

## Investigations on geometrical features in induced ordering of collagen by small molecules<sup>¶</sup>

B MADHAN, ARUNA DHATHATHREYAN, V SUBRAMANIAN and  
T RAMASAMI\*

Central Leather Research Institute, Adyar, Chennai 600 020, India  
e-mail: clrim@giasmd01.vsnl.net.in

**Abstract.** Binding energies of the interaction of collagen like triple helical peptides with a series of polyphenols, viz. gallic acid, catechin, epigallocatechingallate and pentagalloylglucose have been computed using molecular modelling approaches. A correlation of calculated binding energies with the interfacial molecular volumes involved in the interaction is observed. Calculated interface surface areas for the binding of polyphenols with collagen-like triple helical peptides vary in the range of 60–210 Å<sup>2</sup> and hydrogen bond lengths vary in the range of 2.7–3.4 Å. Interfacial molecular volumes can be calculated from the solvent inaccessible surface areas and hydrogen bond lengths involved in the binding of polyphenols to collagen. Molecular aggregation of collagen in the presence of some polyphenols and chromium (III) salts has been probed experimentally in monolayer systems. The monolayer arrangement of collagen seems to be influenced by the presence of small molecules like formaldehyde, gluteraldehyde, tannic acid and chromium (III) salts. A fractal structure is observed on account of two-dimensional aggregation of collagen induced by tanning species. Atomic force microscopy has been employed to probe the topographic images of two-dimensional aggregation of collagen induced by chromium (III) salts. A case is made that long-range ordering of collagen by molecular species involved in its stabilisation is influenced by molecular geometries involved in its interaction with small molecules.

**Keywords.** Collagen; polyphenols; tanning; assemblies.

### 1. Introduction

Functional properties of tissues are generally influenced by the structure of assemblies associated with supramolecular systems.<sup>1</sup> Collagen participates in several supra molecular assemblies.<sup>2,3</sup> Supramolecular assemblies of collagen seem to vary with the role of the protein in the specific connective tissue. Ever since the report of triple helical structure of collagen by Ramachandran and co-workers nearly fifty years ago<sup>4,5</sup> there has been considerable interest in the study of molecular properties and structure of the protein.<sup>6</sup>

The implication of structural modification of collagen in connective tissue disorders like inflammation and arthritis on the one hand and in manufacture of the leather on the other has added further interest to understanding of the interaction of the protein with small molecules like water, polyphenols and chromium (III) salts.<sup>7</sup> Overwhelming influence of water structure and hydration on the molecular aggregation and properties of

<sup>¶</sup>Dedicated to Professor C N R Rao on his 70th birthday

\*For correspondence

collagen has been recognized early.<sup>8</sup> Whereas the secondary structure of native collagen exhibits repeat periodicity of 640 Å, an increased long-range order is observed when collagen is interacted with specific chromium (III) species.<sup>9</sup> It is now established that several molecules bearing flavanoid and other polyphenolic structures elicit favourable influence on patients suffering from arthritic conditions by reducing inflammation.<sup>10</sup> The ability of flavanoids and polyphenols to stabilize skin against biodegradation leading to tanning is well documented.<sup>11</sup> An attempt has been made to probe in greater details the interactions of collagen with some chromium (III) complexes and selected organic molecules. The importance of interaction areas as a geometric tool for measuring the extent of protein recognition sites has been analysed based on X-ray diffraction data.<sup>12</sup> The importance of geometric features in recognition of sites in protein–protein and protein–DNA interactions has been examined.<sup>13–15</sup> On similar lines, geometric features in the stabilisation of collagen on account of interactions with select organic molecules and chromium (III) salts have now been examined in this work using both molecular modelling and experimental investigations. Correlation of maximum surface pressure with interface molecular areas and energies of binding of four polyphenols to collagen-like triple helical peptides with interfacial molecular volumes is presented.

## 2. Materials and methods

### 2.1 Computational details

Four representative polyphenol molecules, viz. catechin, epigallocatechingallate, gallic acid and pentagalloylglucose have been selected. Molecular models have been built using builder tools outfitted with Silicon Graphics O2 workstation. Consistent Valence Force Field (CVFF) has been assigned to all atoms of the polyphenol molecule. The energy expression for the consistent valence force field (CVFF) consists of bond stretching (Morse type), angle bending, torsional energy, van der Waals interactions with the Lennard–Jones function and Coulombic interactions. In order to mimic the interaction in water medium, a dielectric constant of 4.0 has been applied to the polyphenol. The geometry of the polyphenol has been minimised using steepest descent method followed by conjugate gradient algorithm. Molecular electrostatic potentials (MESP) are useful in understanding the weak and non-covalent interactions taking place in a complex system. Through the electrostatic topography of a polyphenol molecule, possible interacting sites of polyphenol has identified for its interaction with collagen.

*2.1a Probing interactions of polyphenols with collagen triple helix:* Restricting the number of repeating units is necessary in the modelling and simulation of a large molecule like collagen. In the present study, 24-mer collagen triple helix is constructed by Object Technology Framework (OTF) using the GENCOLLAGEN package.<sup>16</sup> The 24-residue long triple helix constructed corresponds to the residues 193 to 216 (2 $\mathbf{a}$  and 1 $\mathbf{a}$  chains) of the native type I collagen except residue 204 of the  $\mathbf{a}$  chain, where Ala of native collagen is replaced by lysine in order to study the interaction of polyphenolic molecules with the side chains of basic amino acids.

