SOLUBILISING CURCUMIN, SYNTHESIZING GOLD NANOPARTICLES AND THEIR ANTI-OXIDANT PROPERTY
Curcumin is a phytochemical that gives yellow color to turmeric and is responsible for most of the therapeutic effects of turmeric. The keto-enol-enolate equilibrium of this heptadiene-dione moiety determines its physiochemical and antioxidant properties. However, solubility of curcumin is very low in water at neutral pH and room temperature, which decreases its bioavailability. The mechanism behind its solubility issues at various temperatures and the influence of interplay of temperature, dielectric environment and intra/intermolecular H-bonding in curcumin leading to aggregation-disaggregation in various temperature regions is investigated. A remarkable change in the temperature dependent electronic transition behavior of the molecule in water is observed. The study indicates that it is perhaps the breaking of the intra-molecular hydrogen bonding which leads to the exposure of the polar groups and hence responsible for the dissolution of curcumin at higher temperature. We believe that formation of inter-molecular aggregates might be responsible behind a better room temperature stability of the molecules after cooling its aqueous suspension from 90°C to 25°C.

The synthesis of gold nanoparticles using curcumin in water around 90 °C is carried, thereby increasing the possibility of enhancing the bioavailability and drug-action. In this method curcumin acts both as a reducing agent and as a capping agent. The nanoparticle size (~20 nm) is quantified by transmission electron microscopy. The synthesized nanoparticles are very stable and show good anti-oxidant activity.
The study in this chapter began as an endeavor to functionalize gold nanoparticles with a strong anti-oxidant such as curcumin, which would have multiple advantages. This chapter comprises of two parts: **Part I** deals with the study of the temperature-dependent spectroscopic behavior of curcumin in water and **Part II** comprises of the use of the aqueous curcumin to synthesis gold nanoparticles and their characterization.

### 5.1.1. PART I: Study of dissolution of curcumin in water

Turmeric is a wonder drug¹ which has been therapeutically used in the Indian subcontinent for various ailments including wound-healing, skin-lightening (as a cosmetic), anti-inflammatory and antimicrobial since time-immemorial.² Interestingly, it was also included as an inseparable part of the Indian/Asian cuisine in the form of spice and coloring agent since time immemorial. Curcumin or diferuloylmethane (1, 7-bis [4-hydroxy-3-methoxyphenyl]-1, 6-heptadiene-3, 5-dione), is a major component (2-6%) of turmeric, isolated from rhizomes of the perennial herb *curcuma longa* and is being recognized as being responsible for most of its therapeutic effects.³⁻⁵ Curcumin, also called as the Indian solid gold, has highly active medicinal properties such as anti-oxidant and anti-inflammatory, anti-cancer, anti-tumor, inhibitor of angiogenesis and neuroprotective, to name a few.⁶⁻⁸ Owing to its wondrous actions in protecting the human body, the molecule is being recently revisited using modern science and technological tools, and is revived back from the age-old ayurvedic medicine to validate the age-old practices in a scientific way. Neurodegenerative diseases like the Alzheimer’s disease are associated with the formation and accumulation of proteins like amyloid-beta (Aβ) plaque in this case, that leads to declined memory and other neurocognitive functions of the brain.⁹ (Figure 5.1)
Curcumin has shown potent anti-amyloidogenic effects for Alzheimer’s amyloid fibrils \textit{in vitro}\textsuperscript{10} as well as \textit{in vivo}\textsuperscript{11}. It is reported that the low molecular weight and the hydrophobic nature of curcumin allows it to penetrate the blood brain barrier effectively and bind to the beta amyloids.\textsuperscript{11} Recently scientists have established a direct link between its anti-neurodegradation properties as well as relatively lower number of neurological diseases in Indian subcontinent (such as Alzheimer’s and Parkinson’s disease)\textsuperscript{11, 12} and have attributed it to its direct intake as part of the regular diet in Indian curries. Curcumin has also shown to reduce induced-memory deficit in rat\textsuperscript{13}. An extensive research on curcumin and its biomedical pathways reveal that curcumin possesses strong inflammatory and anti-oxidant properties, contributing to antiseptic and analgesic, anti-bacterial\textsuperscript{14, 15} and anti-fungal\textsuperscript{16}, anti-malarial and insect repellant properties.\textsuperscript{17} It is also recently reported that curcumin kills tumor cells, suppresses lipid peroxidation and angiogenesis\textsuperscript{18} by affecting the action of a wide variety of growth factors and enzymes. Curcumin has been shown to down-regulate the activity of human EGFR-2 (called HER2/neu), a growth factor...
receptor closely linked with cancer of the breast, lung, kidney and prostate.\textsuperscript{19} It is reported to be cancer preventing and cancer curing as well.\textsuperscript{6, 19, 20} Noting its curing abilities, curcumin is studied for widespread clinical applications.\textsuperscript{21} The immunomodulatory effects of curcumin has been studied in depth and shown that it exerts either null or beneficial effects on cellular immune functions \textit{in-vivo}.\textsuperscript{22} Various studies have shown that it is safe to use curcumin even at high doses. In spite of all the medicinal properties exhibited by curcumin, it is not yet a pharmacological drug due to its very low bioavailability. Owing to extremely low solubility of curcumin in water, it has a very poor bioavailability.\textsuperscript{23} In humans, after 1h of administration of 4-8 g of curcumin, showed peak plasma levels of 0.41–1.75 μM\textsuperscript{24} whereas in an oral dose, the peak plasma level of curcumin was at 11.1 nmol/L.\textsuperscript{25} Despite its lower bioavailability, the therapeutic efficacy of curcumin against various human diseases, including cancer, cardiovascular diseases, diabetes, arthritis, neurological diseases and Crohn's disease, has been widely documented as mentioned above. The potential of therapeutic applications has triggered an interest in manipulating the solubility of curcumin in water by chemical modifications of the molecular structure. However, its stability and solubility in water still remains poorly understood. Its promise as an efficacious and safe drug is precluded due to low aqueous solubility and consequently, poor bioavailability \textsuperscript{23, 26} hindering the direct use of curcumin as a biomedicine. The strong hydrophobicity of the conjugated alkene chain and the unavailability of polar group render the molecule insoluble or sparingly soluble in polar solvents. Curcumin is practically insoluble in water at neutral pH. However, in slightly acidic media and possibly in the interior of cell membranes, it is likely to exist in the keto form. This form appears to favor H-atom transfer reactions,\textsuperscript{27} thus playing a crucial role in the antioxidant action of curcumin. For ages, curcumin has been consumed in the form of powdered turmeric added into oil, milk or water for Indian curries while cooking at high temperatures. Enhanced bioavailability of curcumin as a drug in the near future is likely to bring this promising natural product to the forefront of therapeutic agents for treatment of human disease. Henceforth, several methods of conjugation have been adopted for enhancing the solubility and/or its bioavailability (Part II). It is important to note that the expected phase transfer from organic phase to aqueous phase in the human body.
is quite less and administration of the drug in the aqueous phase may help in increasing its bioavailability.

