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Circular dichroism spectroscopic studies reveal pH dependent binding of curcumin in the minor groove of natural and synthetic nucleic acids†

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For the first time, an interaction between the non-toxic, cancer chemopreventive agent curcumin and both natural and synthetic DNA duplexes has been demonstrated by using circular dichroism (CD) and absorption spectroscopy techniques. Upon addition of curcumin to calf thymus DNA, poly(dG-dC)·poly(dG-dC) and poly(dA-dT)·poly(dA-dT) solutions, an intense positive induced CD band centered around 460–470 nm was observed depending on the actual pH and Na^+ ion concentration of the medium; no CD signal was obtained, however, with single stranded poly(dC). Interaction of curcumin with calf thymus DNA was observed already at pH 6.5 in contrast with poly(dG-dC)·poly(dG-dC) which induces no extrinsic Cotton effect above a pH value of 5. The protonated, Hoogsteen base-paired structure of poly(dG-dC)·poly(dG-dC) is necessary for curcumin binding while the alternating AT-rich polymer formed complexes with curcumin only at certain Na^+ concentrations. Evaluation of the spectral data and molecular modeling calculations suggested that curcumin, this dietary polyphenolic compound binds in the minor groove of the double helix. The mechanism of the induced CD activity, the effects of the pH and Na^+ ions on the ligand binding and conformation of the double helix are discussed in detail. As well as being an essentially new phenolic minor groove binder agent curcumin is also a promising molecular probe to study biologically important, pH and cation induced conformational polymorphisms of nucleic acids.

Introduction

The powdered rhizome of the members of *Curcuma* species (ginger family of herbs) known as turmeric has been used for thousands of years in Asian countries in medicines as well as in cooking.[1,2] The chemical structure of the main yellow biologically active phytochemical compound of turmeric called curcumin has been known since 1910[1] but systematic studies of curcumin and curcuminoids on their potential uses in medicine started only in the eighties. The ongoing research and clinical trials proved this natural phenolic compound to possess great and diverse pharmacological potencies. Beside its effective antioxidant,[3] antiinflammatory[4] and antimicrobial/antiviral[5–8] properties, the compound is also considered as a cancer chemopreventive agent.[9,10] Mechanisms by which curcumin prevents cancer were attributed to several effects including anti-angiogenic action, up-regulation of enzymes detoxifying carcinogens such as glutathione S-transferase, inhibition of certain signal transduction pathways critical for tumor cell growth, suppression of cyclooxygenase expression and neutralization of carcinogenic free radicals.[9–11] However, the molecular basis of the various medical actions of curcumin is far from complete elucidation and research conducted in this area focused only on proteins of the potential macromolecular targets of curcumin.

Using circular dichroism (CD) and ultraviolet-visible (UV/Vis) spectroscopy methods, we report here for the first time that curcumin directly binds to both synthetic and genomic nucleic acids by a unique, pH and ionic strength dependent manner. Both the spectral data and molecular modeling calculations suggested curcumin to bind in the minor groove of the double helix. Based on these results, curcumin has to be considered as a new phenolic, minor groove binder drug lacking any nitrogen functionalities, and its capability for interaction with nucleic acids may help us to better understand the observed anticancer potential and other pharmacological effects.

Experimental

Materials

Calf thymus DNA (ctDNA, highly polymerized sodium salt) was purchased from Calbiochem (42% G + C, mean molecular mass is about 2×10^7). Sodium salt forms of poly(dG-dC)·poly(dG-dC) (average molecular weight 6.0×10^5), poly(dA-dT)·poly(dA-dT) (average molecular weight 2.3×10^6) and poly(dC) (molecular weight 1.2×10^5) were obtained from Amersham Pharmacia Biotech. All DNA samples were used without further purification. Deionized double distilled water and analytical grade reagents were used throughout.

Preparation and handling of DNA stock solutions. ctDNA stock solution was prepared by dissolving the solid material, normally at 0.3 mg ml^{-1}, in distilled water. Then, the solution was kept overnight at 4 °C. The resulting somewhat viscous solution was clear and particle-free. Working standard solutions were obtained by appropriate dilution of the stock solutions. The stock solution was stored at −20 °C. ctDNA concentration in terms of base pair L^{-1} was determined spectrophotometrically by using the molar extinction coefficient of ε_{max} = 13,200 M^{-1} cm^{-1} (base pair)$^{-1}$ at 259 nm.

Twenty-five A_{260} units of poly(dG-dC)·poly(dG-dC), poly(dA-dT)·poly(dA-dT) and 25 A_{270} units of poly(dC) sodium salt were rehydrated in 5 ml double distilled deionized water, frozen and maintained at −20 °C until just before use. Concentrations of poly(dA-dT)·poly(dA-dT), poly(dG-dC)·poly(dG-dC), and poly(dC) polymers in mole base pairs L^{-1} (c_{bp}) were determined by using molar extinction coefficients of 13200, 16800 and 6800 M^{-1} cm^{-1}, respectively. Curcumin purchased from Sigma (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione, catalog no. C-7727) was dissolved in 100% ethanol. The concentration of curcumin stock solution was determined

† Electronic supplementary information (ESI) available: CD and UV/Vis spectra; molecular models. See http://www.rsc.org/suppdata/ob/b4/b409724f/

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spectrophotometrically at 429 nm using a molar extinction coefficient of 55000 M^{-1} cm^{-1}.[1]

Circular dichroism and UV/Vis spectroscopic measurements

CD and absorption spectra were recorded on a Jasco J-715 spectropolarimeter in a rectangular cuvette with 1 cm optical pathlength at 25 ± 0.2 °C. Temperature control was provided by a Peltier thermostat. All spectra were accumulated four times with a bandwidth of 1.0 nm and a resolution of 0.5 nm at a scan speed of 100 nm min^{-1}. The induced CD is defined as the CD of the curcumin–DNA mixture minus the CD of DNA alone at the same wavelength and is expressed as ellipticity in millidegrees (mdeg).

Setting and measuring the pH of sample solutions

The pH of the samples was set by the addition of μl aliquots of dilute HCl and brought back, if necessay, to a given pH with small aliquots of NaOH solution. Spectra were not corrected for dilution by NaCl, HCl and NaOH since the dilution factor was never more than 3%. The pH of the solutions was measured directly using a digital pH meter with a combined glass electrode (Radelkis, Budapest, Hungary).

Concentrated NaCl solution (5 M) was added to the sample solutions to obtain the desired Na^+ concentration.