Following is the amino acid sequence of the  $\mathbf{a}$  chain of the triple helix, which is represented by standard three-letter codes of amino acids,

[Gly–Glu–Hyp–Gly–Pro–Hyp–Gly–Pro–Ala–Gly–Ala–Lys–Gly–Pro–Ala–  
 Gly–Asn–Hyp–Gly–Ala–Asp–Gly–Gln–Hyp] **a**  
 [Gly–Glu–Hyp–Gly–Pro–Hyp–Gly–Pro–Ala–Gly–Ala–Lys–Gly–Pro–Ala–  
 Gly–Asn–Hyp–Gly–Ala–Asp–Gly–Gln–Hyp] **a**  
 [Gly–Glu–Val–Gly–Leu–Hyp–Gly–Leu–Ser–Gly–Pro–Val–Gly–Pro–Hyp–  
 Gly–Asn–Ala–Gly–Pro–Asn–Gly–Leu–Hyp] **a**.

The 24-mer triple helix is minimized using CVFF with a dielectric constant of 4.0. The geometry of the triple helix has been minimised using steepest descent method followed by conjugate gradient algorithm.

The side chain hydroxyl group of the amino acids, serine and hydroxyproline, carboxyl group of aspartic acid, amino group of lysine and amide group of asparagine are considered as potential interacting sites for the formation of hydrogen bonds with the polyphenols. Five different complexes were generated for each selected polyphenol. Initial positioning of polyphenol to facilitate the formation of hydrogen bond has been selected such that it satisfies the necessary optimum distance and angle parameters for an effective hydrogen bond. Literature is available on the distances and angles ( $\rho$  and  $\Psi$ ) involved in the formation of hydrogen bond between peptide donors or acceptors and bound water.<sup>17</sup> No constraints were, however, imposed between the representative polyphenolic molecule and model collagen triple helix. The potential from Consistent Valence Force Field (CVFF) has been assigned to all atoms of energy minimized structural models of collagen triple helix and polyphenols. To mimic the interaction in water medium, a dielectric constant of 4.0 has been applied to the complex system. The geometry of the triple helix-polyphenol complex has been minimised using the steepest descent method for first two thousand iterations followed by conjugate gradient algorithm. The interaction energy ( $V_{\text{INT}}$ ) of the complex has been calculated as the difference between the total energy ( $TE_{\text{complex}}$ ) of the complex and the sum of the energy of model collagen triple helix and respective polyphenol molecule. The negative of the interaction energy is termed as binding energy ( $V_{\text{BE}}$ ).

$$V_{\text{INT}} = TE_{\text{complex}} - [TE_{\text{modelcollagen}} + TE_{\text{polyphenol}}], \quad (1)$$

$$V_{\text{BE}} = -V_{\text{INT}}. \quad (2)$$

In order to validate the force-field calculations, the results obtained from CVFF have been compared with Hartree–Fock (HF) method using 3-21G\* and 6-31G\* basis sets for simple systems like gallic acid–water and gallic acid–formaldehyde.<sup>18</sup> A comparison of the values obtained from CVFF with *ab initio* showed on an average, a percentage deviation of about 15.

**2.1b Calculation of surface areas:** Coordinates of energy-minimised structures of polyphenols, collagen-like triple helix and inter-molecular complexes of collagen-like peptides with polyphenol have been used to estimate the total and contact surface areas using the Connolly method as implemented in the Insight II software package.

## 2.2 Experimental

**2.2a Studies on two-dimensional organization of collagen: Preparation of solutions for monolayer studies** – Stock solution of collagen was prepared as follows. The protein was

dissolved in a small amount of acetic acid at 25°C for 2 h. The solution was kept at 20°C for 24 h before it was transferred into deionised water (G18.2 MΩ, Milli-Q, Millipore). Concentration of acetic acid/water weight ratio was maintained at 2%. Sodium azide (1 mM) was added to prevent bacterial growth. The collagen concentration in such a prepared stock solution was  $1.0 \times 10^{-3}$  g/ml, which was further diluted to different desired concentrations. Solutions of collagen and chromium (III) salts were respectively clarified using a 0.45 μm Millipore millex filter and a 0.1 μm Whatman filter paper to remove any suspended particles. For investigations on Cr (III) and tannic acid-induced aggregation of collagen, a pre-selected volume of an aqueous dust-free solution of basic chromium sulphate and tannic acid was added drop wise into 2 ml of dust-free protein solution. Protein concentrations employed were  $1.5 \times 10^{-4}$ ,  $4 \times 10^{-4}$ , and  $6.0 \times 10^{-4}$  g/ml.

A NIMA 611 single barrier trough with a Wilhelmy balance for measuring surface pressure (accuracy 0.1 mN/m) was employed and freshly cleaned quartz, and mica (AFM) were used for transferring the films using a horizontal touching method.

**2.2b Atomic force microscopy:** An atomic force microscope (Nanoscope IIIa, Digital Instrument Inc., Santa Barbara, CA) equipped with a 180-μm scanner (*j*-scanner) and a tapping mode etched silicon probe (TESP) was used. The cantilever (160 μm in length) and the probe were an integrated assembly of single-crystal silicon. All the topographic images were recorded in the tapping mode at a constant force. The same solution was used for recording transmission spectra (UV-Vis) as well as for the atomic force microscope. UV-visible spectra in the transmission mode were used to monitor the aggregation process ( $\lambda = 584$  nm). A piece of freshly cleaved mica ( $\approx 1.0$  cm  $\times$  1.0 cm) was dipped into the solution for 2 s and then dried before the film cast was imaged using AFM. To minimize possible contamination of the surface by particulates in ambient air, each sample was freshly prepared just before the AFM study.

