

J. Surface Sci. Technol., Vol 24, No. 1-2, pp. 39-55, 2008
© 2008 Indian Society for Surface Science and Technology, India.

Stabilizing Effect of Chitosan on Curcumin from the Damaging Action of Alkaline pH and Ultraviolet Light

SANKAR P. MITRA

Senior Scientist, Integrated Pharmaceuticals Inc. 310, Authority Dr, Fitchberg, MA 01420, USA.

Abstract —The naturally available biopolymer, chitosan (MW = 10 kD) binds to yellow herbal spice, curcumin with high affinity ($K_a \sim 400 \mu\text{M}$) and moderate capacity ($n \sim 20$ Mole/Mole of Chitosan) at considerably high pH (pH ~ 10.5 , 0.1 M NaBO_3). Binding is pH sensitive and reduces nearly to zero at the acidic range (pH ~ 5.0). The presence of high salt (0.1 M – 2.0 M NaCl) does not alter the binding affinity (K_a) rather increases the capacity $\sim 12\%$. Interestingly, the affinity (K_a) remains unaltered even when the chitosan was coated on powdered Talc surface causing $\sim 40\%$ reduction in binding capacity (n). The chitosan – curcumin complex formed at high pH shows remarkable stability at pH 7.0 - 10.5 and in high salt concentrations (1.0 M – 4.0 M NaCl) showing least dissociation effect. Lowering the pH (< 7.0) enables the complex to dissociate efficiently. The bound curcumin remains chemically unaltered when analyzed by high-pressure liquid chromatography (HPLC). As per logical expectation, the glucosamine unit within chitosan molecule participates in the binding process as evidenced by dose-dependent enhancement of optical density (O.D.) ($\lambda = 440 \text{ nm}$) of curcumin in the presence of glucosamine at pH – 8.5. The thin layer chromatography (TLC) of glucosamine – curcumin complex confirms the chemically unaltered state of curcumin within the complex. The enhancement of O.D. in basic environment by glucosamine was unnoticed in acidic condition, pH ~ 4.8 . Perhaps, protonation ($-\text{NH}_3^+$) of the $-\text{NH}_2$ (s) within sugar moiety in acidic environment hinders curcumin to interact. As for interest, the complexed curcumin molecule in chitosan or glucosamine acquires substantial stability from the alkaline pH (~ 10.5) or UV damage ($\lambda \sim 240 \text{ nm}$).

Keywords : Binding isotherm, Chitosan, Curcumin, Glucosamine, Scatchard analysis, Stability, UV light.

*E-mail : smitra@intepharm.com or sankar_mitra@msn.com.

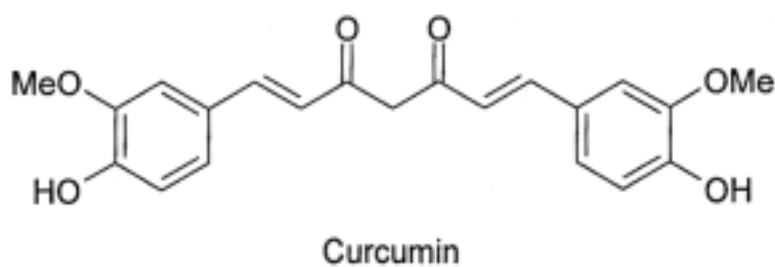
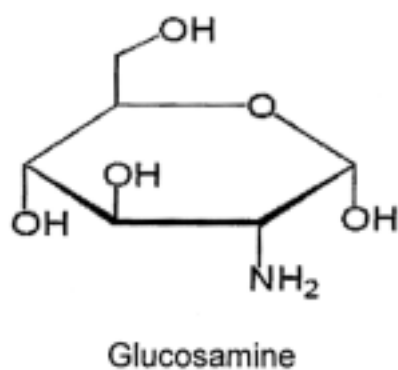
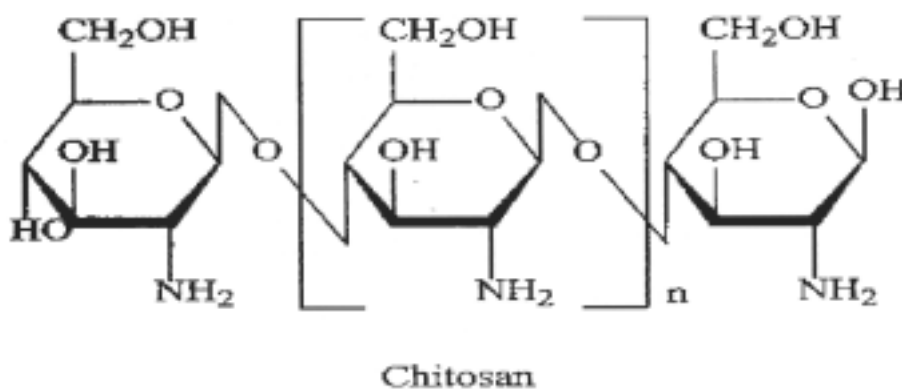
INTRODUCTION

Chitin, the naturally occurring biopolymer of crustacean origin is a linear chain of covalently linked (β - {1 - 4} link) amino-sugars of D-glucosamine units with varying extents of free ($-\text{NH}_2$) or -N - acetylated amines ($-\text{NHCOCH}_3$) [1]. Alkaline de-acetylation removes acetyl (CH_3CO -) groups while freeing the primary amine(s) ($-\text{NH}_2$) and thus creating chitosan [2]. Due to its easy bio-degradability and biocompatibility, chitosan is currently in use for varieties of pharmaceutical and commercial purposes specially during water purification for detoxifying from hazardous metal wastes [3,4]. Its cationic nature is extensively exploited to use for bio-adhesives, wound healing and in regulating plasma cholesterol by sequestering intestinal bile acids preventing absorption and subsequent transformation to cholesterol in liver [5-8, 12-14]. In addition, the polymer expresses strong bacteriostatic plus antifungal effects and thus recently finds applications in preserving fruits, vegetables as well as protecting plants and trees, the fact relates to chitosan's role on regulating various endogenous enzyme actions [12-14].