Molecular modeling calculations

The AutoDock program package[12] was used to study binding of curcumin to the B-form of DNA. The three-dimensional coordinates of a 28 base pair B-DNA element were obtained from the Protein Data Bank (entry PDB code 1AU7). Essential hydrogen atoms were added to the structure by the Sybyl 6.6 program (Tripos Inc.). The starting conformation of curcumin was minimized by the MMFF94 force field. Partial atomic charges were calculated with the Gasteiger–Hückel method for ligand atoms, while Amber charges were applied for DNA atoms. Grid maps were generated with 0.375 Å spacing by the AutoGrid program. 12–10 and 12–6 Lennard-Jones parameters (supplied with the program package) were used for modeling H-bonds and van der Waals interactions, respectively. The Lamarckian genetic algorithm (LGA) and the pseudo-Solis and Wets methods were applied for minimisation using default parameters. Random starting positions, random orientations and torsions were used for the ligand. Subsequent docking of a second ligand to the structure with the lowest energy of the first run was done by the same parameters. All computer modeling procedures were run on a Silicon Graphics Octane workstation under the Irix 6.5 operating system.

Results

UV/Vis spectroscopic properties of curcumin in organic and aqueous solutions

The diarylheptanoid type curcumin (Fig. 1) contains two vinylguaiacol groups joined by a β-diketone unit. The β-diketone moiety undergoes keto–enol tautomerization and the molecule exists as the planar, intramolecularly hydrogen-bonded form both in solution and in the solid state.[13] The enolization allows conjugation between the π-electron clouds of the two vinylguaiacol parts resulting in a common conjugated chromophore. Due the low-energy π–π* excitation of that chromophore, the organic solution of curcumin exhibits a bright yellow color; the main, intense round-shaped absorption band (ε_{max} is between 55000–60000 M^{-1} cm^{-1}, Fig. 2) is centered around 428 nm in ethanol.[1] The very weak, electronic dipole forbidden n–π* transition of the carbonyl group of curcumin is located somewhere above 300 nm but it can not be identified due to the strong masking effect of the neighboring absorption band.

The water solubility of curcumin shows a pH dependence. At alkaline pH values the acidic phenol hydrogens dissociate resulting in a phenolate ion with very good water solubility. In

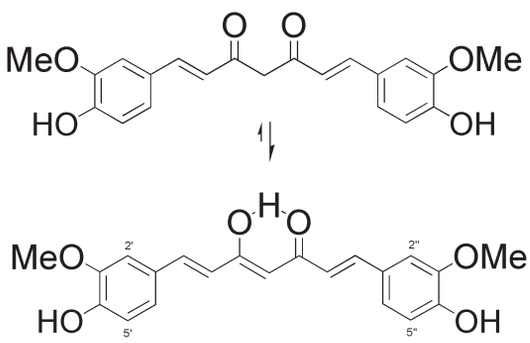

Fig. 1 Chemical structures of keto and enol forms of curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)1,6-heptadiene-3,5-dione).

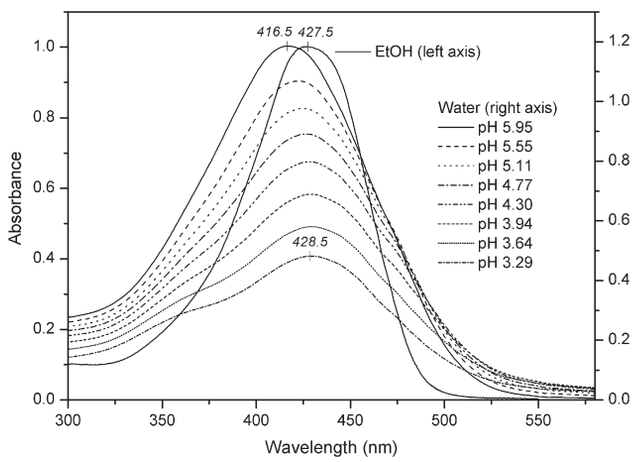

Fig. 2 UV/Vis spectra of curcumin in EtOH and in water at different pH values; c (in EtOH) = 2.0×10^{-5} M, c (in H_2O) = 3.2×10^{-5} M, cell length 1 cm, t = 25 °C.

alkaline solution, however, curcumin molecules are unstable and suffer rapid hydrolytic degradation.[14] 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 (Fig. 2). As the pH values decrease the absorption band loses intensity and its maximum is shifted to longer wavelengths due to aggregation of undissociated curcumin molecules.

It is important to note that curcumin is devoid of any chiral center and in the absence of external chiral influence it shows no intrinsic optical activity either in organic or aqueous solutions (data not shown).

Curcumin–calf thymus DNA

CD and UV/Vis spectra of curcumin in the presence of calf thymus DNA. It was observed through our experiments that the induced CD spectra of curcumin–nucleic acid complexes are very sensitive on the concentration of Na^+ ions in the medium. Due to this fact and the tendency of curcumin to form complexes with salts used in common buffer systems, all spectroscopic measurements were performed in double distilled water with or without added sodium chloride. The pH of the sample solutions was adjusted by addition of small volumes of concentrated HCl or NaOH and was checked by using a digital pH meter.

CD and UV/Vis spectra of curcumin–ctDNA solution were recorded changing the pH value from 6.52 to 3.11 at a constant ligand/base pair ratio of 0.3. In the near UV and visible spectral region of the CD spectrum, where nucleic acids do not absorb light and show no CD activity, a definite positive extrinsic CD band appeared around 472 nm, within the spectral area of the main absorption band of curcumin (Fig. 3). Its amplitude is sharply increased with decreasing pH values between 6.52 and 5.63. The CD maxima showed a wide plateau in the range of pH 5.38–3.87 and was strongly reduced below pH 3.87 (Fig. 4).

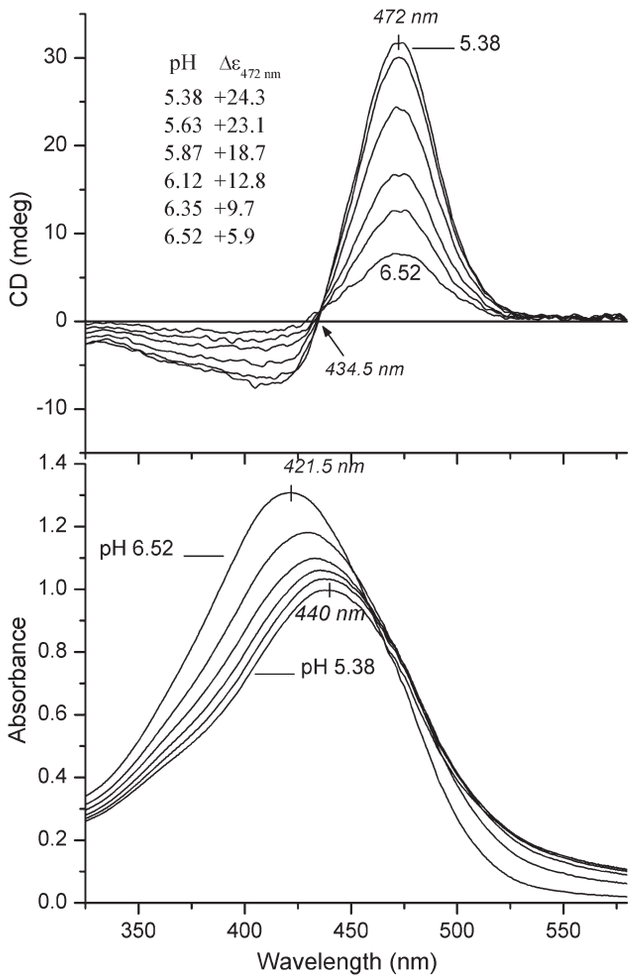

Fig. 3 Induced CD and UV/Vis spectra of curcumin in the presence of ctDNA between pH 6.52 and 5.38 (cell length 1 cm, t = 25 °C, double distilled water). The [curcumin]/[base pair] ratio is 0.3; molar dichroic absorption coefficients ($\Delta\varepsilon$ in M^{-1} cm^{-1}) are shown ($c_{bp} = 1.4 \times 10^{-4}$ M, $c_{curc.} = 3.9 \times 10^{-5}$ M).