### 3. Results

Volume and solvent accessible contact areas of four polyphenols have been computed in this study using a molecular modelling approach and standard software packages. Calculated data on total volumes and solvent accessible contact areas are presented in table 1. Binding of polyphenols, viz. gallic acid, catechin, epigallocatechingallate and pentagalloylglucose to the 24-mer collagen-like triple helical peptide has been probed. A representative example of such an interaction is presented diagrammatically in figure 1. Interactions of the pentagalloylglucose with peptide residues in the main polypeptide chain (figure 1a) as well as the side chain (lysine) residue with gallic acid (figure 1b) have been depicted. Binding energies have been computed for interaction of polyphenols with different sites of collagen-like triple helical peptide. Binding energy data for various interactions are presented in table 2. Hydrogen bond parameters in terms of distance and bending angle around the hydrogen atom involved in the hydrogen bond have been calculated and presented in table 3.

An attempt has been made to analyse the interactions of collagen-like triple helical peptides with polyphenols in terms of extent of contact between local site structures. The total interface area,  $A_T$ , forms an interesting geometric parameter. It provides a useful tool in assessing the extent of interaction of protein with polyphenols. The total interface area,  $A_T$ , has been computed by calculating the total surface area of collagen-like peptides and

polyphenol separately and subtracting the total surface area of collagen-like peptide complex with polyphenol as given by,

$$A_T = T_c + T_p - T_{cp}, \quad (3)$$

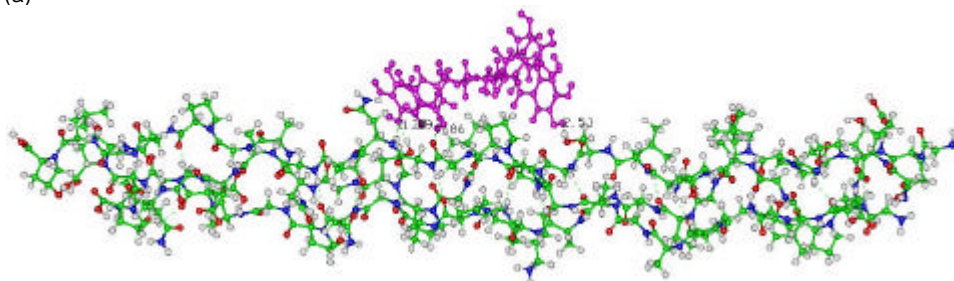
where  $T_c$ ,  $T_p$ , and  $T_{cp}$  are total surface area of collagen-like peptide, polyphenol, and collagen-like peptide–polyphenol complex respectively.

In other words,  $A_T$  is expected to provide an estimate of solvent inaccessible surface area formed during the complexation of collagen-like peptide and polyphenols. Implicit in the calculation of  $A_T$  is the assumption that conformational changes resulting from the interaction of collagen-like peptides and polyphenols, if any, are negligible or can be neglected. In other words, a hard sphere approach has been assumed. Total and solvent

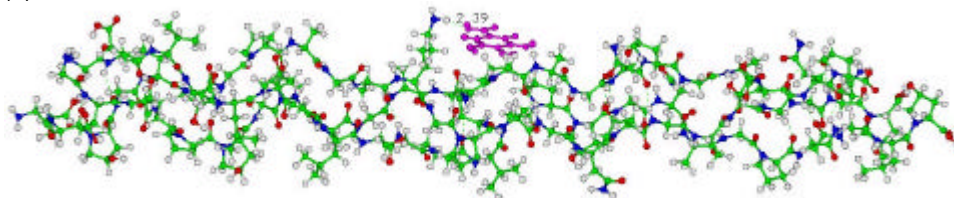
**Table 1.** Size, volume and solvent accessible area of polyphenols.

Polyphenols	Size of the molecule (Å)			Volume (Å <sup>3</sup> )	Contact surface area (Å <sup>2</sup> )
	X-axis	Y-axis	Z-axis		
Gallic acid	7.8	5.5	0	139	84
Catechin	11	7	5.7	263	120
Epigallocatechingallate	12.0	8.5	7.8	404	163
Pentagalloylglucose (tannic acid)	18.5	16.5	10.1	856	275

(a)



(b)



**Figure 1.** (a) Main chain complex of pentagalloylglucose and collagen 24-mer triple helix. (b) Side chain (lysine) complex of gallic acid and collagen 24-mer triple helix.

**Table 2.** Binding energies of various complexes of polyphenol and triple helix.

Binding sites	Binding energy (kcal/mol)			
	Catechin	Epigallocatechingallate	Gallic acid	Pentagalloylglucose
9th residue				
Ser of C-chain ( $\nu_2$ )	22.5	35.2	16.5	56.6
6th residue				
Hyp of A-chain ( $\nu_1$ )	20.8	34.5	14.5	48.4
12th residue				
Lys of B-chain ( $\nu_1$ )	23.8	37.9	19.2	41.1
21st residue				
Asp of A-chain ( $\nu_1$ )	20.0	38.2	18.4	59.8
17th residue				
Asn of C-chain ( $\nu_2$ )	23.7	34.3	14.1	52.8

accessible contact surface areas for collagen-like triple helix, and polyphenols, as well as for the triple helix–polyphenol complex have been worked out. The calculated data on surface areas are presented in table 4.