The polymer is more soluble at warm or boiling water displaying high viscosity with increase in concentrations. The extent of polymerization and solution pH largely contribute to the solubility which is high at low pH. This low cost readily available polysaccharide is eco-friendly and by virtue of its basic nature and additional cross-linking ability with bivalent anions at high pH, chitosan is currently applied in number of drug delivery systems and orthopedic treatments as hydro-gels [15-17].

Curcumin, a widely known spice from turmeric roots used in most Jamaican, Asian or Indian cooking is drawing major interest these days for its preventive role in contracting a number of horrific diseases. The notable ones are Alzheimer and several forms of carcinoma [18-22]. It is a poly-phenolic compound [1,7-bis (4-hydroxy-3-methoxy phenyl)-1, 6 heptadiene - 3, 5-dione; diferuloylmethane] therefore acting as a potent antioxidant [23]. Due to strong reducing nature it scavenges endogenously generated oxidative free radicals normally deleterious to health, the fact established by eminent investigators thus categorizing the spice as preventive medicine [22]. It extends promise also toward cystic fibrosis by hindering the progression [24].

Previously, I reported that curcumin binds with bovine serum albumin (BSA) at neutral or physiologic pH and acquires stability from the damaging effect of alkali or ultraviolet (UV) radiation. The interaction comprises electrostatic as well as hydrophobic effect [25]. In this article I intend to show that curcumin binding to chitosan is possible only at high pH. The binding complex formed is exceptionally stable at high pH ($\text{pH} > 7.0$) and dissociates only under acidic environment. The glucosamine unit within the biopolymer is the sole participant in the binding process. Importantly, the complexation event prevents curcumin from chemical degradation by alkali or exposure to UV light.



EXPERIMENTAL

Materials : Chitosan was purchased from Sigma Chemicals (Lot # 13604PC, MW = 10 kD) and purified further in the laboratory. The analytical grade (> 95%) Curcumin (Fluka, Lot # 1122956) was purified further by slightly modifying the

reported protocol [25]. Unless otherwise mentioned all other chemicals including Talc (particle size 400 mesh, Lot # 03928BE) and Glucosamine (Lot # 116K07031) used were bought from Sigma-Aldrich Corporation and are of analytical grade. The spectral measurements ranging from 300 - 600 nm were performed manually by Hitachi U-2000 spectro-photometer using optically balanced quartz cuvettes [25]. The silica (particle size 0.2 μm) coated TLC plates were supplied by VWR International (Boston, MA).

Methods : Purification of Chitosan — Approximately 4.0 gm of low molecular weight (MW \sim 10 kD) chitosan was added to 400 ml of boiling water along with 10 gm of pure gluconic acid. The solution was stirred with magnetic stirrer without further boiling and left to dissolve all the chitosan particles. Occasional heating often facilitates the dissolution because high viscosity of the chitosan solution imposes immense difficulty during solubilization process. The solution was then cooled to room temperature and the pH was raised to \sim 10.0 by adding 45% KOH solution with continuous stirring until a flocculent white precipitate ceased to appear. After adding ethanol to \sim 50% by volume, the precipitate was collected by filtering over several layers of cheese cloth and washing continuously and successively with 50% ethanol and distilled water to get rid of any remaining alkali. The gelatinous precipitate was freeze dried and washed twice with 50% ethanol and dried further and kept in the refrigerator inside a vacuum desiccator over dri-rites (VWR International, Boston) for future use.

Purification of Curcumin — It was conducted by following the previous protocol [25]. About 10 % solution of curcumin was prepared in HPLC grade methanol. The solution was subjected to slow distillation under vacuum at 40 $^{\circ}\text{C}$ until it reduced to $\frac{1}{4}$ of the original volume. It was then left at room temperature in the dark for crystallization. The crystals were collected by filtration over sintered glass filter under suction, freeze-dried and kept in the dark in a desiccator. The sample purity was 99.99% as assessed by HPLC with a single peak at both UV (260 nm) and visible (440 nm) region.

Binding Assay — The assay was conducted in 2.0 ml poly - propylene Eppendorf tubes at room temperature. In most occasions 500 μl of 2 % Chitosan solution in 0.2 M Gluconate + 0.1 M Borate buffer, pH 5.0, were aliquoted in several Eppendorf tubes. Varying amount of curcumin solution from 0.1 M stock in methanol were then added to each tube and the solutions were vigorously shaken with a vortex mixer. In no case, any cloudiness or precipitate was allowed to appear due to the presence of excessive level of alcohol. The solution pH was adjusted to 10.5 by using 6.0 M NaOH (\sim 5.0 μl). There would be an observable color change of curcumin