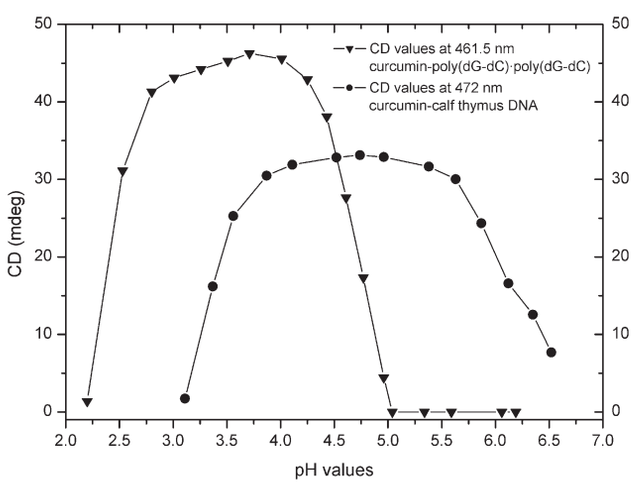

Fig. 4 CD–pH titration curve of curcumin–DNA mixtures. See experimental conditions in Fig. 3 and Fig. 5.

At lower wavelengths, next to the positive Cotton effect a smaller negative CD band developed also around 410–415 nm (Fig. 3); the zero cross-over point between these oppositely signed bands was found at 434.5 nm. The shape and wavelength position of the induced CD bands remained unchanged during the whole experiment.

Except for a small blue shift (5 nm) of the negative peak at 250 nm, the CD spectrum of ctDNA showed no considerable alteration either upon addition of curcumin or pH lowering. The UV absorption band at 260 nm exhibits moderate hypochromism with decreasing pH values (data not shown).

Addition of Na^+ ions into the sample solution exerted a profound effect on the induced CD spectrum of curcumin; the magnitude of the positive band rapidly decreased with increasing ionic strength. Above 0.04 M Na^+ ion concentration only a weak, entirely positive band was measured between 400 and 525 nm ($\Delta\varepsilon_{463\ nm} = +2.2\ M^{-1}\ cm^{-1}$).

Curcumin–poly(dG-dC)·poly(dG-dC)

CD and absorption spectroscopic properties of curcumin–poly(dG-dC)·poly(dG-dC) solution measured in the visible spectral region. Acidification of a curcumin–poly(dG-dC)·poly(dG-dC) solution resulted in three, positive–negative–negative CD bands similar in shape to those measured with ctDNA but they are found at shorter wavelengths by about 10 nm (Fig. 5). The third partially resolved negative peak between 350 and 380 nm is hidden in the CD spectra of curcumin–ctDNA (Fig. 3). However, there are additional spectroscopic differences relative to the curcumin–ctDNA system. The positive induced CD band appeared first at pH 5 and no CD activity was recorded above this value (Fig. 4 and 5). Additionally, higher extrinsic molar CD values ($\Delta\varepsilon$) were obtained with poly(dG-dC)·poly(dG-dC) (*cf.* Fig. 3 and 5).

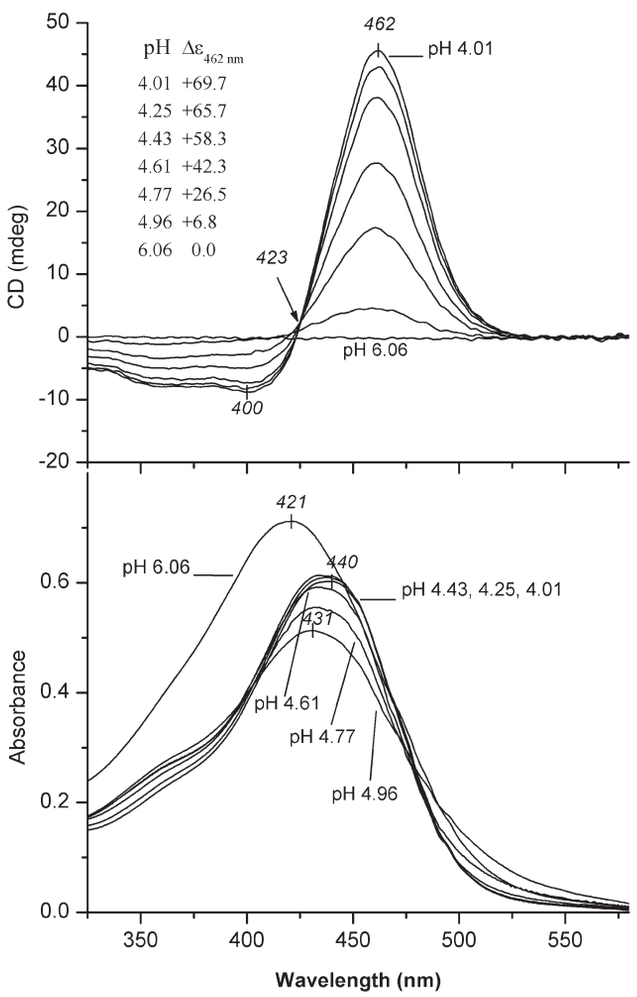

Fig. 5 Induced CD and UV/Vis spectra of curcumin between pH 6.06 and 4.01 in the presence of poly(dG-dC)·poly(dG-dC) (cell length 1 cm, t = 25 °C, double distilled water). The pH values were set by addition of μl volumes of 0.01 or 0.1 M HCl. The [curcumin]/[base pair] mixing ratio is 0.42 ($c_{bp} = 4.8 \times 10^{-5}$ M, $c_{curc.} = 2.0 \times 10^{-5}$ M).

Upon lowering the pH value from 6 to 5, the magnitude of the absorption band decreased steadily, but below pH 5 it began to increase together with the appearance of the extrinsic Cotton effects (Fig. 5). In full agreement with the behaviour of the CD

spectrum, these data indicate that curcumin molecules do not bind to the double helix above pH 5, rather they aggregate due to their low aqueous solubility in that pH range. Below pH 5, however, curcumin molecules start to bind to DNA and the equilibrium shifts towards deaggregation and the formation of curcumin–DNA complexes with consecutive enhancement of light absorption.