For the interaction of interest, total surface area (TSA) and solvent accessible contact surface area (CSA) of the peptide are of the order of 3825 and 1164 Å<sup>2</sup> respectively. Solvent accessible contact areas available to polyphenols as calculated are generally in the range of 84 to 275 Å<sup>2</sup>. Hydrogen bond distances as calculated from the study for various interactions are in the range of 2.7 to 3.4 Å. Interfacial volumes bound between the interacting collagen-like peptide and the polyphenol have been correlated with binding energies for various interactions as shown in figure 2. Binding energies calculated seem to vary systematically with the interacting interfacial volumes. The interaction involving side chain  $\epsilon$  amino groups of lysine and other residues seems to require higher interacting interfacial volumes for binding along the main chain of the triple helix as shown in figure 1.

A plot of binding energies against the effective solvent inaccessible contact volumes,  $B_T$  as calculated from contact surface area (CSA) by substituting CSA by TSA in (3) and hydrogen bond lengths shows a linear correlation as shown in figure 3. The correlation between effective surface area and binding energy has been shown as an inset of figure 3. The nature of dependence of binding energy with interacting volume as calculated from total surface area,  $A_T$  and hydrogen bond distances exhibits a general trend given in (4).

$$Y = \frac{a[\text{volume}]}{b + c[\text{volume}]}, \quad (4)$$

where  $Y$  is binding energy (kcal/mol),  $a$  and  $b$  are proportionality constants with units of kcalmol<sup>-1</sup> and Å<sup>3</sup> respectively,  $c$  is a dimensionless quantity and [volume] is interacting interfacial volume in Å<sup>3</sup>.

A plot of inverse of binding energy with the inverse of interacting interfacial volume (given in figure 2) is approximately linear as seen in figure 4. The slope of the linear plot in figure 4 for interactions involving side chain functionalities like those of lysine amino groups is different from that associated with other sites of collagen. The intercept and the

**Table 3a.** Hydrogen bond sites, distance, angle of gallic acid (Gal), pentagalloylglucose (PGG), complexes with triple helix.

Interaction site	Gallic acid (Gal)			Pentagalloylglucose (PGG)		
	H-bond	Bond distance (Å)	Bond angle (deg)	H-bond	Bond distance (Å)	Bond angle (deg)
9th residue Ser of C-chain ( <i>d</i> <sub>2</sub> )	SerC9-(C)-C-O...H(3)O-Gal	3.02	141	HypC15-(C)-O-H	2.84	177
	AsnA17-N-H...O(19)-PGG	3.17	156	...O(19)-PGG		
	AlaA15C=O...H(20)-PGG	2.76	156			
	SerC9-(C <sub>α</sub> )-C-O...H(10)O-PGG	2.93	121			
6th residue Hyp of A-chain ( <i>l</i> )	AspB21C=O...H(3)O-Gal	2.97	138	GluB2-(C <sub>α</sub> )-C-O-H	3.04	163
	GluB2-(C)-C-O...H(24)-PGG	2.88	139	...O(15)-PGG		
	HypB3C=O...H(23)-PGG	2.96	131			
	HypB3C=O...H(18)-PGG	3.08	157			
	LeuC5-N-H...O(12)-PGG	3.17	149			
12th residue Lys of B-chain ( <i>l</i> )	LysB12-(C)N-H...O(2)-Gal	3.28	126	HypA18-(C)-O-H	3.09	122
	AsnB17-(C)-N-H...O(2)O-PGG	3.12	141	...O(3)-PGG		
21st residue Asp of A-chain ( <i>l</i> )	AspB21-(C)-O-H...O(3)-Gal	2.89	128	AspA21-N-H	2.96	164
	HypB18-C=O...H(6)O-Gal	2.91	147	...O(9)-PGG GlyA19C=O ...H(13)O-PGG	2.83	174
17th residue Asn of C-chain ( <i>l</i> )	AsnA17-(C)-C=O...H(3)O-Gal	2.84	151	AsnB17-(C)-C=O	3.27	159
	HypA18C=O...H(4)O-PGG	2.9	140	...H(8)O-PGG		
	GlyA16N...H(3)O-PGG	3.42	161			

**Table 3b.** Hydrogen bond sites, distance, angle catechin (Cat) and epigallocatechingallate (EGCG) complexes with triple helix.

Interaction site	Catechin (Cat)			Epigallocatechingallate (EGCG)		
	H-bond	Bond distance (Å)	Bond angle (deg)	H-bond	Bond distance (Å)	Bond angle (deg)
9th residue Ser of C-chain ( $\hat{a}_2$ )	SerC9-(C <sub>β</sub> )-O-H...O(2)-Cat	3.04	161	LysA12C=O...H(9)O-EGCG	2.82	132
				SerC9-C=O...H(3)O-EGCG	2.79	148
6th residue Hyp of A-chain ( $\hat{a}_1$ )	HypA 6-(C <sub>β</sub> )-O-H...O(1)-Cat	3.02	127	ProB8-N...H(13)O-EGCG	3.25	142
	AlaB9-N...H(12)O-Cat	3.18	137			
12th residue Lys of B-chain ( $\hat{a}_1$ )	LysB12C=O...H(11)O-Cat	3.1	126	LysB12-(C <sub>β</sub> )-N-H...O(2)-EGCG	3.41	150
21st residue Asp of A-chain ( $\hat{a}_1$ )	AspA21-(C <sub>β</sub> )-C-O-H...O(4)-Cat	3.08	150	AspA21-N-H...O(2)-EGCG	3.3	147
	AlaB20-NH...O(2)-Cat	3.22	133	GlnA23-(C <sub>β</sub> )-N-H...O(6)-EGCG	3.26	146
	GlnA23-(C <sub>β</sub> )-N-H...O(6)-Cat	3.24	164			
17th residue Asn of C-chain ( $\hat{a}_2$ )	GlyA16C=O...H(14)O-Cat	3.00	146	GlyA16C=O...H(12)O-EGCG	2.99	140
	AsnA17-(C <sub>γ</sub> )-C=O...H(11)O-Cat	2.92	151	HypA18C=O...H(9)O-EGCG	2.82	162
			HypA18C=O...H(3)O-EGCG	2.9	156	
			AlaA20-N-H...O(2)-EGCG	3.13	143	