due to the alteration of pH with simultaneous appearances of turbidity by precipitated chitosan. The solution pH was checked by dipping micro – electrode while adding 495 μl of 0.1 M Na-BO₃ buffer, pH, 10.5. The solutions were vortexed frequently in the dark for \sim 5 minutes at room temperature. In the end, the solutions were microfuged (12000 \times g) for 5.0 minutes at 20 °C to separate the precipitate from supernatant. The 300 μl transparent orange red supernatant was diluted by 1.0 ml of 0.1 M HCl; it turned to original yellow curcumin color whose optical density was measured at 440 nm in a spectrophotometer (Hitachi – U2000). A set of separate blanks without chitosan was run at low pH, 5.0 in identical volumes and curcumin concentrations whose optical densities (O.D.) were assessed diluting in a similar way to construct the standard O.D. versus concentration curve for determining the unknown solution strength. [Note : The blank set for standards was kept in acidic pH because of the possible deterioration of curcumin at pH, 10.5 in the absence of chitosan. In other incidence identical experiments were carried out in the same fashion without chitosan and the blanks were left in pH, 10.5 buffers for a short time period (\sim 5 minutes) before mixing with acid. The comparisons were made between the two controls in order to assess any appreciable differences within them. In both cases only \pm 2 - 4 % variations in concentrations were observed, which were ignored during final calculations. This indicates that no significant deterioration of free curcumin occurred during experiment period while in equilibrium with its stable bound phase analogue.] The difference of concentration between the respective blanks and experimental solutions multiplied by the total volume provided the amount of curcumin bound when divided by the exact amount of solid materials of chitosan used, provides the binding value (Γ) often expressed in μg of curcumin/ μg of Chitosan and later converted to mole/mole unit. The bound values increased with the increasing level of equilibrium concentrations and thus provide a binding isotherm as seen in Fig. 1. All measurements were conducted at the same time. The amount bound (Γ) was calculated from the following formula after knowing the initial (C_i) and the final equilibrium concentration (C_f) in $\mu\text{g}/\mu\text{l}$.

$$\Gamma (\mu\text{g of curcumin}/\mu\text{g of Chitosan}) = (C_i - C_f) \times V/W$$

Here, V = Total volume of the binding mixture in μl . W is the dry weight (μg) of the chitosan used in the binding.

Each experiment was conducted three times (N = 3) with duplicate data points and plotted using standard mean error (\pm SEM) of three experiments.

Dissociation Study — It was also conducted at room temperature after performing binding protocol by following the above procedure. The binding complex collected by microfugation was washed twice with 1.0 ml of 0.001 M K-PO₄ buffer pH, 8.0

then vortexed while equilibrating with 1.0 ml of respective buffers of different pH or ionic strengths for varying lengths of time. The reactions were staggered and terminated at the same time by microfugation. The supernatant was collected and assessed spectrophotometrically at 440 nm. The 100 % elution control was accounted by using the elution buffer at pH, 2.5, 0.2 M K-PO₄ and the results were expressed as % dissociation from the bound phase.

Chitosan Coating on Powdered Talc and Binding — The exact amount (0.342 gm) of Chitosan was dissolved in boiling water in presence of gluconic acid (0.2 M final) and H₃BO₃ (0.1 M final). After cooling at room temperature the solution pH was brought to 5.0. At this condition, 15.0 gm talc (400 mesh size) was added with continuous stirring with a high speed vertical motor driven blender and the process was continued for ~ 20 minutes. The suspension was quick frozen and dried by lyophilization. The ratio of talc to Chitosan was 44 : 1.0 gm/gm.

The freeze-dried material was grinded further to eliminate any lumps or grits by using mortar and pestle. The exact amount of fine powder (~ 0.5 gm) was weighed in each microfuge tube and 0.5 ml of Na-gluconate / NaBO₃ buffer pH, 5.0 were added along with varying concentration of 0.1 M curcumin solution in methanol. After vortexing 495 µl of same buffer at pH 10.5 were added and the pH was further adjusted to 10.5 by adding 5.0 µl of 6.0 M NaOH. The vortexing was continued frequently for 5.0 minutes and the solutions were microfuged at room temperature as described above. The supernatant was collected, acidified as above in 0.1 M HCl for optical density measurement (440 nm). As for standards, identical solutions with varying concentrations of curcumin devoid of coated talc were diluted with 0.1 M HCl in above manner for establishing concentration vs optical density standard curve from which unknown curcumin concentration of experimental solutions could be evaluated.

Similar binding experiment to verify whether curcumin by itself can bind with talc only was also conducted as for control.

HPLC Run — The instrument used was from Shimadzu Corporation equipped with peak integrator soft ware and dual range optical detector. The run was isocratic with solvent (50 % CH₃ CN + 50 % CH₃ OH + 0.1% CF₃COOH) at constant flow rate of 0.5 ml per minute using Vydac - C18 (0.3 µm packing) coupled with protective guard column (C18) at 20 °C. In all cases the peak values (retention time, peak area, peak height and % of single peak or purity) were assessed and compared simultaneously by using quality assessment software provided by Shimadzu Corporation through automatic data transfer protocol at both 260 nm and 440 nm in order to quantify any minute damages to the molecule. As per necessity, the

Chitosan-curcumin or Glucosamin-Curcumin complex was acidified in 0.1 M HCl and then diluted with running buffer. After microfuging ~ 10 minutes the solutions were injected for HPLC. In all cases, standards were run back to back for comparison or to observe possible changes of the molecule. The binding complex was made by adding 500 μ l of chitosan solution in the same buffer as mentioned above was mixed with 23.8 mg/ml of curcumin in methanol to attain a final curcumin concentration of 2.38 mg/ml. The medium pH was changed to 10.5 by adding 50 % NaOH. The mixture was left for 30 minutes then spun on a microfuge, washed with 0.1 M K-BO₃, pH, 10.5 buffer three times. The washed precipitate was then dissolved in 250 μ l 0.1 M HCl before shooting on HPLC. As for control curcumin solution (1.2 mg / ml) in methanol was used (Fig - 4B).

Thin Layer Chromatography (TLC) — The procedure was carried on the silica coated glass plate using 50 % CH₃ CN + 50 % CH₃ OH + 0.1 % CF₃COOH as the mobile phase. Exact 2.0 μ l of each sample was placed by Hamilton micro syringe side by side on the same line drawn 2.5 cm from the plate bottom. After sample application the residual solvent from the sample solution were evaporated by a hair drier. The plate was then placed in a metal holder and lowered in the chamber dipping just 1.0 cm of the bottom end. The chamber was covered and the process was allowed to proceed for 1.5 hour. In the end, the plates were removed from the chamber, dried by hot air from the hair drier and the distance traveled by each yellow spots were measured for comparison (Fig - 4A). For complex formation, 400 μ l of 0.1 M K-PO₄ buffer with 0.2 M glucosamine at pH, 8.5 was mixed separately with 50, 100 and 200 μ l curcumin solution 1.01 mg/ml in methanol for 30 minutes before application. Curcumin solution (1.01 mg/ml) was run side by side as control.