CD titration was performed at pH 4 by adding increasing amounts of curcumin to the poly(dG-dC)·poly(dG-dC), the latter being kept at constant concentration (see the CD and UV/Vis spectra in the Electronic Supplementary Information†). At the lowest ligand/base pair ratios two induced CD bands were recorded only: a broad positive one between 390 and 500 nm and a negative one at 355 nm; the 400 nm negative Cotton effect is totally absent from the spectrum. It is important to note that the shape and spectral position of the positive Cotton effect centered at 448 nm is very similar to that of the residual band observed in curcumin–ctDNA solution at high sodium salt concentrations. However, upon further increase of curcumin concentration, the negative CD band did appear at 400 nm and it gradually gained intensity with a concomitant red shift and narrowing of the positive CD peak. The amplitude of the negative band at 355 nm was also increased but its shape and wavelength position showed no significant variations. On the one hand, these results suggest that different mechanisms are responsible for the induction of the 355 nm CD band and the longest wavelength positive–negative CD band pair. On the other hand, an interaction between adjacent, DNA-bound curcumin molecules might account for the development of the separate negative band at 400 nm. Except for a small blue shift, the absorption band of curcumin exhibited no further changes during titration.

Effects of the low pH and the presence of curcumin on the CD and UV spectra of poly(dG-dC)·poly(dG-dC). Alterations of CD and UV spectra of poly(dG-dC)·poly(dG-dC) in the presence of curcumin at different pH values are displayed in Fig. 6. Between pH 5 and 6 the initial changes are manifested in the blue shift and intensity loss of the 252 nm negative band. In more acidic environment the CD spectra are characterized by the formation of a well defined negative band around 302 nm, a positive Cotton effect between 255–285 nm followed by the blue shifted negative band at 244 nm. The spectra showed two isoelliptic points at 235 and 284 nm suggesting equilibria between distinct conformations of DNA. Modifications of the principal UV band of DNA upon acidification involved moderate red shift and hypochromism of the 256 nm band with simultaneous amplification of the extinction in the 280 nm region (Fig. 6). The series of UV spectra also showed two isobestic points in the short- and long-wavelength regions, respectively. It must be noted, however, that neither the CD nor the UV spectra changed further from pH 4 to 2.8 despite the increasing proton concentration (data not shown). The CD and UV spectral alterations observed in our experiments between pH 6 and 4 are very similar to those obtained with hetero G-C polymers in acidic medium under the influence of low ionic strength and temperature. The low pH induced conformational modification in the DNA structure was attributed to the protonation of cytosine residues leading to the formation of the so-called Hoogsten base-paired (H^L-form) structures.[15–17]

It is important to note that addition of curcumin to the acidic solution of poly(dG-dC)·poly(dG-dC) altered significantly the CD bands of the DNA. Three sets of CD spectra measured at pH 3, 4 and 5 before and after the addition of curcumin are shown in Fig. 7. In the spectrum recorded at pH 5 prior to the addition of curcumin no negative band can be seen above 270 nm, rather a positive one which is identical with the corresponding band obtained around pH 6. Upon addition of curcumin, however, the long-wavelength negative band promptly developed, the other bands gained intensity and the resulting spectrum became very similar to the Hoogsteen-type curves reported in the literature. All characteristic bands of the Hoogsteen spectrum already evolved at pH 4 prior to addition of curcumin but their magnitudes were strongly increased in the presence of the ligand. At pH 3, the situation was considerably different; the CD spectrum obtained in the absence of curcumin did not resemble at all to the Hoogsteen-type curve (Fig. 7). Strikingly, however, curcumin was able to transform this spectrum back into the H^L-form. All of these results indicate that binding of curcumin to protonated poly(dG-dC)·poly(dG-dC) in acidic environment is able to induce and stabilize the formation of Hoogsteen-type conformation of the double helix.

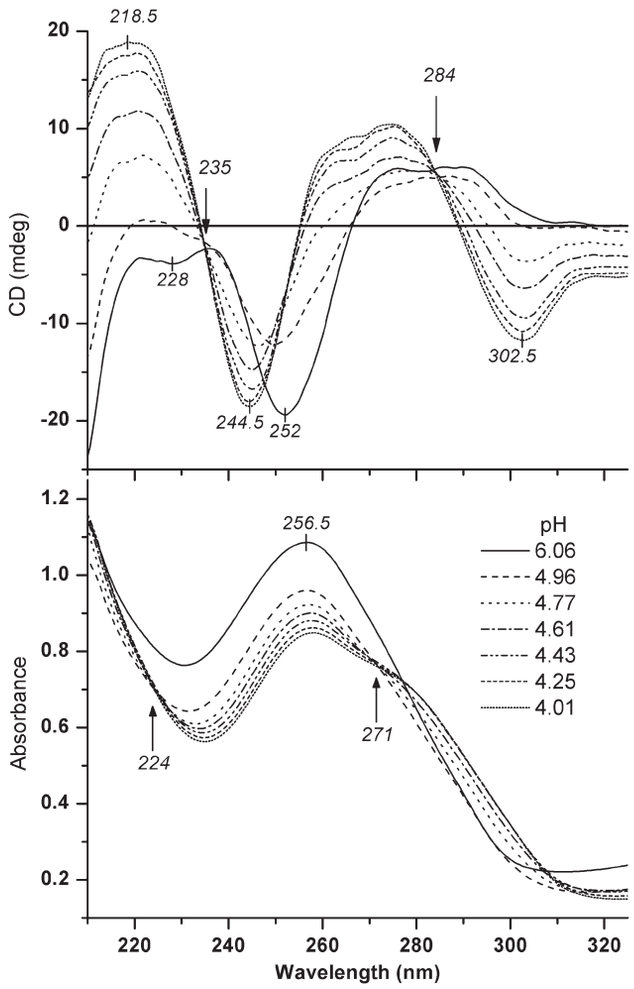

Fig. 6 CD and UV spectra of poly(dG-dC)·poly(dG-dC) in the presence of curcumin between pH 6.06 and 4.01 (cell length 1 cm, t = 25 °C, double distilled water). The pH values were set by addition of μl volumes of 0.01 or 0.1 M HCl. The [curcumin]/[base pair] mixing ratio is 0.42 ($c_{bp} = 4.8 \times 10^{-5}$ M, $c_{curc.} = 2.0 \times 10^{-5}$ M).

It is well known that beside protonation, the ionic strength and salt composition of the medium also have profound effects on the conformation and ligand binding properties of nucleic acids.[18–20] Since the spectroscopic measurements presented above were performed in double distilled water without addition of salt, we investigated the effect of increasing Na^+ concentration on the CD and UV/Vis spectra of the curcumin–poly(dG-dC)·poly(dG-dC) complex. Intensity values of the 460 nm positive induced CD band of curcumin plotted against the Na^+ concentration showed striking differences depending on the actual pH of the solution. The data presented in Fig. 8 show that:

- at pH 4 and 5, increasing concentration of Na^+ ions reduced the magnitude of the extrinsic CD bands to zero;

- sigmoid-like curves with flattening ends were obtained at pH 4 and 5;

- the induced CD band was very stable at pH 3 in the wide range of Na^+ concentration; the band amplitude even increased by 20% at the lowest concentration of NaCl.