slope of linear plots given in figure 4 provide universal parameters, which are useful in the understanding of the interaction of collagen-like peptide and polyphenolic substances through the formation of hydrogen bonds. The inverse of the intercept of the plot in figure 4 provides estimates of upper limits of binding energies for covering solvent accessible surface area of the collagen-like peptide investigated. Various correlations of

**Table 4a.** Total and contact surface areas of the collagen-like triple helix and polyphenols in  $\text{\AA}^2$ .

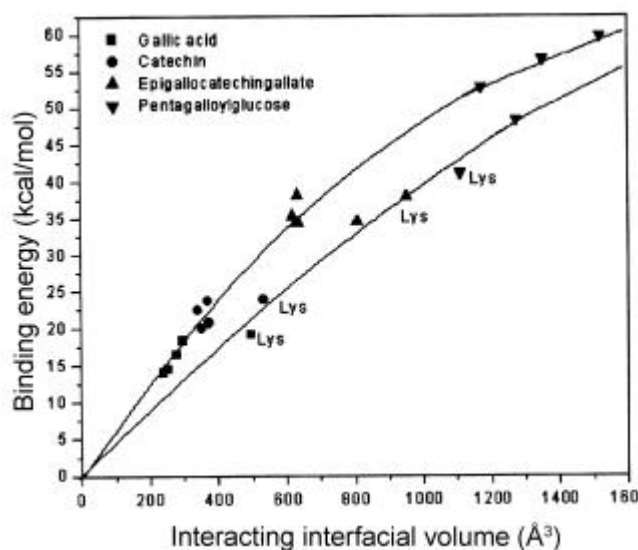
	Collagen (24-mer)	Cat	EGCG	PGG	Gal
CSA	1164	120	163	275	84
TSA	3825	268	382	688	160

CSA – contact surface area; TSA – total surface area

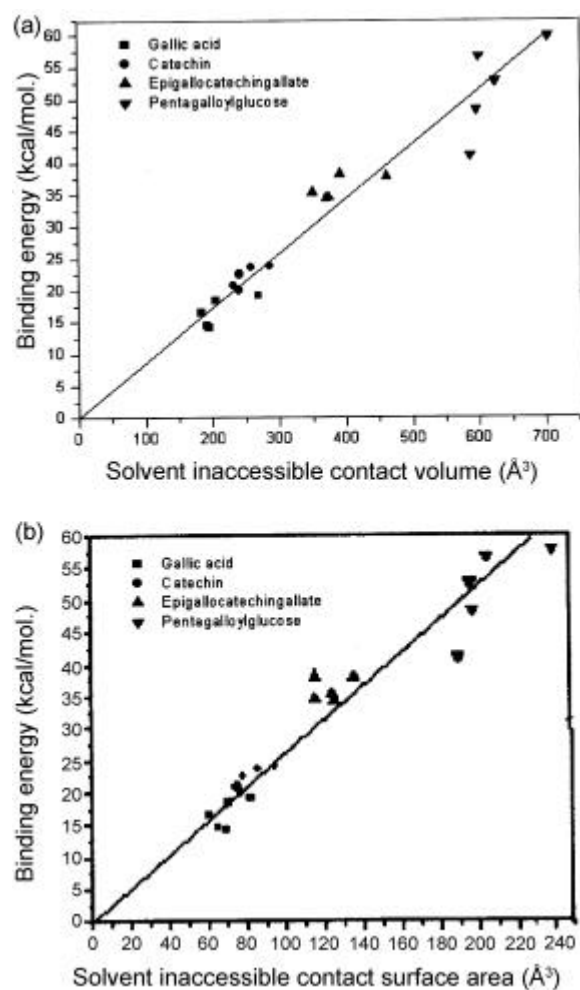
**Table 4b.** Solvent inaccessible surface areas of the complexes in  $\text{\AA}$

	Gal		Cat		EGCG		PGG	
	$A_T$	$B_T$	$A_T$	$B_T$	$A_T$	$B_T$	$A_T$	$B_T$
Ser	92	61	110	78	219	124	462	205
Hyp	85	65	120	75	248	115	421	197
Lys	151	82	176	94	279	135	357	189
Asp	102	71	112	76	186	115	514	238
Asn	84	69	124	86	214	125	368	196

$A_T$  – Solvent inaccessible total surface area;  $B_T$  – Solvent inaccessible contact surface area; TSA of the complexes are in the range of 3840–4160; CSA of the complexes are in the range of 1160–1250