Keeping in mind these challenges, present study involves experiments to solubilize curcumin by heating it in the aqueous medium and performing detailed in-situ UV visible spectroscopic studies to investigate various aspects of dispersion such as the effect of medium on the UV-vis absorption peak, peak shape and the absorption maxima, size of the agglomerates, etc.

Though the UV visible spectroscopy is largely accompanied in the past few decades by Raman Spectroscopy, ESCA studies, NMR spectroscopy, nonetheless it is still a very powerful tool in studying the molecules that absorb in the UV visible radiation. Our goal in this study is to investigate and perform a systematic study on whether curcumin gets dissolved in water by heating it at relatively higher temperature and thereupon the effects of heating on the structural stability as well as long term solubility of the drug. In a previous study by Kurien et al., the mechanism of dissolution of curcumin in water at high temperature is not explained and the quantitative data on its solubility in water is erroneous, as it is calculated from the standard curve of curcumin in methanol, neglecting the corresponding solvent effects in $\lambda_{\text{max}}$ and $\varepsilon$ (molar extinction coefficient), and intensity changes due to the specific preferential solubility in different solvents.

A number of investigators have studied the structure of curcumin, solutions of curcumin in different organic solvents, predictions of the electronic and vibrational excitations and their experimental data; however, a detailed study of curcumin in water at different temperatures is not reported. It is observed that the spectrum of curcumin in water is complicated, uniquely temperature-dependent and different from what is obtained in organic solvents.

### 5.1.2. Results and discussions

The purified curcumin used in this study was a kind gift from Dr. K. J. S. Rao and Dr. P. Srinivas, Central Food Technological Research Institute (CFTRI), Mysore, India. The purity of the as-received curcumin is tested by using techniques such as UV-vis spectroscopy (Figure 5.4), FTIR spectroscopy (Figure 5.10), NMR spectroscopy (Figure 5.9) and HPLC (Figure 5.11).
5.1.2.1. Temperature dependent UV-vis absorption spectroscopy of curcumin in water and effects of intra/inter-molecular H-bonding on the electronic transitions

In Figure 5.2A, we have shown the structure of curcumin. Here, the β-di-ketone moiety undergoes keto-enol tautomerisation\(^\text{29}\) and the molecule exists in a planar, intra-molecularly hydrogen-bonded form; both in solution as well as in the solid state.\(^\text{30}\)

![Curcumin, Demethoxycurcumin, Bis-Demethoxycurcumin](image.png)

**Figure 5.2.** Structure of (A) curcumin, (B) demethoxycurcumin and (C) bis-demethoxycurcumin.

Curcumin is totally hydrophobic in nature due to the lack of any polar groups in the molecule as well as due to the stretch of conjugated back-bone (heptadiene) of curcumin.

The visible and ultraviolet spectra of organic compounds (like curcumin) represent the transitions between the electronic energy levels. These transitions are generally between a bonding or lone-pair orbital and an unfilled non-bonding or anti-bonding orbital. The maximum absorption wavelength of the spectroscopy of the compound is a measure of the separation between the energy levels of the orbitals concerned. An isolated double bond / lone pair of electrons gives rise to a strong absorption
maximum around 190 nm $^{31}$ whereas presence of conjugation reduces the energy separation between the orbital occasioned by absorption at longer wavelengths. In organic solvents, the enolisation of the diketone group in curcumin allows conjugation between the $\pi$-electron clouds of the two vinylguaiacol parts. This leads to a common conjugated chromophore, resulting in reduction in energy. Due to the low-energy $\pi-\pi^*$ excitation of that chromophore, the solution of curcumin in organic solvents (primarily ethanol or methanol) typically absorbs around $\sim$420 nm thus exhibiting a bright yellow color. The $n-\pi^*$ transition, due to excitation of an oxygen lone-pair electron to the anti-bonding $\pi$-orbital of the carbonyl group in curcumin is observed around 262 nm in methanol.