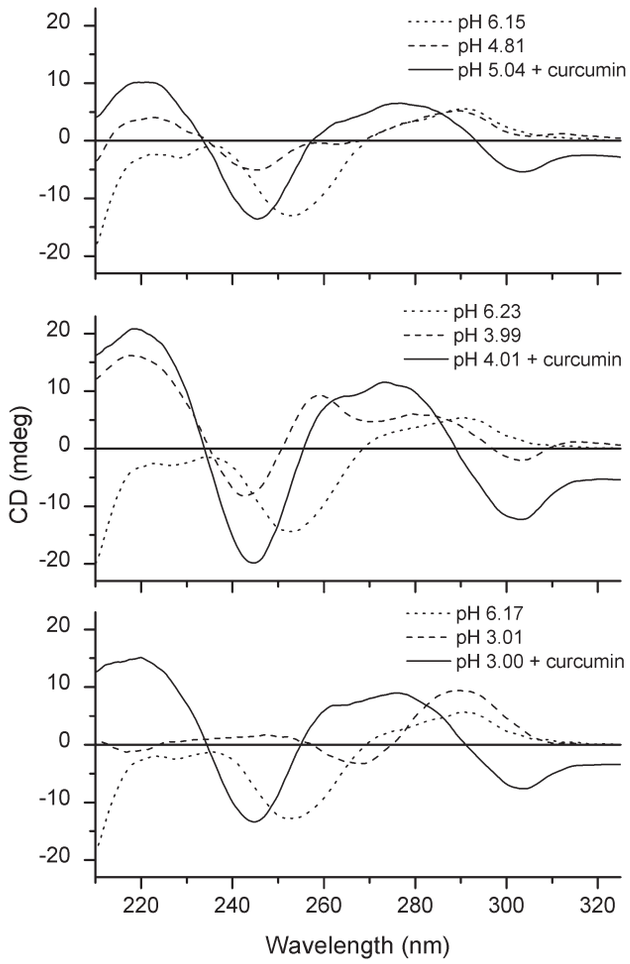

Fig. 7 Comparison of far-UV CD spectra of poly(dG-dC)·poly(dG-dC) measured at pH 3, 4 and 5 before and after addition of curcumin (cell length 1 cm, t = 25 °C, double distilled water). For all spectra the [curcumin]/[base pair] ratio and curcumin concentration are 0.4 and 2×10^{-5} M, respectively ($c_{bp} = 4.6–4.7 \times 10^{-5}$ M).

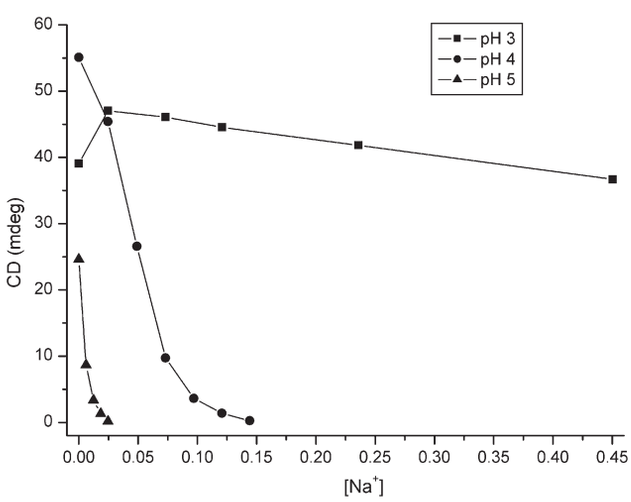

Fig. 8 Effect of the increasing Na^+ concentration on the magnitude of the positive CD band of curcumin induced by poly(dG-dC)·poly(dG-dC) at different pH values (cell length 1 cm, t = 25 °C, double distilled water). For all data points the [curcumin]/[base pair] ratio and curcumin concentration are 0.4 and 2×10^{-5} M, respectively ($c_{bp} = 4.6–4.7 \times 10^{-5}$ M).

These data strongly suggest that H^+ and Na^+ ions have opposite effects on the stability of the complex formed by curcumin with poly(dG-dC)·poly(dG-dC).[16] The higher the proton concentration is, the more stable the complex is and more Na^+ ions are needed to disrupt it. Furthermore, the change of the main absorption band of curcumin upon increasing Na^+ concentration clearly indicated that the loss of the CD activity was caused by dissociation of the complex; the intensity of the band decreased with the increasing ionic strength as a consequence of the advancing aggregation of deliberated curcumin molecules (spectra not shown).

CD curves measured at pH 4 in the UV region indicated that the increasing amount of Na^+ ions caused major conformational changes of DNA; the Hoogsteen-type spectrum was gradually transformed back into the spectrum characteristic of the B-form of the double helix. Concomitantly, the UV band was blue shifted and the shoulder around 280 nm disappeared resulting in an absorption band identical to that obtained in the absence of Na^+ ions at pH 6 (spectra not shown).

Curcumin–poly(dA-dT)·poly(dA-dT)

The synthetic heteropolymer poly(dA-dT)·poly(dA-dT) was also the subject of our spectroscopic investigations regarding its interaction with curcumin. In marked contrast with poly(dG-dC)·poly(dG-dC), this polynucleotide did not induce any CD signal upon changing the pH from 6 to 3 in distilled water (Fig. 9). The absorption band of curcumin exhibited very similar behavior to that found in acidic aqueous environment (*cf.* Fig. 2). Taken together, the CD and UV/Vis spectral features indicated no intermolecular interaction between curcumin and poly(dA-dT)·poly(dA-dT). However, the addition of a small amount of Na^+ at pH 3.62 dramatically changed the CD spectrum; the familiar induced CD band pattern appeared instantly suggesting curcumin to bind to the double helix (Fig. 9). The behavior of the absorption band of the ligand further supports the formation of a curcumin–DNA complex. Upon addition of Na^+ the absorption peak gained intensity, shifted to higher wavelength and its shape became very similar to that measured in ethanolic solution (see Fig. 2). All of these changes clearly indicate deaggregation of curcumin molecules and their binding to the polynucleotide template. Thus, the main conclusion is that Na^+ ions are essential for the binding of curcumin to poly(dA-dT)·poly(dA-dT). It should be noted that all DNA samples contained Na^+ ions since they were purchased in sodium salt form. In the case of poly(dA-dT)·poly(dA-dT) this "intrinsic" salt gave about 2×10^{-4} M Na^+ ion concentration but this amount proved to be insufficient to initiate the curcumin–DNA interaction.