**Figure 2.** Plot of interfacial interacting volume vs binding energy of the complex.



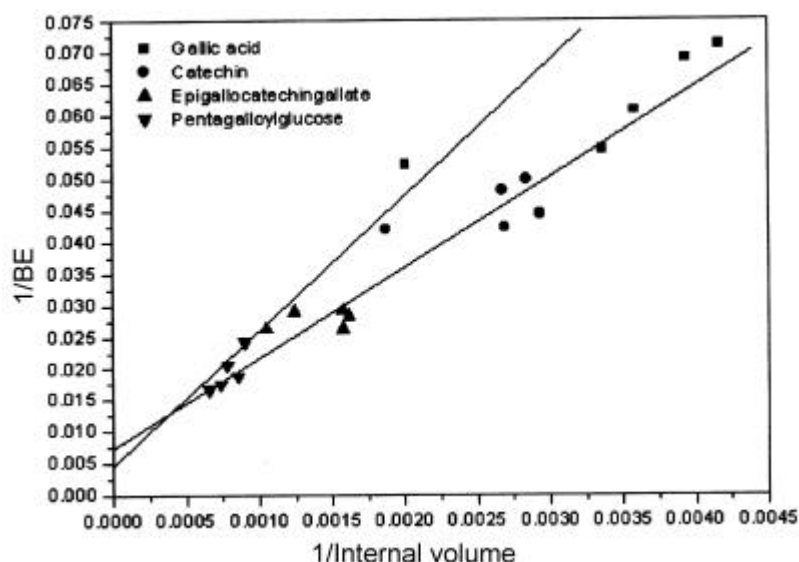
**Figure 3.** (a) Plot of effective solvent inaccessible contact volume vs binding energy of the complex. (b) Plot of effective solvent inaccessible contact surface area vs binding energy of the complex.

binding energy with interfacial volumes presented in this work illustrate the importance of geometric features and interacting interfacial volumes in the binding of small molecules by collagen-like peptides. Aspects like number hydrogen bonds and molecule specific variations in the strength of the hydrogen bonds may be important. However, their binding energies correlate well with interacting interfacial volume. Extent of inaccessibility of the bound surface to the solvent is of interest. The results of the modelling approaches are further supported by observations from experimental investigations involving soluble collagen and series of structure inducing substances, viz. formaldehyde, glutaraldehyde, tannic acid and basic chromium (III) salts.

Two-dimensional organization of native collagen in weak and acidic solutions has been investigated under monomolecular conditions. The influence of formaldehyde, glutaraldehyde, tannic acid and basic chromium (III) sulphate salts on the two-dimen-

sional organisation of collagen has been investigated. Isotherms of films of collagen spread at air/water interfaces as well as solutions of structure-inducing agents namely basic chromium sulphate, tannic acid, formaldehyde and gluteraldehyde have been presented in figure 5. The average area per molecule could be estimated from the isotherms. Concentration dependence of area/molecule for the various structure-inducing salts listed above has been examined. There is a relatively negligible effect of concentration of the agents selected on area/molecule in the range of concentrations investigated.

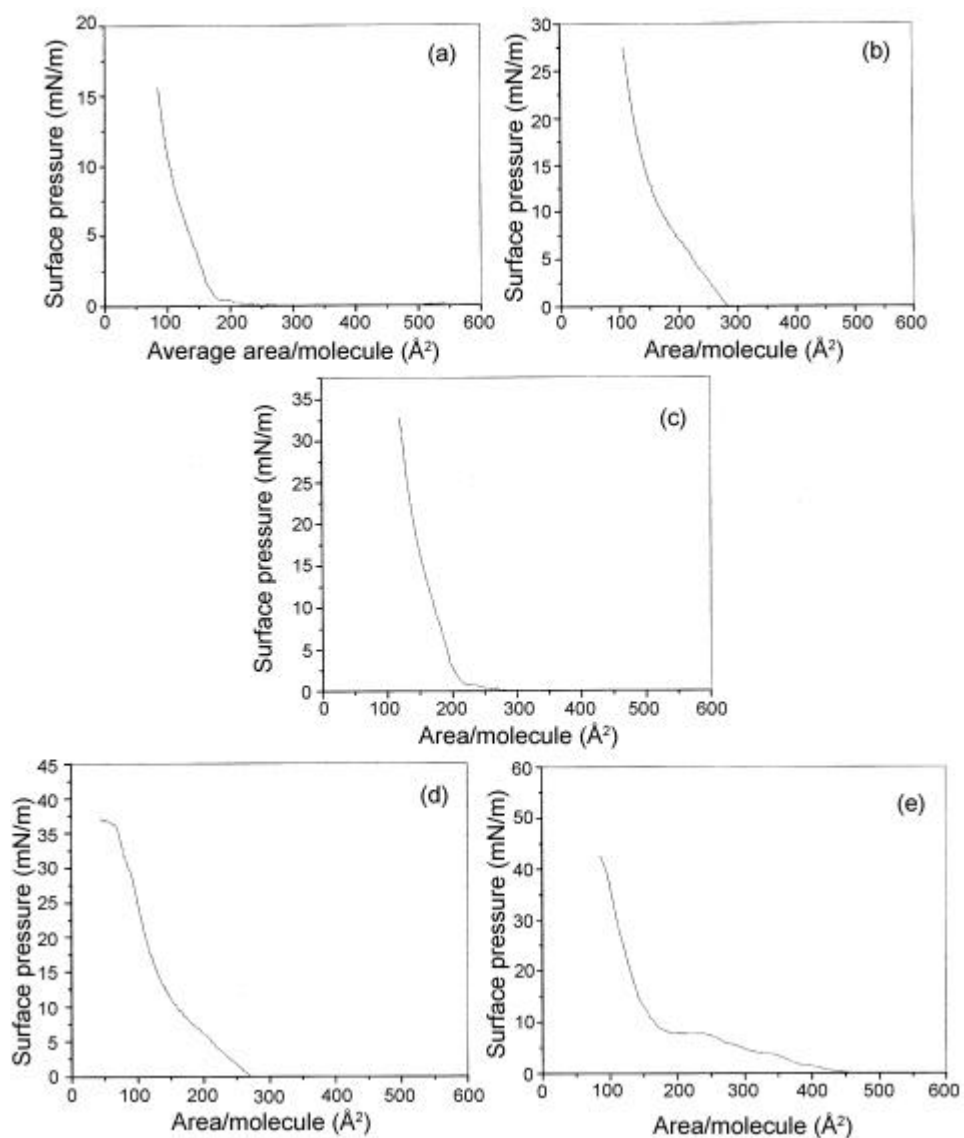
Collagen films were transferred onto freshly prepared mica and AFM studies were carried out under water in the tapping mode. AFM of collagen solutions in the absence and presence of basic chromium sulphate are presented in figure 6. Maximum surface pressure and surface area for collagen as well as the protein complexes with structure forming agents at the interface have been evaluated. Data are presented in table 5. At high dilutions of the protein and basic chromium sulphate, the resulting structure of the protein films established the formation of fractal structures as shown in figure 7.