However, in aqueous systems like water, it is understood that at alkaline pH, the acidic phenol group in curcumin donates its hydrogen, forming the phenolate ion that enables curcumin into dissolution in water. However, the molecule is not stable for long at neutral and alkaline pH and gets easily degraded into compounds like vanillin, ferulic acid, etc. Below pH 7, curcumin is stable but parallel with the decreasing pH values, the dissociation equilibrium shifts towards the neutral form of very low aqueous solubility. Due to this process, significant change of the UV-vis absorption spectrum of curcumin can be observed at acidic pH values. However, in neutral pH, it is practically insoluble at room temperature.

To check the effect of temperature on the solubility and stability of curcumin in water, we took about 2 mg of curcumin in 3 ml of deionized water (purified through a Millipore Milli-Q system with a resistivity of 18 MΩ/cm) at neutral pH and performed an in situ temperature dependent absorption spectroscopy up to 95 °C, with spectrum acquisition at an interval of every 5 °C rise in temperature. Figure 5.3A shows the spectra of curcumin in water as the temperature is increased from 25 °C to 95 °C. We observe that as the temperature is increased, the peak intensity also increases around wavelengths 237 nm, 345 nm and 419 nm and the color of the solution turns turmeric yellow. The increase in intensity of the peak above 400 nm is plotted as a function of temperature in Figure 5.3B. A comparision of the spectrum of curcumin in water and the spectrum of curcumin in methanol is shown in Figure 5.3C. This will help in understanding the following explanation.
We observe solvatochromic shifts in the temperature-dependent absorption spectroscopy of curcumin in water as we compare the spectrum of curcumin in methanol (see Figure 5.3C) and in water (more polar compared to methanol) (Figure 5.3A). In general, the solvent effects on the $\pi-\pi^*$ and the $n-\pi^*$ transitions could be red-shifted or blue-shifted depending upon the polarisabilities of the solute-solvent interactions and thereof their effects on the electron reorganization in both the solute and the solvent. Most transitions result in an increase in the polar nature in the excited state than the ground state. Distortions of the chromophore may also lead to red or blue shifts depending on the nature of the distortion.

![Figure 5.3. (A) Temperature dependent UV-Visible spectra of curcumin in water, (B) Change in intensity (of absorption at $\lambda_{\text{max}} \sim 420$ nm) vs. temperature and (C) Comparison spectrum of curcumin in water with curcumin in methanol.](image)
In the case of weak forbidden n-\(\pi^*\) transitions of the oxygen lone-pair in ketones, the solvent effect is due to the lesser extent to which the solvents can H-bond to the carbonyl group in the excited state. In methanolic solution of curcumin, the absorption maxima of the n-\(\pi^*\) transition is at 262 nm, whereas in aqueous solution the maximum is at 237 nm. Clearly the n-\(\pi^*\) transition is blue-shifted as we move from methanol to water. The effect of temperature seems not to affect the structure of this electronic transition and thus blue-shift is due to the solvent effect.

However, the \(\pi-\pi^*\) transitions of curcumin in water is complicated unlike the n-\(\pi^*\) transition. In case of the \(\pi-\pi^*\) transition, generally the opposite effect occurs where the dipole-dipole interactions with the polar solvent molecules will lower the energy of the excited state than that of the ground state. Thus, we expect a red shift for the \(\pi-\pi^*\) transition as we go from methanol to water. However, we note that the absorption peak for the \(\pi-\pi^*\) transition in methanol is at 420 nm and in water, we observe two peaks at about 345 nm and 419 nm; note here that the intensity of the peak at 345 nm is more than that of the peak at 419 nm. The evolution of two absorption peaks for the \(\pi-\pi^*\) transitions of curcumin in water indicates change in the tautomeric form of the keto-enol-enolate group in curcumin, due to both the solvent effect and the temperature. Temperature, \textit{per se}, does not affect the signature of the peaks in the absorption spectroscopy. We see that the absorption spectrum of curcumin in methanol (boiling point of methanol is 64.7 °C) does not change with varying temperature, as shown in Figure 5.4, except mild decrease in intensity with increase in temperature due to the Boltzmann distribution of the populations at ground state at different temperatures.\(^{32}\)

It appears that the resonance stability in the keto-enol-enolate is changed sharply as the temperature is increased. The thermal energy provided by heating could break the intra-molecular H-bonding of the keto-enol-enolate group in curcumin exposing the polar groups to the solvent. However, instead of a red-shift caused by more dipole-dipole interactions between curcumin and water molecules, we observe a remarkable blue-shift in the peak at 345 nm.
The blue-shift could be due to two factors:

(1) the high temperature of the solution could hinder the interaction between the solute and the solvent, preventing them to form inter-molecular H-bonding, and hence reducing the dipole-dipole interactions and increasing the energy, and
(2) the opening of the cyclic moiety (keto-enol-enolate group) in curcumin, going into its keto form, has lowered the conjugation effect of the molecule and thus increasing the energy.

