$$\frac{F_o}{F} = 1 + K_D[Q] \qquad (5)$$

where, F_o and F are the intensities of the fluorophore (chitosan-curcumin) in the absence and presence of the quencher Q, respectively and K_D is the Stern-Volmer quenching constant and [Q] is the molar concentration of the quencher.

However, in order to understand the relative population of curcumin in different layers of chitosan, the fluorescence intensity changes of the flurophore in the presence of the quenchers were treated with equation (6) which is a modified form of the Stern-Volmer equation (5)

$$\frac{F_o}{\Delta F} = \frac{1}{f_a K_{D'}[Q]} + \frac{1}{f_a} \qquad (6)$$

Here, $\Delta F = F_o - F$ is the difference between the fluorescence intensities from the fluorophore (chitosan-curcumin) in the absence and presence of quencher Q at any point in the quenching titration, f_a is the fraction of the total fluorophores to the quencher, and K'_D is the Stern-Volmer constant.

Figure 4 shows the Stern-Volmer plot for fluorescence quenching of curcumin-chitosan by potassium iodide. On fitting the data obtained from quenching in the presence of potassium iodide to Equation (6) a linear plot has been obtained with a squared correlation coefficient of 0.995. The fitted parameters were found to be f_a=0.30±0.01 and K'_D=17.54± 2.26 M^{-1} for potassium iodide quenching. Similarly, fitting the data obtained from quenching in the presence of acrylamide to equation (6) a linear plot has been obtained with a squared correlation coefficient of 0.997and f_a value of 0.38±0.01 and K'_D = 20.6±4.43 M^{-1} at *p*H 7.4 . From the results obtained it has been observed that there is significant quenching by both of the quenchers, but the quenching by the hydrophobic quencher is slightly greater than that by the hydrophilic quencher. This fact confirms that curcumin is non-uniformly distributed into different regions of chitosan in the physiological *p*H of the medium. We have used a chitosan sample with a high degree of deacetylation (80.5%) and with an increase in DD, the number of amino groups in the polymer increases and as a result a highly cationic chitosan is obtained. The presence of large numbers of amine groups facilitates electrostatic interaction between the cationic groups located on the polyglucosamine chains of the polymer and the negatively charged anionic curcumin. On the other hand, the polymer is a high molecular weight polymer, and thus the polymer configuration in solution becomes a nearly spherical providing hydrophobic environment to entrapped curcumin. In high chitosan concentrations, most of the neutral curcumin partitioned to the compartments made up of chitosan chains.

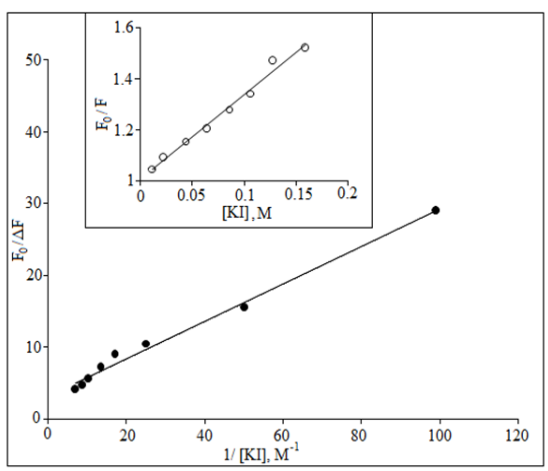


Figure 4. The plots of fluorescence quenching of curcumin-chitosan solution by KI at *p*H 7.4, line shows fitting to equation (6); Inset shows the data for the quenching of curcumin fluorescence by KI fitting to equation (5).

Kinetics of the binding of curcumin with chitosan. In the phosphate buffer medium at *p*H 7.4 or above, deprotonation of curcumin occurs first.Curcumin is initially deprotonated during the course of hydrolysis and results in *trans*-6-(4′-hydoxy-3′-methoxypenyl)-2,4-dioxo-5-hexenal. The hydrolysed product then further degredated to smaller molecular components such as vanillin, feruloyl methane, and ferulic acid. The contribution from these molecules to the absorption maximum of 425 nm of curcumin in aqueous buffer medium is negligible. More than 90% of curcumin decomposed rapidly in buffer systems at physiological *p*H (*p*H 7.4) conditions For the kinetic study of degradation of curcumin, the absorbance changes of aqueous (25% MeOH) curcumin solutions in presence and absence of chitosan have been monitored as a function of time. Since, the maximum absorption occurred at 422 nm in presence of chitosan, the studies have been carried out monitoring at λ_{max} 422 nm.