To determine more precisely the experimental conditions under which induced CD bands appear, series of measurements were taken at three different pH values by varying the Na^+ ion concentration of the sample solution. Below pH 4, the induced Cotton effects appeared first at 0.025 M sodium ion concentration and reached maximum intensities at 0.05 M. Upon repeated additions of aliquots from the 5 M NaCl stock solution, the induced CD values began to decrease but significant CD activity was measured even at tenfold cation concentration. In contrast, at higher pH values lower Na^+ concentrations (0.005 and 0.0025 M) were sufficient to provoke the induced CD activity. In addition, these CD bands were more sensitive to the increase of the ionic strength and they completely vanished at 0.06 M (pH 4.5–4.7) and 0.0025 M (pH 5.8–6.0) Na^+ concentrations (in these cases the CD spectra became time dependent and the band amplitudes decreased continuously to zero within five minutes at constant ionic strengths).

Curcumin–poly(dC)

By using single-stranded poly(dC), spectroscopic measurements were performed to test whether the double helix is essential for the interaction with curcumin. Obviously, this single polynucleotide chain does not form any groove but its bases are stacked and it exhibits definite, right-handed helicity. In pH 6.16 water solution, strong positive and negative DNA CD bands were measured at 288 and 266 nm with a zero cross-over point at 275 nm. CD and UV/Vis spectra of curcumin–poly(dC) solution were recorded at different pH values ranging from 6.08

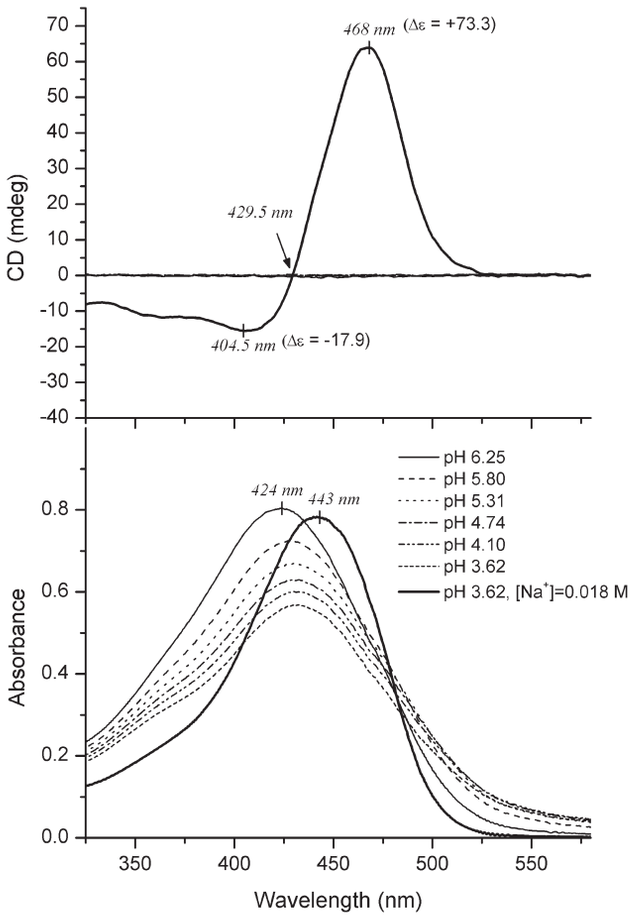

Fig. 9 Effect of pH and Na^+ addition on the CD and UV/Vis spectra of curcumin-poly(dA-dT)·poly(dA-dT) solution between pH 6.25 and 3.62 (cell length 1 cm, t = 25 °C, double distilled water). The [curcumin]/[base pair] ratio is 0.22 (c_{bp} = 1.2 × 10^{-4} M, $c_{curc.}$ = 2.6 × 10^{-5} M).

to 2.75 in the absence and in the presence of Na^+ ions. However, no induced CD bands were found between 210 and 600 nm (spectra not shown). Large hypochromism and a red shift of the visible absorption band of curcumin was observed at low pH values indicating self-association of the molecules. All of these spectroscopic data suggested the lack of any interaction between curcumin and poly(dC).

Molecular modeling of curcumin–DNA interaction

It is notable that curcumin has several structural features which are common in both natural and synthetic minor groove binder drugs. There are potential hydrogen donor groups at both ends and the molecule has a crescent shape with a concave and a convex edges, respectively (see the Electronic Supplementary Information†). The crescent shape of classical minor groove binders is complementary to the natural curvature of the minor groove of B-DNA.[21,22] The radius of the curvature of curcumin was calculated to be 12.8 Å which is similar to the values of known minor groove binder agents.[23] Linear lengths of minor grooves formed by 3 or 4 base pairs are ≈13 and ≈17 Å, respectively; the length of the curcumin molecule is 17 Å. It should be noted that the energy-minimized conformation of the curcumin molecule is not completely planar; the steric clash between the aromatic (6′ and 6″) and the vinyl hydrogens results in a 15° twist of the benzene rings relative to the plane of the middle enol moiety.

The molecular model obtained by automated docking of the curcumin molecule to the B-form of the X-ray structure of d(CTTCCTCATGTATATACATGAGGA)$_2$ (PDB code is 1AU7) revealed that curcumin fits tightly within the minor groove (the model can be found in the Electronic Supplementary Information†). The binding site is 4 base pairs long and involves A-T residues; the docking energy was −12 kcal mol^{-1}. Extensive van der Waals contacts were found between the docked curcumin and the floor and walls of the minor groove suggesting that this type of interaction contributes significantly to the stabilization of the complex. Assuming free rotation around the C–OH bonds, the phenolic OH groups of curcumin might adopt favorable spatial positions to establish H-bonds with the 3′-oxygen atom or adjacent phosphate groups. The aromatic rings are rotated out of the plane of the central enol moiety exposed for solvent molecules; the dihedral angles C(6′)–C(1′)–C(1)–C(2) and C(6″)–C(1″)–C(7)–C(6) are −16.5° and +15°, respectively. The distance measured between the center of the docked curcumin molecule and the long axis of the helix is about 5 Å.

It is important to note that two or more curcumin molecules bound adjacently in the minor groove of the double helix form a chiral supramolecular array (Fig. 10). The basic unit of the array is two neighbouring curcumin molecules bound in the minor groove of the double helix; within this pair, the long axes of the molecules close a positive (clockwise) overlay angle with a magnitude of about 70° (Fig. 10).