Spectroscopic Measurements — The interaction study between Glucosamine and curcumin was conducted in 0.1 M K-PO₄ buffers at pH, 8.5 in presence of 0.1 M glucosamine. The methanolic solution of curcumin from the stock was added to the freshly prepared glucosamine containing buffer and in all cases the methanol concentration remains ~ 1 : 1000. The glucosamine-curcumin complex thus formed was left for an hour at room temperature before measuring the optical density starting from 300 to 600 nm. In order to observe the effect of glucosamine's role, curcumin spectrum was taken separately in the same buffer without any glucosamine. The measurements were performed manually and later plotted to observe the difference in peak heights.

Stability Study — The stability of curcumin was studied following the previous protocol [25]. It was measured after exposing the curcumin solution either to different pH or ultraviolet light (λ = 240 nm) for 8 hr. The exposed solutions were diluted

to 1 : 1 with 1 : 1 methanol and glacial acetic acid and measured for optical density at 440 nm. A linear calibration curve was constructed under similar condition at different dye concentrations from which the sample concentrations were evaluated. The percentage of concentration loss in comparison to the freshly prepared known samples was taken as a measure of molecular stability.

20 ml of binding buffer (0.2 M gluconate and 0.1 M Na-BO₃) having 2 % chitosan solution at pH, 5.8 was mixed with 1.0 ml of 0.1 M curcumin solution and the pH was adjusted quickly to 10.5 by adding 4.0 M NaOH. After vortexing for few minutes, the solution was centrifuged at 10,000 × g using J21 rotor. The collected precipitate after thorough wash by suspending three times in Na-BO₃ buffer, pH 8.5 was re-suspended in 20 ml of Na-BO₃, pH 10.5 buffer. 10 ml of each aliquot was subjected to either UV ($\lambda = 240$ nm) or normal room illumination for almost 8 hr. As per control, for measuring the alkaline or UV damage, curcumin was mixed in similar buffer with or without chitosan and the pH was later advanced to 10.5 and kept for same period of time in presence or absence of UV. In other experiments chitosan was replaced by 0.2 M glucosamine while keeping the time and conditions same. At the end 1.0 ml aliquotes were diluted with acidic methanol before making optical density measurements. The molecular integrity of curcumin was further tested by running on HPLC using freshly prepared solution in methanol as control.

Data Analysis — The binding data were analyzed by using GraFit 4.0 supplied by Erthacus Software Ltd. UK. In several occasions MS Excel was used for further analysis and data presentation.

RESULTS AND DISCUSSION

Binding and Dissociation : Fig. 1 represents the binding isotherm of curcumin bound either directly to chitosan or chitosan coated powdered Talc surface. In both the cases affinity remains reasonably the same [$K_a = (433 \pm 51) \mu\text{M}$ and $(384 \pm 42) \mu\text{M}$ for direct and coated] but considerable loss (~ 40 %) in binding capacity (n) is observed in the case of chitosan coated talc particles as revealed by the Scatchard analysis ($n = 88.8 \pm 15.9$ and 54.2 ± 10.2 moles of curcumin/mole of chitosan; $N = 3$ independent measurements; $p = 0.002$).

By nature, the binding is fully non-cooperative (Hill Coefficient, $h = 0.98$) and reaches to saturation level near 100 mM equilibrium concentration of curcumin in both the cases. The lowering of binding capacity (n) can be attributed to possible interaction between silicate network ($-\text{SiO}_4$) of talc ($\text{Mg}_3 \text{Si}_4 \text{O}_{10} (\text{OH})_2$) and $-\text{NH}_2$ of chitosan when coated on the powdered surface (by itself talc does not interact with the curcumin at this condition). Presumably, the hydrogen-bonding between electron

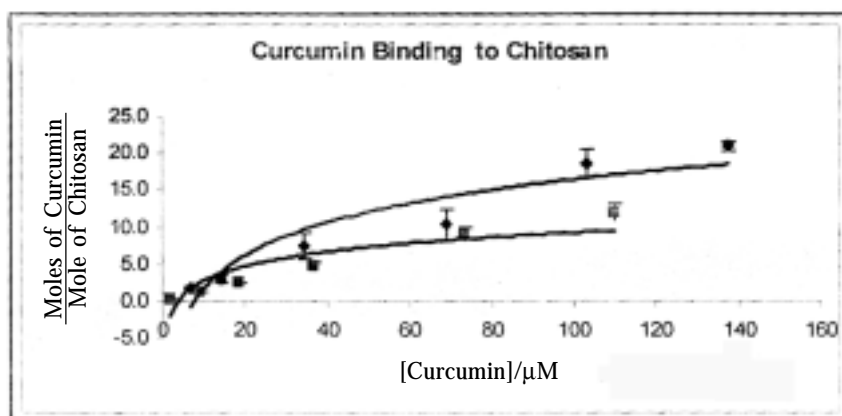
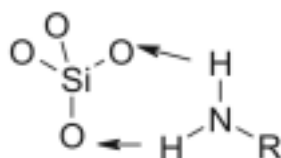