As shown in Figure 1 there is a substantial decrease in the UV-visible absorption curve of curcumin as a function of time at *p*H 7.4 (phosphate buffer). However, in presence 10μM of chitosan the degradation of curcumin has been suppressed to a larger extent as the decrease in absorption maxima is negligible compared to the original value. The linearity of the time dependent degradation of curcumin (figure not included) shows that the reaction is of pseudo first order. The pseudo first order rate constant has been determined using the equation introduced by Sengupta *et. al*.As shown in inset of Figure 1 the linear curve has been obtained with a squared correlation coefficient of 0.991. The rate constant for the degradation of curcumin in phosphate buffer medium has been obtained as 0.20 min^{-1} at *p*H 7.4. In presence of chitosan (10μM), the rate constant has been obtained as 0.076 min^{-1}.Thus, the degradation occurs approximately 3 times slower in presence of chitosan (0.1mM). Hence, the yield of suppression of degradation by chitosan (10μM) is 62 ± 5%. In chitosan-Tergitol-15-S-7 system, the rate constant of degradation of curcumin has been obtained as 0.009 min^{-1} and thus the yield of suppression of degradation is 95.5 ± 5%. The above observations strongly indicate that, chitosan-nonionic system can effectively diminish the degradation process of curcumin at physiological *p*H condition.

The thermodynamic parameters for interaction. The energy efficiency of the curcumin- chitosan system has been studied by monitoring the change in the binding constant, K values as a function of temperature in the temperature range between 298 and 313 K. ΔG^o has been determined from the equation,

$$\Delta G^o = - RT \ln K \quad (7)$$

ΔG^o value has been obtained as − 5.64 kcal/mol. ΔH^o and ΔS^o have been determined using the van't-Hoff equation.

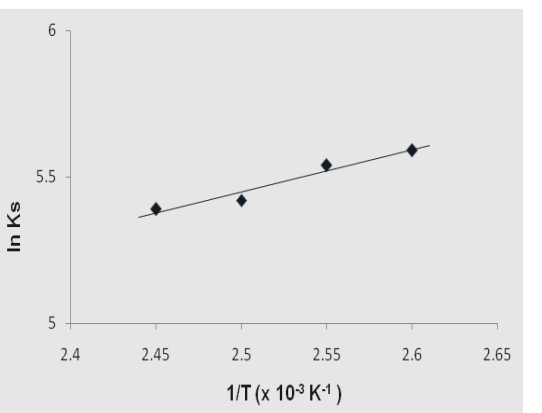


Figure 5. The van't- Hoff plot for the interaction of curcumin with chitosan at *p*H 7.4 (phosphate buffer).

It has been found that K values significantly decreases with increasing temperature. The van't-Hoff plot for the interaction of curcumin with chitosan at *p*H 7.4 is shown in Figure 5. The values ΔH^o and ΔS^o have been determined as − 21.52 kcal/mol and 23.08 cal/mol/K. The ΔH^o values for the transfer of curcumin from aqueous phase to chitosan rich colloidal phase were larger than the total free energy change, ΔG^o, indicates that the process is enthalpy driven, although there is a little contribution of small positive entropy changes. Thus, the strong interactions between curcumin and chitosan are driven by both hydrophobic force and hydrogen bond formation between the hydroxyl group of the glucosamine chains of chitosan and curcumin. And the rest of curcumin free in solution as anionic curcumin interacts with the cationic polymer.

CONCLUSION

The present work clearly shows that, curcumin strongly interacts with chitosan even at the physiological *p*H of the system and the interaction is more pronounced in presence of surfactants. It was observed that the value of the binding constant of chitosan-curcumin binding in chitosan- Tergitol-15-S-7 system is large compared to its value in chitosan.. Fluorescence quenching studies clearly signifies that one fraction of curcumin occupies the hydrophobic interior of chitosan, while the other fraction of curcumin as anionic curcumin occupies the cationic centres of the polymer. Chitosan exhibit the ability to suppress the hydrolytic degradation of curcumin with an impressive yield of about 77 ± 5%. On the other hand, in presence of Tergitol-15-S-7 the yield is increased up to 95.5 ± 5%. Thermodynamic studies reveal that the binding process is driven by both enthalpy and entropy indicating both hydrophobic, electrostatic and hydrogen bond formation between curcumin and chitosan. This study suggests that chitosan -surfactant systems could be effectively used to stabilise curcumin and other water-insoluble bioactive molecules.

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