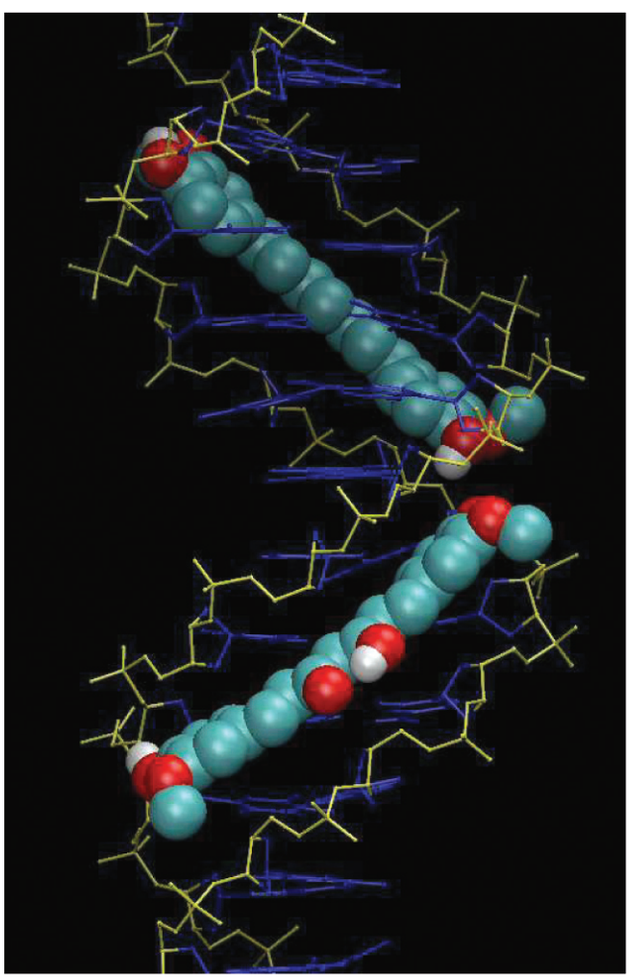

Fig. 10 Relative spatial positions of two curcumin molecules bound adjacent to each other in the minor groove of the right-handed helix (sugar-phosphate backbones: yellow; base pairs: dark blue).

Discussion

Origin and nature of the induced CD activity of curcumin–DNA complexes

The results reported here give evidence that curcumin is able to bind to either AT or GC alternating heteropolymers and mixed DNA tracts. It is also apparent from our data that the double helix is essential for this interaction. Small molecules interacting with double stranded DNAs can be classified according to their binding modes: intercalators insert themselves between the base pairs while nonintercalators bind in the minor or major groove of DNA.[24] The sign, shape and wavelength positions

of induced CD bands of intercalator molecules sensitively depend on the relative spatial orientation between the transition moments of the ligand and of the proximal base pairs.[25] However, the extrinsic CD spectra of curcumin obtained with three different nucleic acids were practically identical (Fig. 3, 5 and 9). Theoretical calculations predicted a positive induced CD band for molecules whose electric transition moments are oriented along the minor or major groove in which they are bound.[25] The induced CD spectra of curcumin are quite similar to those of known minor groove binders.[26–28] Furthermore, the high sensitivity of the induced CD spectrum of curcumin to the increase of Na^+ concentration (Fig. 8 and 9) is also characteristic of groove binding.[29] The absorption spectrum of curcumin lends further support to the concept of groove binding. Strong hypochromism accompanies intercalation of a molecule into the base stack but no such spectral change was observed in the UV/Vis spectrum of curcumin complexed with either synthetic or natural nucleic acids, rather significant hyperchromism of the visible absorption peak was seen in the presence of poly(dG-dC)·poly(dG-dC) (Fig. 5).[29]

When curcumin binds in the minor groove of the double helix, the ligand and the DNA bases are coupled excitonically (non-degenerate exciton interaction).[25,30] Since the bases and the ligand are asymmetrically disposed with respect to each other (Fig. 10) this type of the excitonic interaction results in extrinsic CD activity, the large positive induced Cotton effect in the visible absorption region of curcumin. Since the energy separation between the two oscillators is quite large ($\approx$16000 cm^{-1}) the wavelength position of the positive induced CD band would be expected to match with the absorption peak of curcumin. However, such coincidence was seen only at the lowest ligand/base pair ratios and during addition of Na^+ before the total vanishing of the induced CD band. These facts and the presence of the negative CD peak next to the positive one suggest the contribution of a further mechanism in the generation of the extrinsic optical activity. Adjacent binding of two or more curcumin molecules in the minor groove satisfies spectroscopic and stereochemical requirements of the degenerate exciton coupling case:[30,31] the ligands having identical powerful chromophores are held in chiral orientation relative to each other by the right-handed double helix (Fig. 10). The exciton chirality rule elaborated for the degenerate coupled-oscillator model[31] predicts a long-wavelength positive and a short-wavelength negative CD bands for two chromophores being coupled in a right-handed chiral system. In other words, the polarization directions of the two oscillating dipole moments constitute a right-handed screw, *i.e.* the sign of the intermolecular overlay angle between them is positive (Fig. 10). Consequently, intermolecular exciton coupling between curcumin molecules bound in close proximity in the minor groove gives a reasonable explanation for the presence of the neighbouring opposite CD bands.[31,32] Since induced CD bands arising from degenerate and non-degenerate exciton interactions are present simultaneously in a common spectral region their mixing results in unequal band amplitudes and a wavelength shift between the positive Cotton effect and the absorption band. Positive CD bands originating from the degenerate and non-degenerate exciton splittings mutually amplify each other but the negative lobe of the degenerate exciton interaction is attenuated by the more intense neighbouring non-degenerate positive band.

The weak negative CD band around 355 nm probably comes from the n–π^* transition of the enol moiety perturbed asymmetrically by the chiral environment of the polynucleotide hosts.

Interpretation of pH and ionic strength dependent binding of curcumin to synthetic and natural nucleic acids

The spectroscopic data presented above indicate that pH and ionic strenth induced structural alterations of nucleic acids deeply influence the binding of curcumin. It is well established that several parameters are involved in minor groove recognition processes including width, depth, shape and electrostatic potential of the groove, the number of van der Waals contacts between the ligand and the wall of the groove and hydrogen donor–acceptor sites at the edges of A : T and G : C base pairs.[21–23,33] Most of the minor groove binders prefer to interact with AT rich sequences. This preference is explained by three facts:[33–35]

1) The distribution of electrostatic potential is sequence dependent and it is most negative at the floor of the minor groove AT sites.

2) AT rich sequences exhibit narrower minor grooves (3–4 Å) than GC sites and promote good non-bonded van der Waals contacts between the ligand and the walls of the minor groove.

3) The minor groove floor of an A/T tract of sequence can be approximated by a single smooth curve, whereas at the floor of a G/C tract the exocyclic amino group of the guanine base protrudes and sterically interferes with the binding of small molecules.

As has been demonstrated, Na^+ ions were essential for binding of curcumin to poly(dA-dT)·poly(dA-dT) (Fig. 9). In an attempt to understand this special sensitivity it must be borne in mind that the minor groove of A–T rich sequences has been established as the principal binding site for Na^+ and other monovalent metal ions, and several lines of evidence suggested that there is a close correlation between the entrance of Na^+ ion into the minor groove and groove narrowing.[18–20,35] It appears that minor groove narrowing is facilitated by interactions of cations with opposing phosphate groups. The minor groove is narrowest when Na^+ is at the outer edge of the groove, making direct contact with phosphate oxygens.[20] So, it can be assumed that prior to addition of Na^+, the minor groove of poly(dA-dT)·poly(dA-dT) was not narrow enough to provide suitable van der Waals contacts for curcumin. At this point, it is important to recall that curcumin carries no cationic group, so van der Waals interactions are likely dominant factors in its DNA binding. Upon addition of Na^+, the minor groove narrows increasing the number of nonbonding contacts of curcumin with the groove walls. It was found, however, that there is an actual ion concentration range for optimal binding of curcumin and too high Na^+ concentrations result in the dissociation of the complex. It may be hypothesized that parallel with the increasing Na^+ concentrations more and more cations penetrate into the depth of the minor groove where they interact with the thymine carbonyl group. This ion penetration is accompanied by the widening of the groove[20] and, on the other hand, Na^+ ions may directly compete with curcumin for the same binding room. Consequently, the binding equilibrium shifts toward the dissociation of the complex with the concomitant disappearance of the induced CD activity.