Fig. 1. Binding of curcumin to (\blacklozenge) chitosan and (\blacksquare) chitosan coated on powdered talc surface (zero binding observed on uncoated talc at pH = 10.5 in 0.1 M Na-BO₃ buffer). The affinity constants (K_a) and binding capacities (n) are $K_a = (433 \pm 51) \mu\text{M}$ and $n^* = (88.8 \pm 15.9)$ mole for chitosan and, $K_a = (384 \pm 42) \mu\text{M}$ and $n^* = (54.2 \pm 10.2)$ mole for chitosan coated on talc [$p^* = 0.002$]. Hill coefficients (h) = 0.98 in both. [Results \pm SEM, N = 3 experiments with each data point in duplicate.] K_a ($\sim 400 \mu\text{M}$) is independent of ionic strength (NaCl = 0.0 to 2.0 M) but $\sim 12\%$ increase is observed at 0.5 to 2.0 M [$p = 0.001 - 0.002$, N = 3 Expts].

rich oxygen atoms of $-\text{SiO}_4$ net works and $-\text{NH}_2$ (s) within chitosan lower the accessibility to interact with curcumin resulting in the reduction of binding sites in the polymer. Therefore increasing the chitosan level for coating talc surfaces enhances the binding capacity depending on the dose (data not presented for official reasons).



Possible interaction between silicate network and primary amine

Besides curcumin a number of pharmaceuticals bind to chitosan at basic pH (7.0 - 11.0) [26]. Either H-bonding or electrostatic charge interaction plays the major role in those events. Additional reports further indicate that chitosan often help enhance the solubilization of drug regimen [26, 27].

The dose-dependent addition of NaCl does not alter K_a significantly but raises the capacity $\sim 12\%$ at 2.0 M concentration. Conceptually, either the high ionic strength may reduce intra-chain interaction within biopolymer itself or with surrounding solvated water molecules or even the combination of both, thereby unleashing the masked sites allowing few more ligands to bind while un-altering the affinity, $K_a \sim 400 \mu\text{M}$.

Changing the pH toward acidity drastically reduces binding (data not shown). Around pH 4.5 almost no binding is noticed. At pH 6.5 – 7.0 only 30 % binding is observed whereas at higher pH 9.0 – 10.5 or above (pH, 10.5) maximum binding is achieved. In all events the affinity constant remains unchanged ($K_a \sim 400 \mu\text{M}$ in the range of pH 7.0 – 11.0). It is possible that ionization of phenolic ($\text{p}K_a \sim 9.95$) or enolic – OH ($\text{p}K_a \sim 11$) may be necessary for furthering the binding capacity. As per binding characteristics, it is logical to presume that curcumin interacting sites are homogenous by nature.

As seen in Fig. 2A, the binding complex formed at pH ~ 10.5 is also stable at pH, 8.5 and can only dissociate to a negligible extent ($\sim 5\%$) over 90 minutes

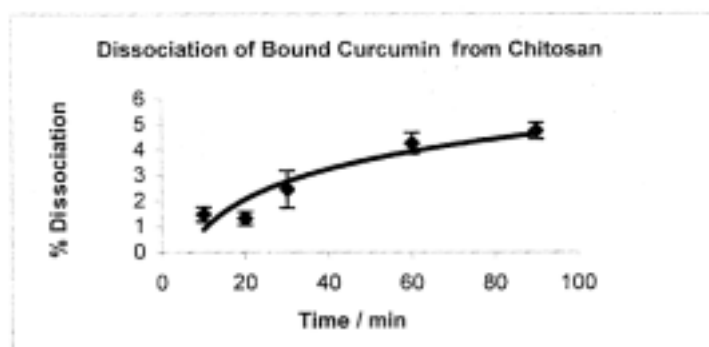


Fig. 2A. Time dependent dissociation of bound curcumin from chitosan suspension ($N = 3$ expts. \pm SEM) at pH 8.5..

period of time. The presence of high NaCl concentration of 1.0 to 4.0 M does not affect the stability level (data not shown). On the contrary, lowering the medium pH produces a significant effect (Fig. 2B). Almost 100% dissociation occurs at pH 4.7 whereas only 20 % dissociates at 5.8 and less than 10 % at pH 6.7 – 8.5 when studied within 30 minutes time period. It is reasonable to assume that at a pH below or near $\text{p}K_a$ of $-\text{NH}_2$ in chitosan ($\text{p}K_a \sim 6.5$), the chitosan - curcumin complex

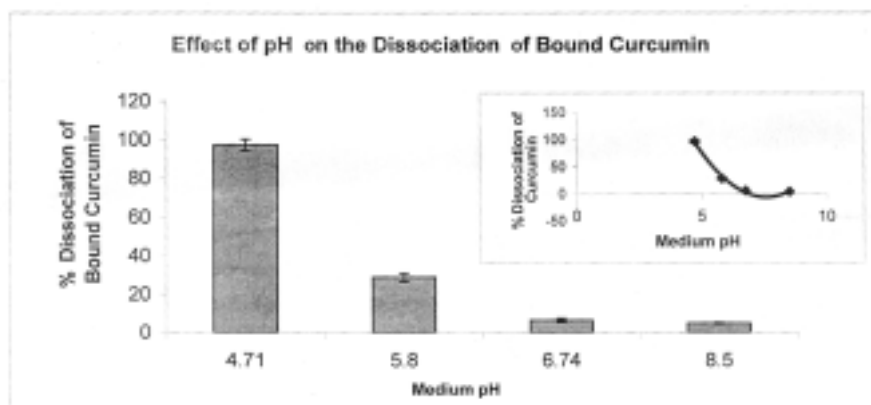
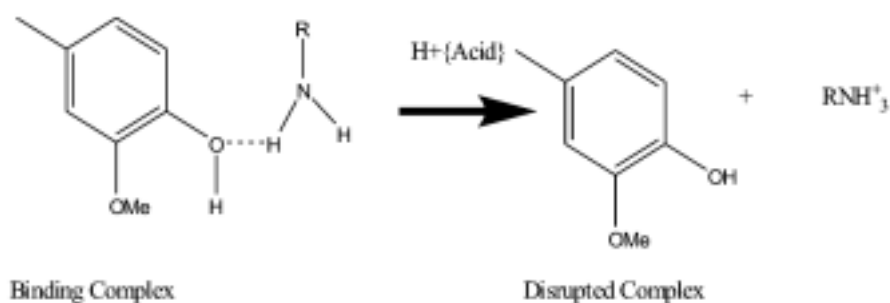


Fig. 2B. pH effect on the dissociation of chitosan-bound curcumin (N = 3 expts \pm SEM).

becomes vulnerable whereas at high pH (7.0 onward) the complex tends to stay intact [25]. So it is inferred that protonation ($-\text{NH}_3^+$) of $-\text{NH}_2$ is the most certain cause for disruption.