Thus, as the temperature of the sample is increased, it is observed that the positions of the uncoupled absorption peaks of the $\pi-\pi^*$ transition moves to higher frequency. This is considered to result from a decrease in the H-bond strength between the water molecules and curcumin, at high temperatures, where the keto-enol/keto groups of curcumin could have been available for interaction with water molecules (due to breakage of intra-molecular H-bonding). As the temperature increases, the H-bond strengths become progressively weaker as a result of increased distortion and elongation of the H-bonds. With respect to the absorption characteristics of the molecule, it is evident that there are environmental changes caused by the breakdown of the intra-molecular H-bond in the keto-enol tautomer as the temperature is raised, leading to the change in the electronic transition energy. And
as the conjugation decreases, the wavelength of the absorption maximum also decreases. Both non-specific dipolar interaction and specific H-bonding interaction play an important role in the position of the absorption and fluorescence maxima of any sample.

**5.1.2.2. Effect of temperature on the spectrum of curcumin in water, solvent effect and its proposed mechanism**

To understand the process involved, we acquired in situ UV-vis spectroscopy of curcumin in water with heating, cooling and re-heating, as shown in Figure 5.5A. Upon heating to about 90 °C, we obtain the spectrum (black curve) similar to what we saw in Figure 5.3A. However, upon cooling, to room temperature (25 °C), there is a remarkable change in the absorption spectra (red curve).

![Figure 5.5(A)](image)

**Figure 5.5.** (A) UV-Visible Spectroscopy of curcumin in water; the graph shows spectra taken at 90 °C, cooled back to room temperature ~ 25 °C and reheated to 90 °C. Inset: (i) Optical transmission of curcumin in water at 90 °C and (ii) Turbid solution of curcumin in water at 25 °C.

The peaks observed at 90 °C are 237 nm, 345 nm and 420nm and after cooling, the peaks observed are 275 nm, 431 nm and 500nm. The n- π* transition is red-shifted
upon cooling owing to aggregation and less polar microenvironment caused due to aggregation and clearly, there seems a phenomenal change in the $\pi-\pi^*$ transition. And this change is a physical change, as the peaks gain back the respective position (237 nm, 345 nm and 420 nm) upon re-heating to 90 °C. Also, the shift from 431 nm to 420 nm upon heating indicates breaking of the interactions therein. Therefore, the spectrum analysis implies the picture of curcumin structure that could be visualized as one in which the structural clusters of curcumin flicker into and out of one form to another form on a rather rapid time scale, maintaining an equilibrium between the different states that is dependent on the temperature of the system. Thus the structural model of curcumin is proposed, based on the spectroscopic data, to be composed of a relatively small number of distinguishable (by absorption spectroscopy) species. The structural arrangements for the species vary with respect to the temperature and solvent effect. An increase in temperature results in an increase in the number of the H-bonds broken and therefore, a variation in the type, size and/or number of the structural units. Conditions which would change the distribution of the strength of the H-bonds would necessarily change the absorption maximum of the molecule.

Thus, depending upon the temperature of the system, the discussion of the results obtained is as follows: as explained before, upon heating, the breaking of the intra-molecular H-bond leads to the absorption maximum at 345 nm. This is one population of curcumin molecules with their polar groups exposed, existing in the solvent at that temperature along with the other population whose distribution contributes to the $\lambda_{\text{max}}$ at 420 nm, where the intact molecules are not much affected by the polar water environment. The peak at 420 nm is characteristic of curcumin in almost all organic solvents (except like ethyl amine, which is highly basic). Upon cooling down, the formation of H-bonding, non-specific dipolar or hydrophobic interactions takes place. Thus, where there was a sharp peak at 345 nm when the sample was hot, there exists a small shoulder upon cooling to room temperature. The species of curcumin existing in this state is converted to other forms (whose transitions are detected at 431 nm and 500 nm). Since more molecules are converted into the form absorbing at 420-430 nm, where molecules already exist, we note an increase in the intensity. Thus the intensity of the 420 nm peak is shot up with a red-
shift to 431 nm after cooling. Once the polar groups in the molecule are exposed by heating, when it is cooled back, there are possibilities of intra-molecular H-bonding, inter-molecular H-bonding and hydrophobic interactions between curcumin molecules and inter-molecular H-bonding between curcumin and water molecules. Owing to these interactions the small red-shift could be explained at 431 nm. However, we also observe another shoulder at 500 nm, which could be due to larger aggregates of curcumin molecule formed upon cooling. This peak is definitely absent upon re-heating the solution. There are both empirical and theoretical attempts made to relate the principal or longest wavelength maximum with chain length; as evidenced, the increasing value of $\lambda_{\text{max}}$ alludes in a conclusive manner for increasing length of the conjugated curcumin molecule when it is present in the aggregated state. When the sample is re-heated, the intensity is increased more than before due to the obvious reason that now more molecules are exposed due to the intra-molecular H-bond breaking in the system.