In the case of poly(dG-dC)·poly(dG-dC) experimental results suggest that the protonation induced conformational change of the double helix is the decisive step in binding of curcumin. As mentioned above, in the canonical B-form of DNA, the amine group of guanine bases protrudes into the minor groove and prevents ligand binding by steric hindrance.[22] However, in acidic solution poly(dG-dC)·poly(dG-dC) is known to undergo a protonation induced structural transition.[15–17] N-3 of cytosine has the highest pK_a (4.5–4.6) among the bases and in mild acidic conditions, protonation of alternating GC heteropolymers results in the formation of Hoogsteen base pairs in which two hydrogen bonds are formed between guanine and protonated cytosine bases (Fig. 11). However, Hoogsteen base pairing removes the amine group from the minor groove, so the groove becomes sterically available for curcumin molecules. Thus, the extrinsic Cotton effects of curcumin–poly(dG-dC)·poly(dG-dC) complex measured at low pH is tentatively attributed to the binding of curcumin in the altered minor groove of the Hoogsteen type conformation of the double helix.

As has been found, increase of the Na^+ concentration decreased and canceled the induced CD activity of the curcumin–poly(dG-dC)·poly(dG-dC) complex and reverted the

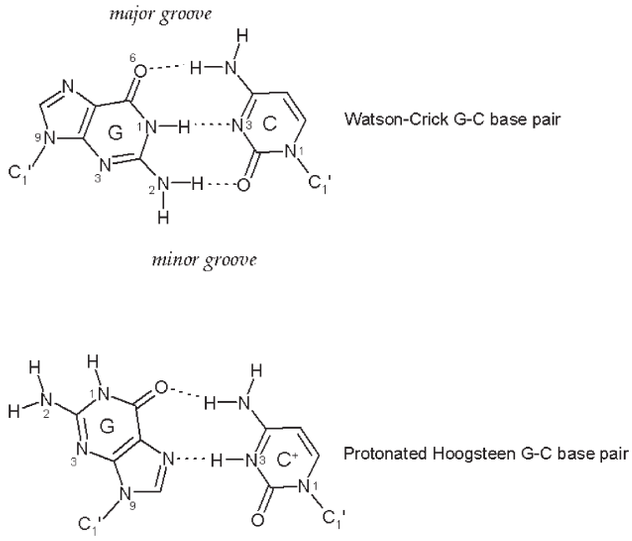

Fig. 11 G-C base-pairs with Watson–Crick and Hoogsteen hydrogen bond schemes.

Hoogsteen base pairs back to the Watson–Crick type suggesting that high salt concentration hinders the protonation of cytosine residues. The same result was obtained by Muntean and Segers-Nolten who studied the effects of Na^+ and Mg^{2+} ions on the low pH induced structural changes of ctDNA.[36] A plausible explanation for this antagonistic effect of Na^+ is that the negative charges of the backbone phosphate groups are progressively shielded by the increasing amount of the cations, so the double helix becomes electrostatically less inviting for protons, and thus protonation of cytosines is efficiently suppressed.[37]

Calf thymus DNA is a nearly equal mixture of GC and AT base pairs containing short GC and AT rich segments, respectively. On this basis it is not surprisingly that spectroscopic properties of curcumin–ctDNA complexes proved to be a kind of mixture of those obtained with the two alternating heteropolymers. The induced CD bands appeared at high pH values (AT like feature) and were stable in time (GC like) but their amplitudes significantly increased upon acidification of the solution (GC and AT like). Due to the small GC rich sequences, conformational changes of the double helix are much less pronounced upon protonation than of poly(dG-dC)·poly(dG-dC) in agreement with minute alterations in the CD spectrum of ctDNA.

The most important conclusion, however, is that curcumin binds to the genomic DNA in mild acidic conditions, near to the physiological pH value.

Significance of the results and potential perspectives. ***A new, polyphenolic type minor groove binder has been discovered.*** Apart from the structurally very different aureolic acid group of antitumor antibiotics mithramycin and chromomycin A_3,[38] curcumin is the first polyphenol compound found to bind in the minor groove of nucleic acids. The overall shape of the curcumin molecule resembles typical minor groove binders although it contains no basic center demonstrating that the presence of nitrogen atom(s) is not an inevitable condition for groove binding. Investigation of natural curcuminoids, synthetic derivatives and related compounds (*i.e.* capsaicin, gingerol) might lead to the identification of further molecules showing specific sequence recognition and/or higher DNA binding affinity.

It is important to note that pH induced conformational polymorphism of DNA might occur *in vivo* as suggested by literature evidence pointing out the significant role of intra- and extracellular pH in the regulation of gene expression for both normal and pathological cells.[39–42] The ability of curcumin to bind to acidic pH induced polymorphic DNA structures might merit further investigations regarding its mechanism of action.

The diarylamidine antibiotic pentamidine is currently in widespread clinical use for treatment of leishmaniasis, trypanosomiasis and *Pneumocystis carinii* pneumonia.[43] There is considerable evidence for its direct interaction with the pathogenic genome. It binds selectively to the minor groove of DNA at AT-rich sites and interferes with the normal functioning of the pathogen topoisomerases.[21,22,24] Interestingly, the non-toxic curcumin and other diarylheptanoid derivatives were also found to be effective against trypanosomiasis and leishmaniasis, respectively.[2,6,7]

Curcumin is a promising, sensitive spectroscopic probe to study conformational polymorphism of nucleic acids. DNA can adopt a variety of secondary structures of which exact biological roles are under intensive studies.[44] As has been shown, the induced CD spectrum of curcumin is very sensitive to subtle changes in the secondary structure of both natural and synthetic polynucleotides. Since the main absorption band of curcumin is well separated from the UV band of nucleic acids, the intrinsic CD activity of the double helix does not interfere with the extrinsic Cotton effects of curcumin. Therefore, curcumin is a suitable label to study pH and ionic strength induced conformation diversity of the double helix and molecular recognition properties of the minor groove under different physico-chemical conditions.

Abbreviations

CD, circular dichroism; CE, Cotton effect; ctDNA, calf thymus DNA; H^L-form, Hoogsteen base-paired structure of DNA; UV/Vis, ultraviolet-visible; PDB, Protein Data Bank.

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