Spectral Analysis and Interaction with Glucosamine : Fig. 3A shows the spectral difference of curcumin at different doses in presence and absence of 0.1 M glucosamine. When compared at $\lambda_{\text{max}} = 440 \text{ nm}$, the dose-dependent enhancement is noticeable through out the concentrations range (1.0 – 30.0 μg curcumin/ml) at pH, 8.5 (Fig. 3B). Interestingly, no such increment is noticed at pH, 4.5 (Fig. 3C). Since glucosamine is a monomeric unit of polymeric chitosan, thus exposing free $-\text{NH}_2$ (s) may find similarity with chitosan interaction. Logically, at pH 8.5 the free or uncharged $-\text{NH}_2$ is the likely participant in forming binding complex with the electron rich oxygen atom(s) of phenolic ($-\text{OH}$) group(s) in curcumin. Perhaps that augments the optical absorption [25]. At further high pH in addition to the phenolic $-\text{OH}$ s' ionization, the enolic ($-\text{OH}$, $\text{pK}_a \sim 11$) form at tautomeric equilibrium with

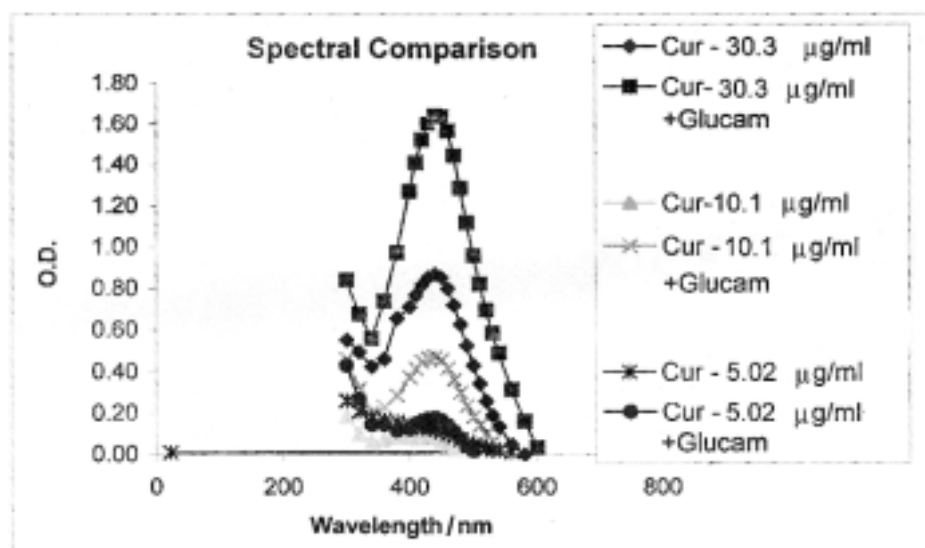


Fig. 3A. Spectral absorption ranging from 300 to 600 nm wavelength of different curcumin concentrations in presence or absence of 0.1 M Glucosamine (Glucam) in 0.1 M K-PO₄, pH, 8.5 at room temperature 20 °C. Picture represents one of the 4 expts.

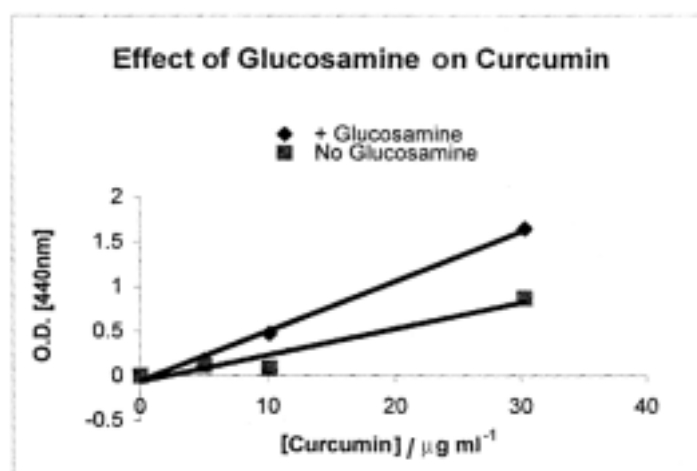


Fig. 3B. Effect of glucosamine on optical density elevation of different curcumin concentrations at pH 8.5.