**Figure 4.** Plot of inverse of interacting interfacial volume (1/int. vol.) vs inverse of binding energy (1/BE) of the complexes.

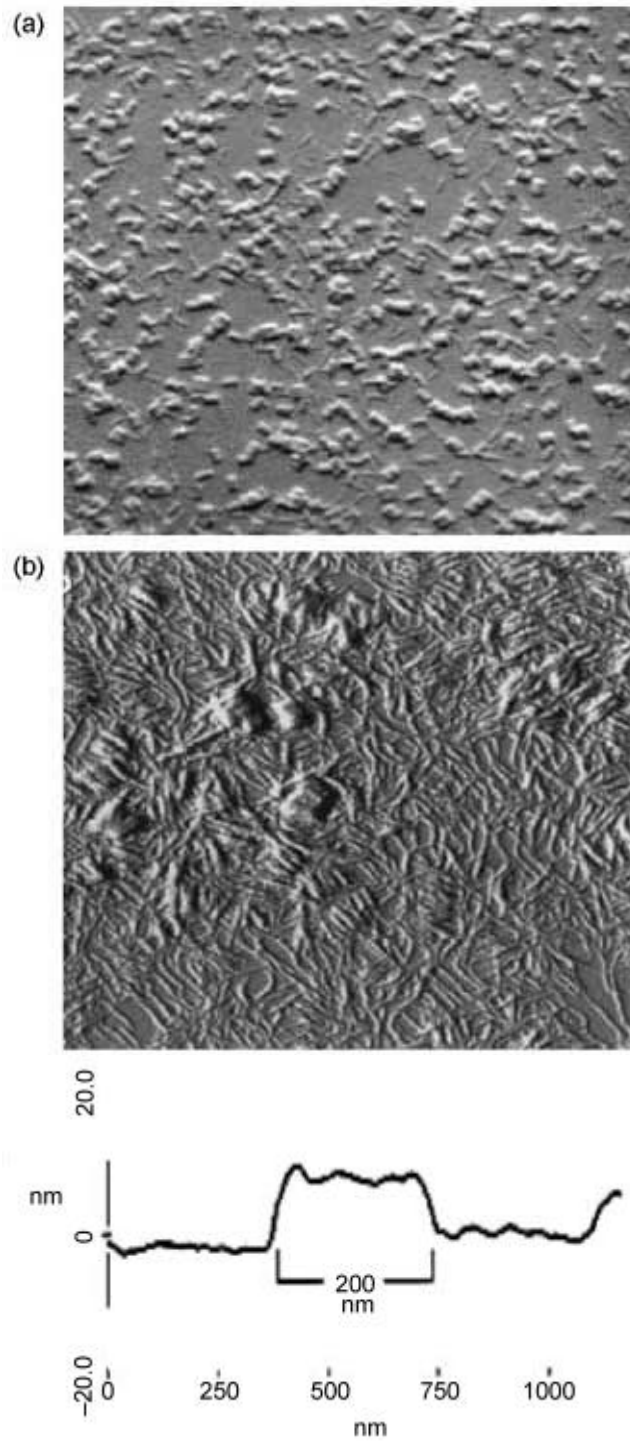
**Table 5.** Surface pressure and surface area of collagen solution and collagen treated with various tanning agents.

Sample at air/water interface	Max. surface pressure (mN/m)	Average surface area ( $\text{\AA}^2$ )
Collagen	15.5	85.4
Collagen + formaldehyde	27.5	108.0
Collagen + gluteraldehyde	32.5	120.9
Collagen + tannic acid	37.5	131.2
Collagen + BCS	42.2	137.2