More proof of the proposed hypothesis is shown in the inset of Figure 5.5A, where the optical transmission of the sample is exhibited. Sample vial (i) is that of curcumin in water at 90 °C, and it is an optically transparent solution. The same sample is cooled to room temperature (ii), and we observe that there is turbidity in the sample. Here, as seen in the picture, owing to the milky appearance of the sample, we suspect that the spectrophotometry of this turbid suspension of curcumin particles in water involves a large scattering contribution. We can infer from this temperature dependent behavior of the sample that the aggregation of the molecules take place upon cooling. It is also important to note that curcumin dissolved in other solvents obey Lambert-Beer’s law suggesting the absence of aggregation of molecules, however, in water, curcumin fails to obey the same. Figure 5.5(B) shows a schematic of the mechanism explained above.
Additionally, to understand the dependence of these peaks of curcumin in water on the solvent also, we took equal amounts of curcumin in different ratios of water and methanol percentage. The absorption spectra of the sample is shown in Figure 5.6, where at 50% of methanol and water mixture, the principle peak is at 420 nm alone though there is also a weak shoulder around 350 nm. As the percentage of water increases, we note that the intensity of the peak at 350 nm increases however it remains relatively lower than the peak at 420 nm unlike the spectrum of curcumin in water alone at high temperatures. Note that in water, at high temperature, the peak was obtained at 345 nm, where as in water-methanol mixtures it is 350 nm, in accordance with the fact that the mixture is less polar. Though the presence of methanol in water reduces the solvation energy required for curcumin to dissolve in the mixture, water molecules being highly miscible with methanol easily forms strong H-bonds with methanol and hence it does not reduce the energy required for the π- π* transition. Therefore, we observe blue-shift. This implies that the peak observed in water around 345 nm is solely due to the solvent effect; wherein the structure of the curcumin molecule in that state gives rise to this signature.
In our study, we did not use half-curcumin, 4(4-hydroxy-3-methoxyphenyl)-3-buten-2-one (hC), a close analog of ferulic acid as a potential model of curcumin, due to the obvious difference in the spectral properties between curcumin and hC in water.

To further study this system, we filtered the solution, containing 0.5 g of curcumin in 325 ml water, after heating it up to 95°C, using Whatman filter paper (retention size ~ 11 micron); much of the insoluble curcumin remained as residue owing to its sparingly soluble nature even at high temperature. The filtrate is a stable yellow solution of curcumin dispersed homogeneously in water as explained before. Then, immediately, while it is still hot at > 95°C, we filtered the same solution using 20 nm filter. Surprisingly, we found that when filtered immediately, the dilute solution of curcumin filtered out as shown in vial (i) in Figure 5.7. However, upon filtering the curcumin solution, after cooling back to room temperature, most of the color in the solution was lost as shown vial (ii) in Figure 5.7, indicating that the curcumin molecules, though in solution, has aggregated into larger sizes and are not filtered out by a 20 nm filter, supporting the explanation given above. Though this is a simple physical test, it strongly supports the mechanism.
This is an important observation, in accordance with our absorption data, revealing that as the temperature begins to reduce, curcumin mostly precipitates back to its stable form of keto-enol-enolate tautomer, recrystallising into few monomer units. The more intriguing observation is the fact that not all of the curcumin precipitates back immediately. We have observed that for more than a month’s time, the curcumin in water remains stable, which can be used for any further assays or applications. Hence, without the use of a co-solvent, or other chemical means to modify the molecule and thus affecting its activity, we note that curcumin gets into the water phase and remains stable.

5.1.2.3. Establishing and estimating solubility of curcumin in water

‘Solubility’ of curcumin in water upon heating is an ambiguous term to use in this study. As observed and established in the above explanations, the tests reveal that curcumin is more dispersed in the solvent (water) than dissolved, though it is readily available for biochemical reactions and drug actions. And the amount of curcumin in the solvent is extremely low even after heating it in water. Establishing the amount of curcumin that goes into the solution phase with acceptable accuracy is difficult. That explains the lack of any work relating to work on curcumin alone in water so far in literature. After repeated tests to estimate the amount of curcumin that is in

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**Figure 5.7.** Picture of curcumin in water after filtering through a 0.22 μ filter at different temperature. (i) Color of solution filtered immediately after dissolution by heating and (ii) Color of solution filtered after cooling.
water, we could roughly calculate 1-10 μg/ml, using gravimetric analysis. However, since decrease in temperature could lead to the molecule precipitating out of water to form aggregates of solid curcumin, ensuring the level of curcumin in solution phase could be erroneous. Hence we cannot accurately calculate the molar extinction coefficient of curcumin in water, as reported in some previous studies, since aggregates are formed and Lambert Beer’s law cannot be applied. Moreover, for conjugated dienes, $\varepsilon_{\text{max}}$ cannot be predicted with accuracy.

5.1.2.4. Addressing the stability of curcumin in water

To determine the stability of curcumin in water, we used UV-vis spectroscopy, FTIR spectroscopy, NMR spectroscopy and HPLC techniques. The aqueous sample was filtered through a Whatmann filter paper to remove particulates, dried to remove the water and re-dispersed in the desired solvent for all the techniques. For UV-visible spectroscopy study, the dried sample was dissolved in methanol and the spectrum was acquired as shown in Figure 5.8A. There is no change in the spectrum of curcumin sample due to the heat treatment as evidenced in the spectrum.

Note that the amount of curcumin treated in water and re-dissolved in methanol is less compared to the amount of untreated curcumin in methanol as seen in difference in the intensity of $\lambda_{\text{max}}$ and the color contrast in Figure 5.8. Curcumin dried in a similar way was dissolved in deuterated methanol and the sample was submitted for proton NMR spectroscopy.
**Figure 5.8.** UV-vis spectra of curcumin in methanol. The solid curve shows the absorption spectra taken after dissolution of curcumin in water (till 100°C) and thereafter drying it and resuspending in methanol. The broken curve shows the absorption spectra of curcumin in methanol without heat treatment. The picture in inset shows the color of the curcumin in methanol. We have shown two different cases where the darker contrast in the second tube is due to higher amount of dissolved curcumin in methanol.
Figure 5.9. Proton NMR spectra of curcumin in CD$_3$OD. The first spectrum is that of curcumin without any heat treatment and the second spectra is that of curcumin with heat treatment in water.
The $^1$H NMR spectra were run on a 400 MHz Bruker AVANCE instrument using a broad band probe with a z-gradient coil. Chemical shifts ($\delta$) are quoted in ppm and are referenced to solvent CD$_3$OD. The NMR spectra shown in Figure 9 also revealed that there was no change in the molecule.