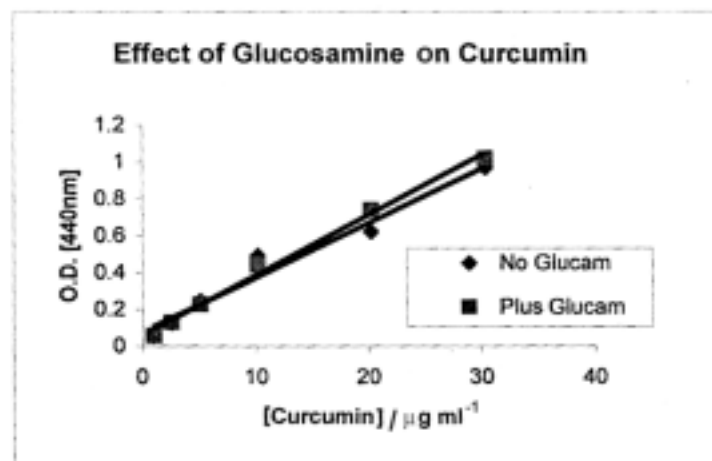


Fig. 3C. Effect of glucosamine on optical density elevation of different curcumin concentrations at pH 4.5.

the keto analogue also tends to dissociate furthering the chances to interact with free $-\text{NH}_2$ (s). More likely, the $-\text{NH}_2$ sets up a hydrogen bond with the anionic oxygen of either fully or partially ionized phenolic or enolic group(s) created nascently. The solubility enhancement of curcumin at higher pH by BSA may lend support in favoring the argument [25].

Although a conjecture at present, but logic insists that the entire binding episode proceeds via electrostatic interaction and the binding complex formed helps retain the original chemical structure of curcumin as evidenced by the chromatographic analysis of the disrupted complex in acid environment (Fig. 4A and 4B). It is also a possibility that any intermediate state(s) formed before reverting to the quinone formation of phenolic group(s) within curcumin is quickly stabilized by the immediate complexation with chitosan $-\text{NH}_2$, which in turn save curcumin molecule from the permanent damage.

Thin Layer Chromatography (TLC) and HPLC Analysis : The analysis of curcumin – glucosamine complex by TLC (Fig. 4A) confirms chemical integrity of the curcumin molecule within complex as visualized by attaining the similar heights of non-complexed/control and curcumin complexes with glucosamine. HPLC analysis of curcumin – chitosan complex also supports the same fact (Fig. 4B). In either situation the complex dissociates in acid environment separating curcumin molecule in tact. Thus one can infer that the complex is ionic by nature and imparts stability to the spice from the damage by alkali.

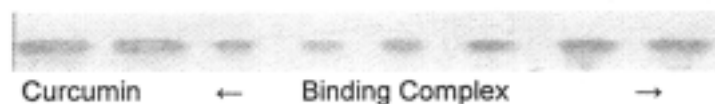


Fig. 4A. TLC analysis of curcumin and glucosamine-curcumin complex. From left, two lanes are free curcumin ($1.0 \mu\text{g} / \mu\text{l}$). The others are complexed with 50, 100 and 200 μl curcumin in 500 μl 0.2 M glucosamine applied in duplicate. [N= 4 expts].

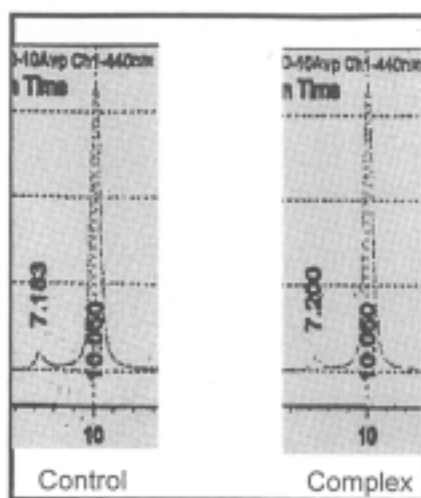


Fig. 4B. HPLC analysis of curcumin-chitosan complex with free curcumin as a control, detection at $\lambda = 440 \text{ nm}$.

Stability and Ultraviolet (UV) Radiation Damage : The complexation event also protects curcumin from the exposure to UV radiation ($\lambda = 240 \text{ nm}$) besides the damage by alkali [25]. After a long exposure at short wavelength $\sim 60\%$ of the curcumin molecule remains intact (Fig. 5). At this condition the uncomplexed or rather free curcumin deteriorates $\sim 100\%$. In general UV exposure of aqueous media often generates free radicals or reactive oxygen species that are harmful to number of organic and biological materials like DNA [28–30]. Scavenging them enhances the longevity and that beneficial event is often applied to the health industry for protecting lives from dreadful diseases including cancers. The 60% retention of original curcumin thus offers valuable information regarding its pharmaceutical uses to protect skin from deep sunrays. In all cases the $-\text{NH}_2$ offers stability by bridging the phenolic group(s) via H-bonding as proposed in the previous report [25].

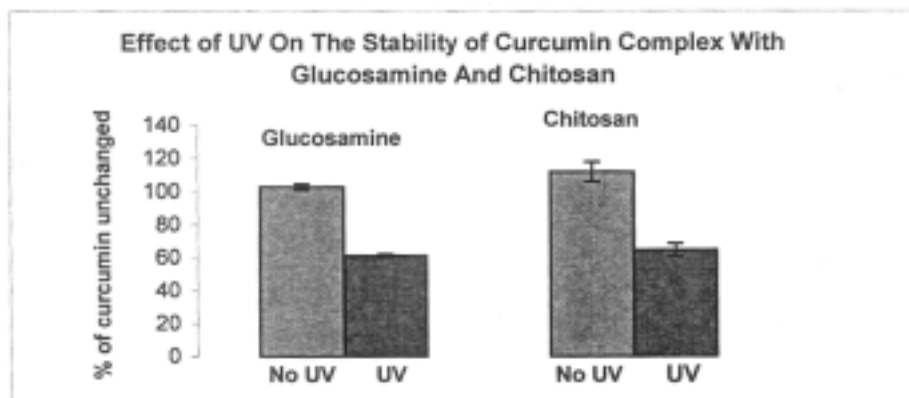


Fig. 5. Stability of curcumin molecule within chitosan–curcumin and glucosamine–curcumin complex under UV ($\lambda = 240$ nm) and normal illumination for 8.0 hr. ~ 100 % destruction observed in the absence of chitosan or glucosamine (pH 10.5) without the UV exposure as analyzed by HPLC. [Results \pm SEM, N= 3 observations].