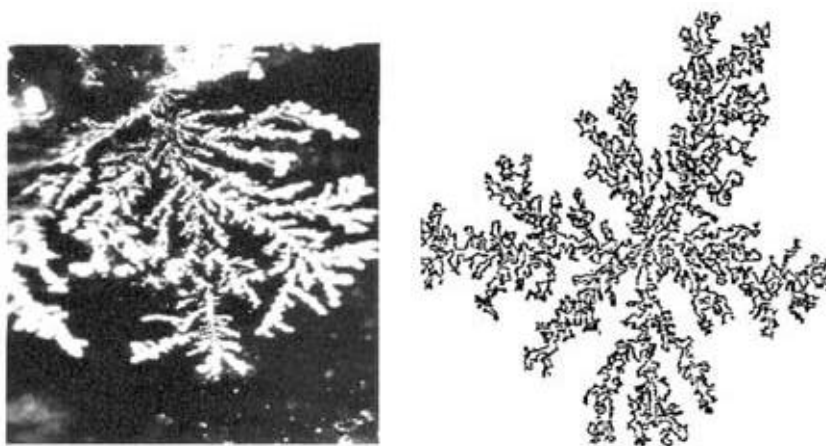


**Figure 5.** Isotherms of films of native collagen spread at air/water interfaces and collagen treated with various structure stabilizing agents. (a) Collagen 1; (b) collagen/formaldehyde; (c) collagen/gluteraldehyde; (d) collagen/tannic acid; and (e) collagen/BCS.

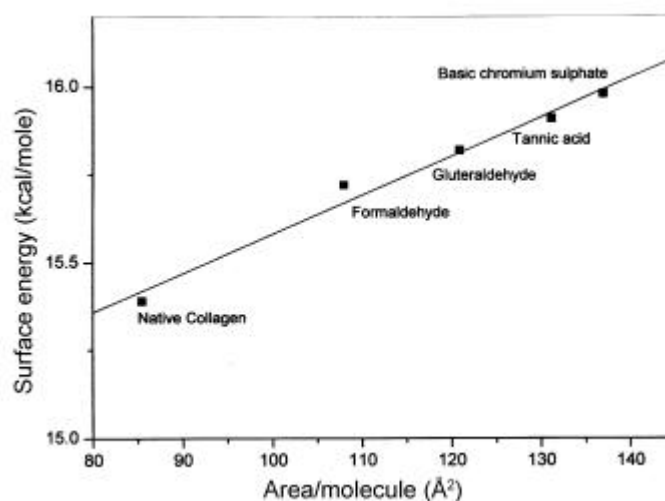
Surface pressure and areas per molecule of collagen with various tanning agents are correlated in figure 8. This correlation is relevant for the two-dimensional interaction of collagen with the tanning systems. A near-linear correlation of surface pressure with average area per molecule for the complexation of collagen with different molecular species is of special interest. The linearity disregards more processes, which involve



**Figure 6.** AFM of native collagen solutions (a) and collagen solution treated with basic chromium sulphate (b).



**Figure 7.** Fractal structures of collagen film induced by basic chromium sulphate.



**Figure 8.** Plot of surface pressure vs areas per molecule of collagen in the presence of various tanning agents.

short-range covalent and other interactions. Interaction of formaldehyde and gluteraldehyde to the protein are expected to implicate the covalent binding of aldehydes to the amino groups. Tannic acid is known to interact with collagen through H-bonding. Species contained in basic chromium (III) sulphate are known to complex with the carboxyl sites in collagen. Thus wide variations are expected in specific modes of binding and yet surface pressure varies linearly with contact surface area/molecule.

A molecular level process at distances of angstrom unit is expected to vary with the nature of the specific kind of bonds formed. However in two-dimensional aggregation processes, the surface energy and adsorption processes seem to dominate. The initial binding and two dimensional assemblies of collagen molecules in a solution seem to be

influenced by macroscopic parameters like surface pressure and the resulting surface energy rather than molecular events like cross-linking. The behaviour of the small molecules, viz. formaldehyde, glutaraldehyde, tannic acid and chromium (III) species, in binding to collagen appears to be similar to the gas phase adsorption on molecular surfaces, where surface energy plays the most important part. Intermolecular cohesion of collagen molecules in a film seems, to be influenced by the geometrical features of the collagen-small molecule complex. The long-range ordering of collagen induced by small molecules like tannic acid and chromium (III) species is evident from atomic force microscopy and photomicrographs. Slope of the plot shown in figure 8 is 0.5 mN/molecule/Å<sup>2</sup>. In energy terms, equivalent of 0.5 mN/molecule/Å<sup>2</sup> is 140.5 cal/mol/Å<sup>2</sup>. Both AFM and optical micrographs presented in figures 6 and 7 show that small molecules with surface areas 100 to 600 Å<sup>2</sup> are able to induce long-range ordering in a connective tissue protein-like collagen.

#### 4. Discussion

The present study has presented data and arguments in the form of ligation phenomena in collagen being influenced by surface pressure and geometric parameters such as interacting interfacial volume. It is conceivable that in complexation of collagen with small molecules, there may exist some minimum geometrical sizes and binding energies for influencing the long-range ordering processes in the protein. Within the range of molecules and substances investigated, binding of collagen seems to be influenced by interacting interfacial volumes and surface areas rendered inaccessible to solvent on account of binding. It is relevant to discuss the implications of the salient features of results presented in this work.

Organic molecules with flavanoid structures are known to elicit favourable response on arthritic conditions in human subjects.<sup>19</sup> Inflammation is an aspect of the arthritic condition of connective tissues in which volume changes in collagen on account of hydration and solvation are relevant. Similar increases in hydrothermal stability of skin gained by tanning are also related to long-range ordering in collagen induced by small molecules.<sup>7</sup> Anti-inflammatory behaviour and ability of polyphenol bearing flavanoid structure in management of arthritis and tanning may well result from their ability to reduce accessibility of solvent (water) to molecular surfaces of collagen.

The present investigation offers the possibility of understanding phenomena associated with protein-protein and DNA-protein interactions in general based on interfacial volume and contact surface areas.

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