For FTIR spectroscopy, the sample while heating was taken and coated onto piranha-treated silicon wafers, dried and data acquired from a Perkin-Elmer spectrophotometer. We observe that upon heating neat curcumin, there is no observable vibrational change as shown in the IR spectra below (Figure 5.10).

![Figure 5.10. FTIR spectra of curcumin at different stages of heating in water.](image)

To further analyze, we obtained HPLC chromatograms (Figure 5.11) on the sample for verification. The HPLC system used was Waters Delta 600 series and a wavelength of 425 nm was used for detection. Chromatographic conditions used are as follows: the elution was carried out with gradient solvent systems with a flow rate of 1.0 mL/min at ambient temperature. The mobile phase consisted of methanol (A), water (B), and acetonitrile (C). The sample was determined using the above solvents programmed linearly from 45 to 65% acetonitrile in B for 0-15 min. The gradient then went from 65 to 45% acetonitrile in B for 15-20 min, with a constant of 5% A. The compounds were analysed using HP ChemStation software.
The HPLC analysis of as-received curcumin in methanol shows a peak at retention time of 4.3 min, which corresponds to curcumin (Figure 5.11A). Also there is a small peak at around 3.75 min which corresponds to bisdemethoxycurcumin ((1E, 6E)-1,7-bis(4-hydroxy phenyl)hepta-1,6-diene-3,5-dione) (zoomed view). Bisdemethoxycurcumin is another curcuminoid present in turmeric whose structure (Figure 5.2B) is similar to that of curcumin. Since these two curcuminoids acts synergically, there will always be a trace of bisdemethoxycurcumin along with curcumin. The HPLC analysis of heat treated curcumin (Figure 5.11B) shows that curcumin has not undergone any chemical change or degradation because of heat treatment. However, the intensity of the peak corresponding to bisdemethoxycurcumin is enhanced little bit; this can be explained by the absence of non-polar methoxy group in this molecule and hence enhancing its availability in polar water as compared to curcumin with two methoxy groups. Further
quantification into these processes is required; however, due to the sparingly low availability of the compound (note the intensities in the HPLC chromatogram) in the aqueous solvent even after heating, it is difficult to analyze.

5.1.2.5. Fluorescence of curcumin in water

In Figure 5.12, we have shown the photoluminescence spectrum (excitation wavelength is 420 nm) of curcumin in water at room temperature, after dissolution at high temperature and filtering it through the Whatman filter paper. As evident from the emission spectra, curcumin emits quite strongly in water.

![Photoluminescence of curcumin in methanol (1) and water (2) at the excitation wavelength of 420 nm.](image)

**Figure 5.12.** Photoluminescence of curcumin in methanol (1) and water (2) at the excitation wavelength of 420 nm.

In a previous report, Chignell et al.\textsuperscript{26} showed that both intensity and the wavelength of the most intense emission peak of curcumin are highly sensitive to the nature of the solvent in comparison to the absorption spectra. In our study we note that at least between changing the solvent from water to methanol, the emission spectra does not change a lot. It is important to note in our results that the emission peaks of curcumin in water is tending to more symmetrical as compared to that of in methanol; this shows that the curcumin and solvent aggregates are not non-symmetrical in nature.

From Chignell et al.’s observation that the position of most intense absorption band in spectrum of curcumin is solvent independent and its fluorescence intensity is solvent dependent, we see in our study that the fluorescence absorption is solvent independent. This could be because of the packing of curcumin molecules into
closed structures and thus the feeble difference in the spectra in spite of the large
difference in the interaction of curcumin with water and the interaction of curcumin
with methanol.

5.1.3. Conclusions
Little or no spectroscopic work is reported about the behavior of curcumin in
aqueous system. We present a detailed spectroscopic data on the interaction of
curcumin with water in a temperature dependent manner. By contrast, considerable
attention has been given to the spectra of curcumin in various organic solvents in
various reports, where the absorption contributions are assigned to the molecular
structure with respect to the solvent in which it is dissolved. Electronic transitions
are characteristic of molecular structure of the molecule. A change in the electronic
transition therefore represents a change in the molecular structure; however, there are
other factors that could affect the transition, such as the solvent effect, presence of an
auxochrome, temperature, aggregations of molecules, etc. To clearly establish the
stable conformation of the molecule in aqueous solution, computational predictions
are required and further detailed experimentation is needed. At this stage, it is not
possible to state the correct conformation of curcumin in water at high temperature.
We observe in our study that the solvent plays a major role in the absorption
spectrum of curcumin and we make use of this interaction to understand the process
of dissolution. In this work, we have shown that curcumin is dispersed in water at
high temperatures and aggregates upon cooling, with some precipitation out of the
solvent. Based on our detailed spectroscopic measurements at various temperature
conditions, we propose that it is the breakage of intra-molecular H-bonding that
probably leads to the increased availability of curcumin molecule in water. As the
temperature is increased, the thermal energy helps in breaking the bonds and exposes
the polar hydroxyl (—OH) and keto (>C=O) group which enhances the solubility of
the amphiphilic molecule. Also, our gravimetric analysis estimates its amount to 1-10
μg/ml at ~ 95 °C. Further study is also carried out to investigate the changes, if any,
upon heating curcumin in aqueous phase and we observe that there is no degradation
of the molecule. The curcumin dissolution at high temperature in water is made use
of synthesizing gold nanoparticles as explained in the next section.
This study would help further research on curcumin in water and its applications in biological systems and pharmaceutical industries. Possible enhancement in its bioavailability cannot be overlooked considering the promising utility of this report.
5.2.1. PART II: Synthesis of gold nanoparticles using aqueous curcumin