CONCLUSION

Curcumin binds to chitosan at higher pH range with considerable affinity. The event proceeds via electrostatic interaction between $-\text{NH}_2$ of chitosan and the phenolic $-\text{OH}$ of curcumin. The binding complex retains original chemical structure of curcumin. Remarkably, the complex provides significant stability protecting curcumin from the damaging effect of alkali or UV lights.

ACKNOWLEDGEMENT

Author gratefully acknowledges sincere and moral supports from all the colleagues at Integrated Pharmaceuticals Inc. Special thanks to Dr. Rajesh Kumar Barur for his exceptional help in allowing frequent use of his laboratory.

REFERENCES

1. A. B. Foster and R. H. Hackman., *Nature*, 180, 40 (1957).
2. L. Illum, *Pharm. Res.* 15 (9), 1326 (1998).
3. P. A. Sandford and G. P. Hutchings, "Industrial Polysaccharides : Genetics Engineering. Structure/Properties Relations & Applications" M. Yalpani (Ed), Elsevier Science B.V. Amsterdam, pp. 363 – 376 (1987).

4. T. Mitani, C. Nakanima, I. E. Sugkano and H. Ishii, *J. Environ. Sci. Health Part A. Environ. Sci. Eng. Toxic.*, **30**, 669 (1995).
5. A. F. Kotze, H. L. Luessen, M. Thanou, J. C. Verhouf, A. G. de Boar, H. E. Junginger and C. M. Lehr, In "Bioadhesive drug delivery systems" E. Mathiowitz, D. E. Chickering III and C. M. Lehr (eds), Marcel Dekker Inc. N. Y. pp. 341 – 385 (1999).
6. P. He, S. S. Davis and L. Illum, *Int. J. Pharm.*, **166**, 75 (1998).
7. G. Biagini, R. A. A. Muzzarelli, R. Giardino and C. Castaldini, in "Advances in Chitin and Chitosan, Vol – 1" C. J. Brine, P. A. Sandford, J. P. Ziakis (Eds), Elsevier Science, Barking, pp. 16 – 24 (1992).
8. H. Ueno, T. Mori and T. Fujinaga, *Adv. Drug. Deliv. Rev.*, **52**, 105 (2001).
9. R. A. A. Muzzarelli, *Cell. Mol. Life. Sci.*, **53**, 131 (1997).
10. X. F. Liu, Y. L. Guan, D. Z. Yang, Z. Li and K. D. Yao, *J. Appl. Polym. Sci.*, **79**, 1324 (2001).
11. D. Koga, *Chitin Enzymology*, R. Chen and H. C. Chen (Eds) in "Adv. Chitin Sci.", **3**, 16 (1998).
12. E. Wuolijoki, T. Hirvala and P. Ylitalo, *Methods Find Exp. Clin. Pharmacol.*, **21**, 357 (1999).
13. Y. Fukuda, K. Kimura and Y. Ayaki, *Lipids.*, **26**, 395 (1991).
14. L. K. Han, Y. Kimura and H. Okuda. *Int. J. Obs. Relat. Metab. Disord.*, **23**, 174 (1999).
15. D. Capitani, A. A. De Angelis, V. Crescenzi, G. Masci and A. L. Segre, *Carbohydr. Polym.*, **45**, 245 (2001).
16. V. R. Patel and M. M. Amiji, *Pharm. Res.*, **13**, 588 (1996).
17. M. V. Risbud, A. A. Hardikar and R. R. Bhonde, *J. Biosci.*, **25**, 25 (2000).
18. R. S. Sharma, S. A. Euden, S. L. Platton, D. N. Cooke, A. Shayafat, H. R. Hewitt, T. H. Marrczylo, B. Morgan, D. Plummer, S. M. Hemingway, M. Primohamed, J. A. Gescher and W. P. Steward, *Clin. Canc. Res.*, **10**, 6847(2004)
19. C. V. Rao, A. Rivenson, B. Simi and B. S. Reddy, *Canc. Res.*, **55**, 259 (1995)
20. T. Kawamori, R. Lubet and V. E. Steele. *Canc. Res.*, **59**, 259 (1999).
21. A. L. Cheng, C. H. Hsu and J. K. Lin, *Anti Canc. Res.*, **21**, 2895 (2001).
22. G.P. Lim, T. Chu, F. Yang, W. Beech, S. A. Frautschy and G. M. Cole, *Neurosci.*, **21**, 8370 (2001).
23. J. L. Funk, J. N. Oyarzo, J. B. Frye, G. Chen, R. C. Lantz, S. D. Jolad, A. M. Solyom and B. Timmerman, *J. Nat. Prod.*, **69**, 351 (2006).
24. M. Egan, M. Pearson, S. A. Weiner, V. Rajendran, D. Rubin, J. Glockner-Pagel, S. Canny, K. Du, G. Luckas and M. J. Kaplan, *Science.*, **304** [5670], 600 (2004).

25. S. P. Mitra, *J. Surf. Sci. Technol.*, **23**, 91 (2007).
26. K. A. Alkhamis, W. M. Obeidat and N. M. Najib, *AAPS PharmSciTech*, **2**(1), Article 3 (2001).
27. T. Imai, S. Shirashi, H. Saito and M. Otagiri, *J. Pharmaceutics.*, **67**, 11 (1991).
28. K. Fabio, D. P. Felipe, E. A. Bernard and J. C. F. Moreira., *Photochem. Photobiol. Sci.*, **2**, 856 (2003).
29. M. Murata and S. Kawanisih, *J. Biol. Chem.*, **275**, 2003 (2000).
30. X. Zhang, B. S. Rosenstein, Y. Yang, M. Lebowhl, and H. Wei, *Free Radical Biol. Med.*, **23**, 980 (1997).