Among other possible ways to overcome this problem of bioavailability of curcumin, nanoparticles, specifically metal nanoparticles, seem to overcome this problem in aqueous media, providing enhanced activity due to (1) conjugation onto a large surface to volume moiety, (2) increased half-life, (3) enhanced stability and (4) resistance to metabolic processes. Previous reports of use of nanoparticles as carriers of curcumin, circumventing the hydrophobicity of the molecule as a hindrance in its solubility involves a polymer-based nanoparticle. However, these results proved little advantage in terms of enhanced solubility/availability in water and potent anti-oxidant activity.

We have shown in our recent paper that the curcumin heated to increase its solubility is stable enough to retain its chemical nature and anti-oxidant activity [Ramya Jagannathan, et al. (communicated)]. In the present paper a new and a very promising tool against various disorders and diseases (such as neurodegenerative & cardiac problems) has been proposed, which aims at an improved pharmacokinetic activity of curcumin using noble metal nanoparticles. The high surface areas of these small particles allow them to be solubilised into the bloodstream where micro particles or larger particles cannot. Gold nanoparticles have been proved to be effective drug carriers and diagnostic probes. Biocompatibility of gold nanoparticles is well reported. This work demonstrates the promising use of curcumin in conjugation with gold nanoparticles as a good showcase of drug delivery. We have used curcumin in situ to synthesis gold nanoparticles in aqueous system, where curcumin acts as a reducing agent.

The anti-oxidant activity of the curcumin reduced gold nanoparticles being functional; it could act as a promising pharmacological drug for human use. With the efforts to enhance the solubility and availability of curcumin, this work has paved a new way of using the curcuminoids towards possible drug delivery and therapeutics. Extensive research is carried on and a plethora of review articles are available on the use of gold nanoparticles for drug and gene delivery.
5.2.2. Results and discussions

The synthesis of gold nanoparticles comprises of a facile mixing of the reactants around a temperature of 90 °C as explained. The deep red color of the reaction mixture indicates the formation of gold nanoparticles. We tried the reduction of various concentrations of chloroauric acid by curcumin, which helped us in determining the optimum concentration of chloroauric acid by measuring the absorbance in the UV-NIR region. For the synthesis of gold nanoparticles using curcumin, the aqueous solvent (500 ml) was heated on an oil bath up to 95 °C, when chloro auric acid (optimized concentrations is $1.5 \times 10^{-4}$ M) followed by curcumin (4 mg) was added under constant stirring. The high temperature of the reaction mixture was to solubilise curcumin in water and also to kinetically control the nucleation of the gold nanoparticles so as to achieve monodisperse spherical particles. Immediately after mixing the reactants, heating was stopped and the reaction mixture was allowed to cool to room temperature. Within few minutes of mixing, deep red color of gold nanoparticles is formed.

![UV-Visible Spectroscopy of gold nanoparticles at different concentrations](image)

Figure 5.13. UV-Visible Spectroscopy of gold nanoparticles at different concentrations: (1) $1 \times 10^{-4}$ M, (2) $1.5 \times 10^{-4}$ M, (3) $2 \times 10^{-4}$ M, (4) $5 \times 10^{-4}$ M and (5) $5 \times 10^{-5}$ M, synthesized by curcumin.
The pH of the sol is 5.67. It is known that curcumin is a superb H-atom donor, based on the rate constants of H-atom transfer reactions. Therefore, it appears that it is better than well-known, “classical” H-atom donors as thiol\(^2\). This explains the ready reduction of chloroaauric acid into gold nanoparticles. In vivo also curcumin is reported to undergo extensive reduction, followed by conjugation\(^2\). Thus, in view of this, curcumin probably readily reduces gold ions to zero oxidation state gold and also caps them to form stable sol. The bioconjugation onto the gold nanoparticles makes it a very stable sol and the results confirmed that the bioconjugation of curcumin on gold nanoparticles showed an improved bioavailability as compared to curcumin alone. Figure 5.13 shows the surface plasmon resonance of these gold nanoparticles synthesized at different concentrations of chloro auric acid; we observe that unlike our previous reports using different biomolecules for synthesis of gold nanoparticles,\(^{40, 41}\) we do not observe any anisotropic growth here. After the synthesis of colloidal gold solution, we dialyzed the solution to remove any unbound curcumin molecules as well as unreduced gold ions by using a cellulose tube (MW cutoff 12 400 D) against 1 L of deionized water for 9 h at 30 °C. We repeated the dialysis process to make sure the removal of unbound curcumin molecules as well as unreduced gold ions was complete. We used the dialyzed curcumin synthesized gold nanoparticles for various characterizations as discussed.

The isotropic SPR of gold nanoparticles is further supported from the TEM micrographs (Figure 5.14A, B) of the nanoparticles where the particles are largely around 20 nm in size (Figure 5.14C). The particles are primarily spherical in shape, though a few equilateral triangles are also seen in Figure 5.14A.
Figure 5.14. TEM micrographs of gold nanoparticles (1.5 x 10^{-4}M) synthesized by curcumin (A), SAED pattern (B) and the particle size distribution (C).

The 20 nm size of these particles is commendable for use to deliver into biological systems. In this study, the optimized concentration of choloauric acid, 1.5 x 10^{-4} M is used for the synthesis and the as-synthesized gold nanoparticles are used for further characterization and analysis. The pH of the sol is ~5.67, and hence forming stable gold nanoparticles, since it is known that curcumin is stable in acidic conditions. It exists in the keto-form under acidic pH; alkaline conditions degrade the molecule. The stable pH-dependence of curcumin molecules could form stable nanoparticles as well as stable conjugated molecules.

To empirically determine the amount of capping on the gold nanoparticles, thermogravimetric analysis was carried out. The weight loss of the capping molecule on the gold nanoparticles is plotted in Figure 5.15. Though the percentage of weight loss is found to be around 63±10%, it is not conclusive of the amount of the capping agent, since the melting point of gold nanoparticles, corresponding to this size regime (~20
nm) also falls under this temperature range\textsuperscript{42}. Therefore, it would be presumptuous to conclude the weight loss of the capping molecules from the gravimetric analysis. Nonetheless, this experiment provides a depth of the information that could be possibly obtained using such techniques in different systems, for example, as in our case the curcumin synthesized gold nanoparticles.

![Thermo-gravimetric analysis of gold nanoparticles synthesized by curcumin. Curve 1: Curcumin alone and curve 2: Gold nanoparticles synthesized by curcumin](image)

**Figure 5.15.** Thermo-gravimetric analysis of gold nanoparticles synthesized by curcumin. Curve 1: Curcumin alone and curve 2: Gold nanoparticles synthesized by curcumin

Figure 5.16 shows the fluorescence spectra of curcumin and curcumin reduced gold nanoparticles in water. It is known that the effect on chromophore fluorescence in close proximity to the surface of metal nanoparticles is due to the strong electromagnetic field generated at the surface of metal nanoparticles\textsuperscript{43}. The oxidized curcumin molecules on the gold nanoparticles surface interact electronically with the surface to donate electron to the metal, thus quenching the fluorescence by non-radiative pathways available in the metal nanoparticles. Thus we see that the emission peak of curcumin in water, prominent around 550 nm is absent in the gold...
nanoparticles synthesized by curcumin, when excited by the wavelength, $\lambda = 420$ nm.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fluorescence_spectra.png}
\caption{Fluorescence spectra of (1) curcumin in water and (2) gold nanoparticles ($1.5 \times 10^{-4}$ M) synthesized by curcumin in water. The Raman peaks of the water molecule have not been subtracted from the spectra.}
\end{figure}

The anti-oxidant activity of gold nanoparticles synthesized by curcumin was determined using the free radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH'). The radical DPPH' absorbs at 515 nm, which disappears upon reduction by an anti-radical or an anti-oxidant species. Gold nanoparticles synthesized and stabilized by curcumin was reacted with DPPH' and shown to possess anti-oxidant activity, with curcumin (in aqueous phase) alone as control. For each sample tested, the reaction kinetics was plotted as shown in Figure 5.17A. It shows the decrease in the optical density at $\lambda = 515$ nm as the radical DPPH' is quenched by the anti-oxidant.
Chapter 5 - solubilising curcumin, synthesising gold nanoparticles and their anti-oxidant property

Figure 5.17. Anti-oxidant activity by DPPH radical test. (1) Only DPPH in water as control, (2) gold nanoparticles (1.5 x 10^-4M) synthesized by curcumin in water and (3) curcumin in water.

The use of DPPH method provides an easy and rapid method to check the presence of any anti-oxidant property. Since this reaction mechanism is highly solvent dependent, Curve (1) in Figure 5.17A shows plain water with the radical as a control, and we see that there is almost no quenching. And with temporal progression, it is a plateau. Curve (2) is the gold nanoparticles synthesized by curcumin and curve (3) is curcumin mixed with the radical respectively. The observation is a qualitative one owing to the difficulties encountered in quantifying the solubility of curcumin in water and hence a quantitative comparison between the sample and control is not valid here. Hence, the optical density on the ordinate is arbitrary and not comparative for quantifying. We can conclude from the observation of curve (2) and curve (3) that the rate reaction of anti-oxidant activity of gold nanoparticles synthesized by curcumin is progressively enhanced. The slope in curve (2) is more than in curve (3), as evident in Figure 5.16B. We could infer from the anti-oxidant activity that these nanoparticles are functional and could be used for further studies.

5.2.3. Conclusions

In conclusion, gold nanoparticles have been synthesized using curcumin in aqueous phase. These gold nanoparticles, reduced and capped with curcumin, are synthesized in a simple process without harsh chemicals. These curcumin reduced gold nanoparticles exhibit potent anti-oxidant activity, which promises for unlimited
medicinal use. This study warrants future investigation of the potent *in vitro* activity of the bio-conjugated nanoparticles and thereupon pharmacokinetics as well. Further studies such as regarding safety, needs to be guaranteed by performing tests on cells and animals.
5.3. References